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Multiple States of the 5-Hydroxytryptamine$_1$ Receptor as Indicated by the Effects of GTP on [^{3}H]5-Hydroxytryptamine Binding in Rat Frontal Cortex

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SUMMARY

In several monoamine receptor systems, agonist but not antagonist binding is found to be associated with both a high- and a low-affinity state of the receptor. Guanine nucleotides, like GTP, can eliminate the high-affinity receptor state such that only the low-affinity state of the receptor is present. Since [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) is an agonist, its binding may also be associated with multiple states of a receptor. To evaluate this, the binding of [^{3}H]5-HT to rat frontal cortical membranes was measured in the absence and presence of GTP. Three different types of binding experiments were performed: (a) saturation experiments, (b) dissociation experiments, and (c) competition experiments with 5-hydroxytryptamine (5-HT) agonists. When all three types of binding experiments were carried out in the absence of GTP, results from either graphical representations or computer analysis of the data indicated that a two-component model of binding described the data better than a single-component model. By contrast, in the presence of GTP, a one-component model adequately described the data obtained from either saturation or dissociation experiments. Competition of [^{3}H]5-HT (15 nM) binding by three 5-HT agonists (5-methoxytryptamine, 5-HT, and *d*-lysergic acid diethylamide) was adequately described by a single-component system in the presence of GTP as well, even though all three agonists produced biphasic inhibition curves in the absence of the guanine nucleotide. These experimental results are consistent with the idea that the binding of [^{3}H]5-HT in the absence of GTP is associated with multiple receptor states. Since the presence of multiple states can confound the interpretation of inhibition curves of [^{3}H]5-HT binding caused by agonists, it is important to eliminate the high-affinity state of the receptor by including GTP in the binding assay.

INTRODUCTION

Questions concerning the types and subtypes of 5-HT[3] receptors in the central nervous system have developed into a complex issue in the past several years. On the basis of the results of ligand binding studies, Peroutka and Snyder (1) suggested that two 5-HT receptor sites existed. These were designated the 5-HT$_1$ receptor, labeled by the agonist [^{3}H]5-HT, and the 5-HT$_2$ receptor, labeled by the antagonist [^{3}H]spiperone.

Recently, the functional significance of these binding sites has begun to be addressed. For example, recent studies have attempted to determine whether the 5-HT$_1$ or 5-HT$_2$ receptor is involved in mediating either the release of 5-HT (2) or certain responses elicited by the indolealkylamine (3, 4). In these types of studies, the ability of a series of compounds to elicit the response is correlated with their ability to inhibit either [^{3}H]5-HT or [^{3}H]spiperone binding. An issue usually not considered in such studies is the fact that an agonist is being used to label the 5-HT$_1$ receptor. If multiple receptor states exist in the absence of guanine nucleotides, data from studies correlating receptor binding with functional processes may be erroneous, since the radioligand, [^{3}H]5-HT, would have different affinities for these states.

In studies where a labeled agonist is used as the radioactive ligand, it is possible that multiple "states" of a receptor are involved in the binding reaction. In many systems, agonists, but not antagonists, demonstrate com-

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[3] The abbreviations used are: 5-HT, 5-hydroxytryptamine; *d*-LSD, *d*-lysergic acid diethylamide; 5-MT, 5-methoxytryptamine; Gpp(NH)p, 5′-guanylylimidodiphosphate.

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plex binding behavior that cannot be explained by simple mass-action kinetics. Although this has been most clearly described in the *beta*-adrenergic receptor system (5), other receptor systems such as *alpha*$_2$-adrenergic (6), glucagon (7), opiate (8), and dopamine (9) receptors demonstrate similar properties. For example, when labeled antagonists are used as ligands for *beta*-adrenergic receptors, competition experiments show that antagonists produce steep competition curves (with a Hill coefficient close to 1) that are not altered by guanine nucleotides, whereas agonists generate shallow competition curves with a Hill coefficient significantly less than 1 (10, 11). When guanine nucleotides are added, agonist competition curves demonstrate characteristics similar to those of antagonists, i.e., steep slopes with a Hill coefficient of 1. Such data have been interpreted in terms of the ability of agonists, but not antagonists, to form a high-affinity state of the receptor, which is "destabilized" to a low-affinity form by the addition of guanine nucleotides such as GTP. The high-affinity state of the receptor is thought to be a ternary complex consisting of the agonist, receptor, and a guanine nucleotide regulatory protein (*N*) (12).

Receptors coupled to adenylate cyclase are, for the most part, associated with ternary complex formation (13). Therefore, since 5-HT can stimulate adenylate cyclase activity, and this response may be associated with the 5-HT$_1$ receptor (14, 15), we wanted to determine whether [^{3}H]5-HT binding was associated with multiple states of the 5-HT$_1$ receptor. This issue has not been properly addressed even though GTP has been shown to decrease the binding of [^{3}H]5-HT (14). The results of these studies indicate that multiple states of the 5-HT$_1$ receptor exist, consistent with the idea that this receptor participates in ternary complex formation.

METHODS

Tissue preparation. Adult male Sprague-Dawley rats (Ace Animals, Boyertown, Pa.) weighing 250–350 g and housed on a 12-hr light-dark cycle were used in these experiments. They were killed on the day of the experiment by decapitation; the brains were removed rapidly, and the frontal cortex was quickly dissected over ice. Frontal cortical membranes were prepared essentially as described by Nelson *et al.* (16), the primary change in the preparation of the frontal cortical membranes and in the [^{3}H]5-HT binding assay being the use of $MgCl_2$ instead of $CaCl_2$ in the incubation and binding reaction buffers. $MgCl_2$ was substituted for $CaCl_2$ on the basis of the report by Hamon *et al.* (17) that the effect of GTP on [^{3}H]5-HT binding was inhibited to a greater extent by Ca^{2+} than by Mg^{2+}. Furthermore, preliminary experiments showed that 2.5 mM $MgCl_2$ increased specific [^{3}H]5-HT binding to a degree (about 40%) similar to that seen upon the addition of 4 mM $CaCl_2$. Briefly stated, each frontal cortex was homogenized in 7.0 ml of a sucrose-Tris buffer (280 mM sucrose/25 mM Tris/2.5 mM $MgCl_2$, pH 7.4) using a motor-driven Teflon pestle in a glass homogenizing tube. The homogenates were centrifuged at 30,000 × *g* for 10 min. The crude pellets were suspended in 7.0 ml of ice-cold distilled water, to promote lysis, and kept on ice. After 20 min, the samples were centrifuged at 30,000 × *g* for 10 min and the resultant pellet was washed three times with 7.5 ml of preincubation buffer (50 mM Tris/2.5 mM $MgCl_2$, pH 7.4). The washing procedure consisted of homogenizing the tissue and centrifuging the resultant homogenate. Between the second and third washes, the tissue was incubated at 30° for 20 min in a water bath to remove endogenous 5-HT as described by Nelson *et al.* (16). The final pellet was suspended in preincubation buffer such that the final protein concentration was approximately 6 mg/ml.

[^{3}H]5-HT binding assay. [^{3}H]5-HT binding was measured according to the method of Bennett and Snyder (18) as modified by Nelson *et al.* (16). Binding was performed in polystyrene tubes. The final assay volume of 1.5 ml consisted of 100 μl of [^{3}H]5-HT (15 nM; specific activity 24.5–29.1 Ci/mmole; New England Nuclear Corporation, Boston, Mass.); 100 μl of unlabeled 5-HT, competing drug, or incubation buffer (50 mM Tris/2.5 mM $MgCl_2$/10 μM pargyline/5.7 mM ascorbic acid, pH 7.4); 100 μl of GTP (or other nucleotides where indicated) or incubation buffer; 1.1 ml of incubation buffer; and 100 μl of tissue homogenate, which was added last to start the binding reaction. The incubation was carried out for 15 min at 30°, after which time 3.0 ml of ice-cold rinse buffer (a 40% dilution of the incubation buffer without pargyline) was added to each tube. The contents were then rapidly filtered through glass-fiber filters (Schleicher and Schuell, No. 25; Schleicher and Schuell, Inc., Keene, N. H.) mounted on a multiport filtering manifold connected to a vacuum pump. The filters were washed twice (one 3.0-ml wash followed by one 10.0-ml wash), transferred to vials, and allowed to dry overnight. This washing procedure did not cause any loss of specific binding. Budget Solve (4.0 ml) was added to the vials, and the radioactivity was measured by liquid scintillation spectrophotometry (30% efficiency). Specific [^{3}H]5-HT binding was defined as the difference in the amount of radioactivity bound in the absence and presence of 10 μM unlabeled 5-HT. Specific binding in the absence of GTP (using 15 nM [^{3}H]5-HT) averaged about 65% of total radioactivity bound. In the presence of GTP, specific binding was reduced from that seen in the absence of GTP to about 50% of total radioactivity bound.

Competition experiments were performed to compare the ability of serotoninergic compounds to inhibit [^{3}H]5-HT binding in the absence or presence of GTP. Multiple concentrations of each drug were examined for their ability to compete with 15 nM [^{3}H]5-HT for binding sites. Results from competition experiments were transformed and are represented as Hofstee plots together with the inhibition curves of the untransformed data.

Experiments were conducted to compare the kinetics of dissociation of 15 nM [^{3}H]5-HT binding in the absence and presence of GTP. The experiments were carried out at 20° in a large flask containing all ingredients of the binding assay in the same proportion as described previously. Dissociation was begun by adding 250 μl of a concentrated solution of unlabeled 5-HT such that its final concentration was 10 μM. Aliquots of 1.5 ml were removed at multiple time points for each experiment.

Saturation experiments were performed to determine the binding characteristics of [^{3}H]5-HT in the absence and presence of GTP. Multiple concentrations of [^{3}H]5-HT ranging from 0.25 to 50 nM were examined. Results from these experiments were transformed and are represented as Scatchard plots.

Protein concentrations were determined according to the method of Lowry *et al.* (19).

Drugs. Drugs generously donated by the following companies or agencies were metergoline (Farmitalia, Milan, Italy) and *d*-LSD (National Institute of Drug Abuse, Bethesda, Md.). Pargyline HCl, 5-MT, and 5-HT creatine sulfate were purchased from Sigma Chemical Company (St. Louis, Mo.). All drugs were dissolved in 15% ascorbic acid (subsequently diluted in distilled water so that the final assay concentration of ascorbic acid was 0.1%) except for *d*-LSD and pargyline, which were dissolved in distilled water. All other reagents were obtained from commercial sources.

Data analysis. Binding experiments were analyzed simultaneously essentially as described by DeLean *et al.* (20) by an unweighted nonlinear regression analysis program. These analyses were performed using Prophet, an interactive computer system which utilizes the modeling programs MLAB. Computer analysis of binding data involves the fitting of curves to the data points using binding equations which are based on the law of mass action (ref. 21; see also ref. 22). The best

value for the unknown parameters of the given equation are iteratively determined such that the lowest value for the sum of the squares of residuals between the actual data points and the fitted points is obtained. The data are first fit to the simplest model (i.e., one-component), followed by the more complex (two-component) model. The resulting sum of the squares of the residuals obtained can be used to determine whether the more complex model describes the data significantly better by the partial F-test (23), according to the equation $F = [(SS_1 - SS_2)/(df_1 - df_2)]/(SS_2/df_2)$, where SS and df are the sum of the squares of the residuals and degrees of freedom for the one- and two-component models, respectively. It is assumed that the simplest model adequately describes the data until the more complex model reaches a level of statistical significance ($p < 0.01$) (see ref. 24). The 95% confidence limits generated by computer analysis are given along with each parameter value (for a fuller discussion of the parameter error, see ref. 24).

RESULTS

Definition of Nonspecific Binding

Correct definition of nonspecific binding is essential for the accurate analysis of multiple binding components. Therefore, studies were performed initially to determine whether unlabeled serotonin was a suitable compound to use to define specific binding of [^{3}H]5-HT. Although it is generally not a good practice to use the same chemical as the labeled compound to define nonspecific binding (see ref. 25), unlabeled 5-HT has in the past been used routinely to define specific binding (18, 26). To determine whether there was a single concentration of unlabeled 5-HT that could be used to define nonspecific [^{3}H]5-HT binding over a wide range of concentrations of the labeled indolealkylamine, the inhibition of [^{3}H]5-HT binding by unlabeled 5-HT was evaluated at four concentrations of [^{3}H]5-HT ranging from 0.5 to 50 nM. The results of this experiment are shown in Fig. 1. 5-HT inhibited binding at all four concentrations of [^{3}H]5-HT, and a common plateau for the inhibition of binding occurred between concentrations of 5-HT of 5 μM and 25 μM. At higher concentrations of competing drug, nonspecific [^{3}H]5-HT binding began to be inhibited. Therefore, 10 μM unlabeled 5-HT was chosen to define nonspecific binding in subsequent experiments comparing the inhibition it produced with that of other 5-HT agonists or antagonists.

Inhibition of binding of [^{3}H]5-HT (15 nM) produced by 10 μM unlabeled 5-HT was compared with that caused by 3 μM metergoline, a 5-HT antagonist. This concentration of metergoline was selected from preliminary experiments showing it to be on the plateau portion of the inhibition curve (data not shown). The inhibition of [^{3}H]5-HT binding produced by these two compounds was compared in both the absence and presence of GTP (1 mM). As shown in Table 1, both compounds reduced the binding of [^{3}H]5-HT; more important, there was no significant difference in the amount of inhibition of [^{3}H]5-HT binding produced by these concentrations of unlabeled 5-HT or metergoline, in either the presence or absence of GTP. If unlabeled 5-HT correctly defined nonspecific binding, then other 5-HT compounds would be expected to produce the same maximal inhibition of [^{3}H]5-HT binding as produced by 5-HT. Results from experiments examining the inhibition of 15 nM [^{3}H]5-HT binding by 5-HT compounds (e.g., 5-MT, *d*-LSD, methysergide, methiothepin) showed that all compounds maximally inhibited the same amount of specific [^{3}H]5-HT binding as 10 μM 5-HT (data not shown). Therefore, unlabeled 5-HT (10 μM) was used to define nonspecific binding in subsequent experiments.

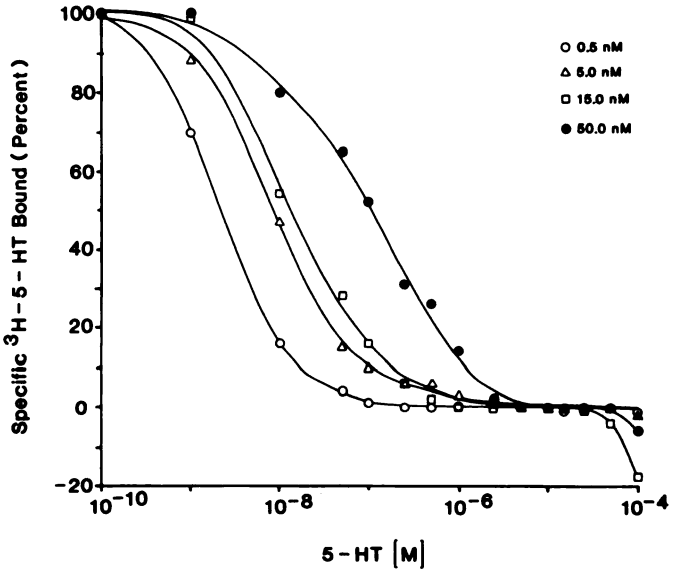

FIG. 1. *Inhibition of specific [^{3}H]5-HT binding by unlabeled 5-HT in rat frontal cortical membranes*

At all concentrations of [^{3}H]5-HT, specific binding was defined as the difference in binding measured in the absence and presence of 10 μM serotonin. The best line through the data points was drawn using a computerized nonlinear regression analysis program. The competition curves shown are representtive of the results from two experiments performed in triplicate.

TABLE 1

Comparison of unlabeled 5-HT and metergoline to define specific binding of 15 nM [^{3}H]5-HT

GTP	[^{3}H]5-HT binding		
	Total	+ 5-HT (10 μM)	+ Metergoline (3 μM)
	fmoles/mg protein		
–	709 ± 27[a] (3)[b]	242 ± 19 (3)	228 ± 15 (3)
+	533 ± 9 (3)	272 ± 16 (3)	250 ± 5 (3)

[a] Values are means ± standard error of the mean.
[b] Number of experiments.

Determination of Optimal Concentration of GTP

Several types of binding experiments were conducted to determine whether multiple states of the 5-HT$_1$ receptor were present. These were saturation, dissociation, and competition experiments. Data supportive of multiple states of a receptor should show, for all three types of experiments, two-component binding in the absence of GTP and one-component binding in the presence of GTP.

Before examining [^{3}H]5-HT binding in the absence and presence of GTP, it was essential to determine the optimal concentration of GTP to use in these experiments. Previous investigators have demonstrated the

ability of guanine nucleotides to decrease [^{3}H]5-HT binding (14, 26, 27). If the GTP-induced decrease in [^{3}H]5-HT binding reflects the elimination of the high-affinity state of the receptor (i.e., ternary complex), some binding to the low-affinity form of the receptor may remain even in the presence of GTP if the dissociation rate of the low-affinity state is slow enough. Initial saturation experiments with [^{3}H]5-HT revealed the presence of a low-affinity binding component with a K_d of about 15 nM, and approximately 50% specific binding (1500–2000 cpm) was present at this concentration of labeled 5-HT (see below). These results indicated that 15 nM [^{3}H]5-HT would be a suitable concentration to use to examine the effect of GTP on binding, as a measurable amount of binding to the low-affinity component should remain in the presence of GTP. Therefore, the ability of GTP to inhibit the binding of 15 nM [^{3}H]5-HT was examined (Fig. 2A). GTP produced a dose-dependent decrease in specific [^{3}H]5-HT binding from 0.002 to 0.75 mM. Concentrations above 0.75 mM did not result in further decreases in binding. The maximal decrease in binding was about 50%, and the EC_{50} value for GTP was approximately 50 μM. Therefore, 1.0 mM GTP was used in subsequent binding experiments, unless noted otherwise.

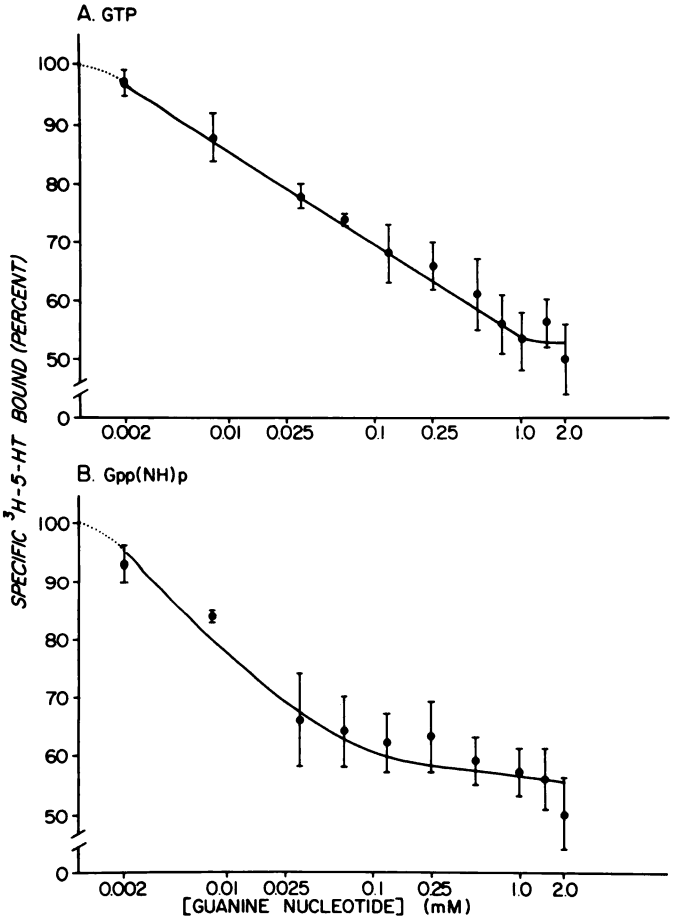

FIG. 2. *Inhibition of specific [^{3}H]5-HT (15 nM) binding to rat frontal cortical membranes by (A) GTP and (B) Gpp(NH)p*

Nonspecific binding was defined by 10 μM unlabeled serotonin. All binding assays were carried out in quadruplicate. Each point and bracket represent the mean ± standard error of the mean (N = 3 to 5) for the percentage [^{3}H]5-HT bound at each nucleotide concentration. The EC_{50} value for GTP is approximately 0.05 mM; for Gpp(NH)p, 0.01 mM.

This concentration of GTP produced no significant inhibition of nonspecific [^{3}H]5-HT binding, i.e., binding measured in the presence of 10 μM 5-HT. This is indicated by the data presented in Table 1. Also, the results of 15 paired experiments randomly selected showed nonspecific binding in the absence of GTP to be 4292 ± 88 cpm (mean ± standard error of the mean). In the presence of 1 mM GTP, nonspecific binding was 4297 ± 90 cpm, which is essentially identical with that measured in the absence of the guanine nucleotide.

The ability of other nucleotides to inhibit [^{3}H]5-HT binding was examined to compare their effect with that of GTP. Multiple concentrations of each nucleotide were used, ranging from 0.002 to 2.0 mM. The results of the experiment examining inhibition of 15 nM [^{3}H]5-HT binding by Gpp(NH)p are shown in Fig. 2B. Although Gpp(NH)p inhibits the same maximal amount of [^{3}H]5-HT binding as GTP (about 50%), it is approximately 5 times as potent as GTP, as demonstrated by its EC_{50} value of 10 μM. Of the other nucleotides examined (data not shown), ATP was the least effective; 1.0 mM ATP inhibited only 15% of specific binding. ITP was also less effective than GTP, since 1 mM inhibited only 25% of the binding of [^{3}H]5-HT. GDP was slightly less effective than GTP in inhibiting the binding of [^{3}H]5-HT.

Multiple States of [^{3}H]5-HT Binding

Saturation experiments. When cerebral cortical membranes were incubated with [^{3}H]5-HT concentrations ranging from 0.25 to 50 nM in the absence of GTP, complex binding resulted as indicated by a curvilinear Scatchard plot (Fig. 3). The slope of the Hill plot was 0.76 ± 0.02 (mean ± standard error of the mean). Computer analysis revealed that a two-component binding model described the data significantly better than a one-component model ($p < 0.001$). The computer-derived parameter values revealed a B_{max} of 230 ± 90 fmoles/mg of protein and a K_d of 0.5 ± 0.3 nM (mean ± 95% confidence limits, as described under Methods) for the high-affinity binding component, whereas the B_{max} and K_d for the low-affinity component were 480 ± 70 fmoles/mg of protein and 9 ± 3 nM, respectively.

To determine the effect of GTP on this two-component binding, the same experiments were performed in the presence of 1 mM GTP. As shown in Fig. 3, when GTP was present during the binding reaction, only a single population of binding sites remained, as revealed by a linear Scatchard plot. Since in the absence of GTP the steeper portion of the curve reflects binding to the higher-affinity component, it is apparent that the addition of 1 mM GTP reduced the high-affinity component of [^{3}H]5-HT binding. In the presence of 1 mM GTP, the Hill slope was 0.92 ± 0.01. When these experiments were subjected to computer analysis, a one-component model was found to describe [^{3}H]5-HT binding adequately; the B_{max} was 615 ± 20 fmoles/mg of protein and the K_d value was 14 ± 1 nM. This value is in reasonable agreement with the K_L value of 9 ± 3 nM for the low-affinity component of binding in the absence of GTP.

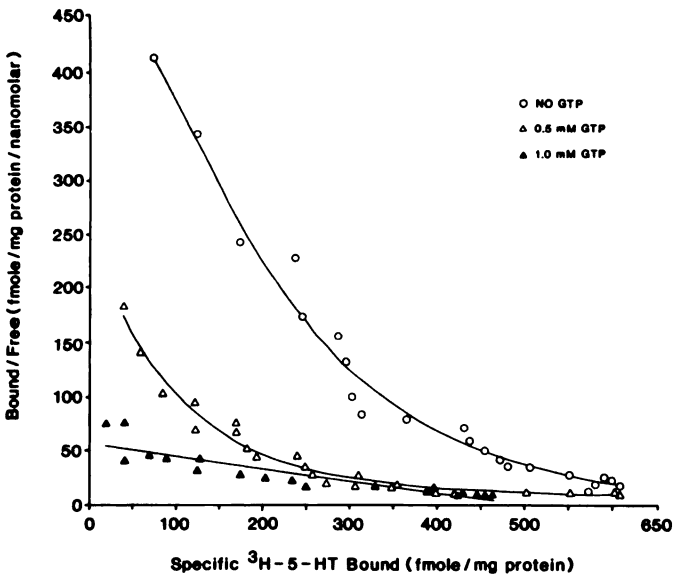

FIG. 3. *Scatchard plots of [^{3}H]5-HT binding to rat frontal cortical membranes in the absence and presence of GTP*

The concentrations of [^{3}H]5-HT ranged from 0.25 to 50 nM. Nonspecific binding was defined by 10 μM unlabeled serotonin. The best line through the data points was drawn using a computerized nonlinear regression analysis program. Each curve represents data from a single experiment performed in quadruplicate and is representative of the results from either two (0.5 mM GTP) or four (no GTP; 1.0 mM GTP) experiments.

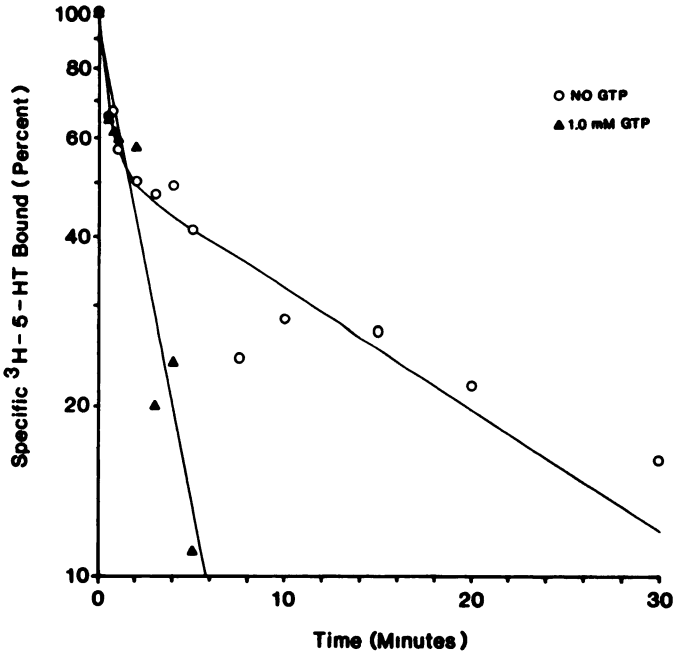

FIG. 4. *Dissociation of [^{3}H]5-HT (15 nM) binding from rat frontal cortical membranes in the absence and presence of GTP*

Unlabeled 5-HT (10 μM) was added to a flask containing the binding reaction mixture to begin the experiments, and the reaction was terminated at various times as described under Methods. Experiments were performed in the absence and presence of 1.0 mM GTP. The best line through the data points was drawn using a computerized nonlinear regression analysis program. Each curve represents the average of three experiments performed in quadruplicate.

To determine whether the effect of GTP was concentration-related, a lower concentration of GTP (0.5 mM) was examined. The results of this experiment demonstrate that 0.5 mM GTP produced a decrease in the high-affinity component of binding (Fig. 3). Computer analysis of these binding data showed that they were still described better by a two-component model ($p < 0.001$) and that the percentage of binding to the high-affinity component decreased from approximately 33% in the absence of GTP to about 20% in the presence of 0.5 mM GTP. Computer analysis of the binding data revealed a K_H of 0.7 ± 0.7 nM and a K_L of 17 ± 7 nM. These values are consistent with those obtained in either the absence or presence of GTP. Therefore, the saturation experiments indicate that GTP, in a concentration-dependent manner, eliminates a high-affinity component of [^{3}H]5-HT binding.

Dissociation experiments. Analysis of the kinetics of dissociation, in the absence and presence of GTP, can provide evidence for multiple states of a receptor. Therefore, cortical membranes were incubated for 60 min at 20°, after which time unlabeled serotonin was added at a final concentration of 10 μM. Aliquots were removed and filtered at multiple time points. In the absence of GTP, a biphasic dissociation curve was obtained (Fig. 4). Computer analysis revealed that a two-component binding model described the data more accurately than a one-component model ($p < 0.001$). The high-affinity component of binding represented 54 ± 3% of the binding.

When the same experiments were carried out in the presence of 1.0 mM GTP, a linear dissociation plot was obtained, indicative of a single component of binding (Fig. 4). The loss of the slower phase of the dissociation curve indicates that GTP eliminated the high-affinity component of binding, as in the saturation experiments. These results are consistent with those of Peroutka *et al.* (14), who showed that GTP accelerated the rate of [^{3}H] 5-HT dissociation. Computer analysis of the data revealed that a two-component binding model no longer described the binding data significantly better than a one-component model ($p > 0.1$).

Competition experiments. Experiments examining the ability of agonists to compete with the labeled compound in the absence and presence of GTP were performed. Cortical membranes were incubated with 15 nM [^{3}H]5-HT and multiple concentrations of a competing agonist, in both the absence and presence of GTP. When GTP was not present during the binding reaction, 5-HT and two other agonists, *d*-LSD and 5-MT, displayed shallow inhibiton curves (Fig. 5). For all three compounds, the Hill coefficients were less than 1 in the absence of GTP, and transformation of the inhibition curves yielded curvilinear Hofstee plots. Consistent with these findings, computer analysis of the binding data revealed that a two-component model described the data significantly better than a one-component model for all three compounds in the absence of GTP ($p < 0.001$).

Different results were obtained when the same experiments were performed in the presence of 1.0 mM GTP. Under these conditions, the inhibition curve for all three compounds became steeper, as shown by Hill coefficient values not significantly different from 1. The Hofstee

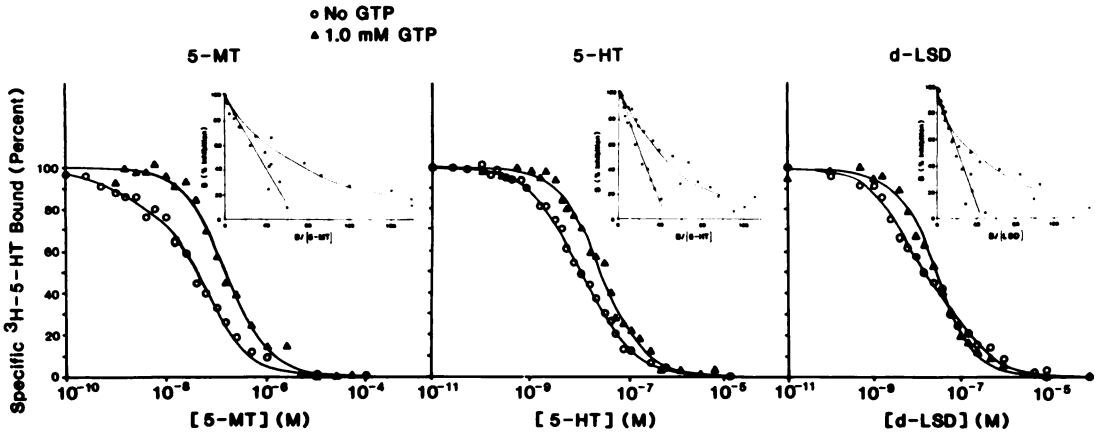

FIG. 5. *Competition curves for the inhibition of [3H]5-HT (15 nM) binding by 5-MT, 5-HT, and d-LSD to rat frontal cortical membranes in the absence and presence of GTP.*

Insets show Hofstee plots of the transformed data. Nonspecific binding was defined by 10 μM unlabeled 5-HT. The best line through the data points was drawn using a computerized nonlinear regression analysis program. Each curve represents the average of three or four experiments performed in quadruplicate. The Hill coefficients (means ± standard error of the mean), as determined by computer analysis for 5-MT, 5-HT, and *d*-LSD, were 0.64 ± 0.02, 0.83 ± 0.03, and 0.58 ± 0.03, respectively, in the absence of GTP, and 1.00 ± 0.04, 0.99 ± 0.08, and 0.99 ± 0.07, respectively, in the presence of GTP.

plots for all three compounds became linear (Fig. 5) owing to the ability of GTP to eliminate the high-affinity component of binding. This can also be seen by a shift of the competition curves to the right. The ability of GTP to shift the competition curve to the right was weaker for *d*-LSD than for either 5-HT or 5-MT. The K_i values for *d*-LSD, 5-HT, and 5-MT were 13 ± 1 nM, 13 ± 1 nM, and 60 ± 5 nM, respectively. The K_i value for 5-HT of 13 nM is essentially the same as the K_d value of 14 nM, which was determined from saturation experiments in the presence of GTP.

DISCUSSION

The results of this study provide evidence that binding of the agonist [3H]5-HT exhibits both a high-affinity and a low-affinity component of binding to frontal cortical membranes of the rat in the absence of guanyl nucleotides. Since the addition of GTP eliminated the high-affinity component in dissociation, saturation, and competition experiments, it seems likely that this high-affinity component represents a high-affinity "state" or ternary complex consisting of agonist, receptor, and guanine nucleotide-binding regulatory component. By analogy with the scheme proposed for *beta*-adrenergic receptors (see ref. 5), GTP may destabilize the high-affinity ternary complex associated with the 5-HT_1 receptor such that only the low-affinity state of the receptor is present.

Consistent with this scheme is the finding that saturation experiments reveal essentially no difference between the K_d of the low-affinity component in the absence of GTP (9 ± 3 nM) and the K_d of the binding component in the presence of 1.0 mM GTP (14 ± 1 nM). Although these values are similar, a perfect agreement is not expected, since the equations that are generally used to obtain parameter values in the absence of guanine nucleotides actually describe a two-component system that is non-interconvertible, i.e., two receptors. Since these equations only approximate the actual binding conditions, slight differences are not unexpected. Consequently, in order to obtain accurate parameter values for the 5-HT_1 receptor itself, i.e., the low-affinity state, it is necessary to include GTP in the binding assay to eliminate the contribution of ternary complex to binding.

Furthermore, we find that even in the presence of concentrations of GTP that eliminate the high-affinity state of the receptor, sufficient binding remains using 15 nM [3H]5-HT to measure accurately the low-affinity binding. This latter finding would not necessarily have been predicted, since in some other receptor systems coupled to adenylate cyclase, the addition of GTP completely eliminates the ability to measure the bound agonist ligand (28).

To demonstrate multiple states of the 5-HT_1 receptor, it was first necessary to show that two components of binding existed in the absence of GTP. This study demonstrates that for all three types of binding experiments (saturation, dissociation, and competition), two components of binding are revealed in the absence of GTP.

Interestingly, it was necessary to perform dissociation experiments at 20° rather than at 30°, the temperature at which all other binding experiments were conducted. At temperatures above 20°, the dissociation rate of [3H]5-HT was too rapid to be characterized adequately in the presence of GTP. When these experiments were carried out at 0–4° to slow the rate of dissociation, the effect of GTP to decrease [3H]5-HT binding was lost. Even at 10°, 1 mM GTP inhibited [3H]5-HT specific binding by only about 10%. This inhibitory effect of lowering the temperature on the decrease in [3H]5-HT binding caused by GTP has been noted in other receptor systems also. For example, Zahniser and Molinoff (29) reported that at 0° GTP was no longer able to produce a shift in the inhibition curve of dopamine for [3H]spiperone binding. In contrast to these findings, Peroutka *et al.* (14) reported that the reduction of 2 nM [3H]5-HT binding by guanine nucleotides in rat frontal cerebral cortex was the same at either 4° or 37°.

The results from competition experiments not only show that GTP eliminates the high-affinity component of binding for all three 5-HT agonists, as indicated by a steepening and a shift of the competition curves to the right, but that the addition of GTP produces the smallest shift for the *d*-LSD inhibition curve as well. Lefkowitz *et al.* (10) originally showed that the extent of the guanine nucleotide-induced shift of the competition curve produced by compounds for the *beta*-adrenergic receptor follows the order: full agonists > weak or partial agonists > antagonists. Since other investigators have suggested that *d*-LSD displays both agonist and antagonist properties (e.g., refs. 1 and 18), our results indicate that a similar phenomenon may exist for the 5-HT_1 receptor.

Previous work examining the effect of guanine nucleotides on [^{3}H]5-HT binding has shown that GTP or Gpp(NH)p inhibits specific [^{3}H]5-HT binding (14, 26, 27). Peroutka *et al.* (14) demonstrated that the guanine nucleotides GTP and Gpp(NH)p inhibited the binding of 2 nM [^{3}H]5-HT in a dose-dependent fashion. Approximately 40% specific [^{3}H]5-HT (2 nM) binding was inhibited in the presence of 1.0 mM GTP and 80% in the presence of 1.0 mM Gpp(NH)p. The EC_{50} values for GTP and Gpp(NH)p were approximately 50 μM for both compounds. When these experiments were performed in our assay system, 1.0 mM GTP and Gpp(NH)p were found to inhibit approximately 80% of the specific binding of 2 nM [^{3}H]5-HT. The finding that GTP and Gpp(NH)p have similar effects in our system may be due to the absence of calcium ions in our assay. This was suggested previously by Hamon *et al.* (17), who proposed that Ca^{2+} decreases the availability of GTP, by either complexation or degradation. Peroutka *et al.* (14) and Mallat and Hamon (27) demonstrated that GTP was able to inhibit [^{3}H]5-HT binding by increasing the rate of dissociation of the radiolabeled agonist and thereby decreasing the affinity of the receptor for [^{3}H]5-HT. Furthermore, Peroutka *et al.* (14) showed that GTP was able to decrease the ability of agonists, but not antagonists, to compete for 2 nM [^{3}H]5-HT binding. However, whether multiple states of the 5-HT_1 receptor were present was not addressed by these investigators. This may be due to their reporting both linear Scatchard plots and steep curves for the inhibition of 2 nM [^{3}H]5-HT by unlabeled 5-HT in the absence of GTP; this would indicate only one component of binding. Other investigators have reported linear Scatchard plots in rat brain as well (27, 30). However, these investigators may not have been able to detect a low-affinity component of binding because of their use of either a limited range of [^{3}H]5-HT concentrations (0.49–7.32 nM) (27) or of only two [^{3}H]5-HT concentrations greater than 4 nM (1, 30). In studies where a wide range of [^{3}H]5-HT concentrations have been used, curvilinear Scatchard plots are obtained. Kienzl *et al.* (31) demonstrated curvilinear Scatchard plots of [^{3}H]5-HT binding in human frontal cortex. The [^{3}H]5-HT concentrations ranged from 0.5 to 35 nM in these studies. In our experiments, at least 18 different concentrations of ligand were used to permit analysis of multiple binding components.

Our results indicate that 2 nM [^{3}H]5-HT, the concentration of [^{3}H]5-HT used by Peroutka *et al.* (14) in their competition experiments, would label primarily the high-affinity state of the 5-HT_1 receptor in the absence of GTP; consequently, multiple components of binding would be difficult to detect using this concentration of the radioligand. In addition, only 20% specific binding remains in the presence of 1 mM GTP using 2 nM [^{3}H]5-HT. The use of a concentration of [^{3}H]5-HT higher than 2 nM in binding experiments is preferable in order to obtain measurable binding to the low-affinity state of the 5-HT_1 receptor. We chose 15 nM as this is the K_d value for [^{3}H]5-HT in the presence of GTP.

Comparison of the effects of other nucleotides on [^{3}H]5-HT binding with that of GTP revealed that GTP was as effective as Gpp(NH)p (the same maximal response was produced by both compounds), although Gpp(NH)p appears to be 5-fold more potent than GTP. One possible reason for these findings is that some hydrolysis of GTP may occur during the assay procedure. Previous studies in other receptor systems have examined the effects of Gpp(NH)p and GTP on radiolabeled agonist binding. Williams and Lefkowitz (32) showed that both Gpp(NH)p and GTP inhibited binding of the *beta*-adrenergic agonist [^{3}H]hydroxybenzylisoproterenol to frog erythrocyte membranes with EC_{50} values of approximately 0.5 and 100 μM, respectively. Gpp(NH)p produced maximal inhibition of binding at 10 μM, whereas the maximal effect of GTP occurred at approximately 1.0 mM. U'Prichard and Snyder (6) demonstrated that GTP and Gpp(NH)p were approximately equipotent in their ability to inhibit either [^{3}H]norepinephrine or [^{3}H]epinephrine binding to calf frontal cortical membranes. They reported EC_{50} values of approximately 1.0 μM for both radioligands. Maximal inhibition of binding occurred at concentrations above 100 μM for both compounds. In addition, De Lean *et al.* (33) have shown that the EC_{50} value for GTP is approximately 20 μM in inhibiting the binding of the dopamine agonist [^{3}H]*N*-propylapomorphine in porcine pituitary. Maximal inhibition of binding occurred at concentrations near 1.0 mM. Therefore, Gpp(NH)p appears to be approximately 10- to 20-fold less potent, and GTP is relatively equipotent in our system as compared with the results found in other receptor systems.

The existence of a 5-HT-sensitive adenylate cyclase has been reported by several groups (15, 26, 34). Previous investigators suggested that the 5-HT_1 receptor is coupled to adenylate cyclase formation, since guanine nucleotides are able to inhibit [^{3}H]5-HT binding (14, 26). Mallat and Hamon (27) proposed that, since the GTP concentration necessary to inhibit binding to the 5-HT_1 receptor (10 μM) is approximately the same as that required to stimulate cyclic AMP formation, the 5-HT_1 receptor is linked to adenylate cyclase. Our results are also consistent with the idea that the 5-HT_1 receptor is linked to adenylate cyclase, since formation of a guanine nucleotide-sensitive ternary complex suggests this type of coupling (13). Recently, Kendall and Nahorski (35) proposed that 5-HT_2 receptors may be linked to adenylate cyclase as well, as based on the ability of GTP to

shift 5-HT-agonist competition curves of [^{3}H]spiperone binding to the right.

The presence of multiple states of the 5-HT_1 receptor has important implications for competition experiments carried out in the absence of GTP; namely, studies that attempt to correlate receptor binding with functional processes using [^{3}H]5-HT in the absence of GTP may be inaccurate (2–4). These studies assumed that [^{3}H]5-HT exhibits a single K_d (approximately 2 nM) in the absence of guanine nucleotides. However, the radioligand has different affinities for the multiple states of the receptor when GTP is not present. Since Michaelis-Menten kinetics is no longer followed, the IC_{50} values obtained may be erroneous.

Another implication of our results is that binding experiments conducted in the absence of GTP cannot distinguish between the presence of multiple states of the same receptor and/or multiple receptor subtypes. For example, other investigators have reported multiple components associated with the binding of [^{3}H]5-HT. Fillion *et al.* (36) found two components of binding to crude bovine brain membranes, one with an affinity of 1–3 nM and the other having an affinity of 10–30 nM. The same authors postulated later (37) that the high-affinity component was present in postsynaptic membranes, whereas the low-affinity component was located in glia. Monroe and Smith (30) showed that two binding sites for [^{3}H]5-HT were present in the spinal cord, with K_d values of 1.4 and 57.8 nM. Vandenberg *et al.* (38) examined solubilized 5-HT_1 receptors in bovine cortical membranes. They reported the presence of two components of binding in both a crude membrane preparation and a soluble/detergent-free fraction but not in the soluble fraction. The K_d values for the high- and low-affinity components were approximately 1.0 nM and 20 nM, respectively. Whether the high- and low-affinity binding components in these studies represent either receptor subtypes and/or multiple states of the 5-HT_1 receptor cannot be determined, since these studies were not carried out in the presence of GTP.

The results of the competition experiments illustrate how erroneous conclusions could be made if experiments are conducted in the absence of GTP. When the guanine nucleotide was not present, all three 5-HT agonists produced shallow inhibition curves of 15 nM [^{3}H]5-HT binding; this might be interpreted as evidence that these compounds are selective for 5-HT_1 receptor subtypes (see ref. 39). However, since only one component of binding is obtained in the presence of GTP, these compounds are actually nonselective for the subtypes of the 5-HT_1 receptor. Therefore, as previously described for the *beta*-adrenergic receptor (40), experiments carried out in the absence of GTP may lead to erroneous conclusions regarding the interactions of 5-HT agonists with the 5-HT_1 receptor.

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