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ACCELERATED COMMUNICATION

[^{3}H]-DOB(4-Bromo-2,5-Dimethoxyphenylisopropylamine) and [^{3}H] Ketanserin Label Two Affinity States of the Cloned Human 5-Hydroxytryptamine$_2$ Receptor

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SUMMARY

The binding properties of the 5-hydroxytryptamine$_2$ (5-HT$_2$) receptor have been the subject of much interest and debate in recent years. The hallucinogenic amphetamine derivative 4-bromo-2,5-dimethoxyphenylisopropylamine (DOB) has been shown to bind to a small number of binding sites with properties very similar to [^{3}H]ketanserin-labeled 5-HT$_2$ receptors, but with much higher agonist affinities. Some researchers have interpreted this as evidence for the existence of a new subtype of 5-HT$_2$ receptor (termed 5-HT$_{2A}$), whereas others have interpreted these data as indicative of agonist high affinity and agonist low affinity states for the 5-HT$_2$ receptor. In this investigation, a cDNA clone encoding the serotonin 5-HT$_2$ receptor was transiently transfected into monkey kidney Cos-7 cells and stably transfected into mouse fibroblast L-M(TK$^-$) cells. In both systems, expression of this single serotonin receptor cDNA led to the appearance of both [^{3}H]DOB and [^{3}H]ketanserin binding sites with properties that matched their binding characteristics in mammalian brain homogenates. Addition of guanosine 5′-(β,γ-imido)triphosphate [Gpp(NH)p] to this system caused a rightward shift and steepening of agonist competition curves for [^{3}H] ketanserin binding, converting a two-site binding curve to a single low affinity binding state. Gpp(NH)p addition also caused a 50% decrease in the number of high affinity [^{3}H]DOB binding sites, with no change in the dissociation constant of the remaining high affinity states. These data on a single human 5-HT$_2$ receptor cDNA expressed in two different transfection host cells indicate that [^{3}H]DOB and [^{3}H]ketanserin binding reside on the same gene product, apparently interacting with agonist and antagonist conformations of a single human 5-HT$_2$ receptor protein. These observations are consistent with the classical view of interconvertible agonist affinity states of GTP-binding protein-coupled receptors and strongly support the "two state" over the "two receptor" model for DOB binding to the 5-HT$_2$ receptor.

The 5-HT$_2$ receptor has been extensively studied by radioligand binding using the high affinity antagonist [^{3}H]ketanserin. Many investigators have observed that agonist competition curves for [^{3}H]ketanserin are shallow, indicating the presence of two binding sites for this radioligand. Some investigators have also reported that GTP and related analogs cause a steepening and rightward shift of this competition curve, indicating the conversion of some agonist high affinity states to the agonist low affinity conformation (1). These observations have led Titeler and colleagues (1, 2) to propose that these two binding sites represent agonist low and high affinity states of the 5-HT$_2$ receptor.

Subsequently, the amphetamine derivative DOB was shown to label a small number of binding sites with properties that matched the predicted properties of an agonist high affinity state of the 5-HT$_2$ receptor. This [^{3}H]DOB binding site displayed antagonist affinities very similar to those seen for [^{3}H] ketanserin binding, but with significantly higher affinities for agonists (2). These data appeared to provide evidence for the first selective ligand for the agonist high affinity state of the 5-HT$_2$ receptor and an indication that hallucinogenic actions of this and related compounds may be mediated by the 5-HT$_2$ receptor. In addition, this observation challenged the roots of the original classification scheme for serotonin receptors, which divided serotonin receptors into two subtypes (5-HT$_1$ and 5-HT$_2$) based on their affinities for the agonist 5-HT (3).

Several problems with this hypothesis were noted by Pierce and Peroutka (4), including the observation that the high affinity sites represented only a small fraction (5%) of the [^{3}H] ketanserin sites in all tissues examined and the fact that efficient interconversion of agonist and antagonist affinity states has not been demonstrated. Additionally, it was found

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; DOB, 4-Bro o-2,5-dimethoxyphenylisopropylamine; DOI, (±)-(2,5-dimethoxy)-4-iodoamphetamine, Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; NECA, 5′-*N*-ethylcarboxamidoadenosine; 8-OH-DPAT, 8-hydroxy-2-di-*N*-propylamino-tetralin; G protein, GTP-binding protein.

that certain tissues appeared to contain only low affinity DOB binding "sites" (4), even when a high specific activity radioligand was used (5). These observations were interpreted to mean that the high affinity agonist (DOB) binding site must represent a unique receptor subtype, which was termed the 5-HT$_{2A}$ receptor, whereas the low affinity site (labeled by [^{3}H]ketanserin but not by [^{3}H]DOB) was termed the 5-HT$_{2B}$ receptor (5).

In order to gain further insight into this issue, we have made use of defined transfection systems expressing a single serotonin receptor cDNA. We have cloned the human 5-HT$_2$ receptor and shown that it exhibits the ^{3}H antagonist binding profile of the 5-HT$_2$ receptor when assayed in a heterologous expression system (6, 7). Results presented here from studies of both agonist and antagonist binding and guanine nucleotide regulation strongly support the hypothesis that the 5-HT$_2$ receptor exists in two affinity states and that the binding site labeled by the agonist ([^{3}H]DOB) is on the same gene product as the site labeled by the antagonist ([^{3}H]ketanserin). A preliminary account of these findings has been previously presented (7).

Materials and Methods

Cloning and expression. A human 5-HT$_2$ cDNA was obtained by homology screening using a probe derived from the rat 5-HT$_{1C}$ receptor sequence, as described previously (6, 7). Of the numerous clones obtained, one cDNA clone contained the entire coding region. This cDNA was inserted into the vector pM05 for expression. This vector was constructed from the plasmids pcDV1 and pL1 (8) and contains the SV40 early promotor in addition to termination sites flanking the cDNA insert. To verify that the cDNA encoded a functional 5-HT$_2$ receptor, Cos-7 cells were transiently transfected using the DEAE dextran technique (9). Subsequently, stable cell lines were produced by cotransfection of the plasmid PM05 containing the 5-HT$_2$ gene with the plasmid PCCneo (which contains the aminoglycoside phosphotransferase gene) into murine fibroblast L-M(TK$^-$) cells (American Type Culture Collection). These stable transfections were accomplished using the calcium phosphate technique (Specialty Media, Inc., Lavellette, NJ). Cells were selected by resistance to the antibiotic G-418 (1 mg/ml; GIBCO) as previously described (10), and were screened for their ability to bind [^{3}H]ketanserin and [^{3}H]DOB.

Membrane preparation. Membranes were harvested from transfected cells by scraping of the cells from the culture dishes at full confluence, as previously described (10). These cells were homogenized by hand in a Wheaton tissue grinder and centrifuged at 200 × *g* for 5 min, followed by pelleting of the low speed supernatant at 40,000 × *g* for 20 min. All preparations were kept on ice and assays were run on the day on which the membranes were collected, because it was observed that the [^{3}H]DOB binding was adversely affected by freezing (manifested as a 50% reduction in the B_{max} for [^{3}H]DOB binding). Protein was determined by the method of Bradford (11).

[^{3}H]Ketanserin binding. Membranes were incubated according to a modification of the method of Lyon and colleagues (2). All assays were run in 96-well microtiter plates. Incubations were performed at 37° for 20 min in a solution containing buffer (50 mM Tris · HCl, 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbate, 10 μM pargyline, pH 7.6), 0.5 nM [^{3}H]ketanserin (64.9 Ci/mmol; DuPont-NEN, Wilmington, DE), 20–40 μg of protein, and drugs. The total reaction volume was 0.25 ml. The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters (presoaked with 0.5% polyethyleneamine, pH 7.4), using a Brandel 48R cell harvester (Brandel, Gaithersburg, MD). Filters were washed for 5 sec with iced buffer to reduce nonspecific binding. Dried filters were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting (Beckman LS 1701; Beckman Instruments, Fullerton, CA). Ready Organic (Beckman) was used as the scintillant and the counting efficiency was 50%. Nonspecific binding was defined by addition of 1 μM mianserin. Specific binding was 95% of total binding for the transiently transfected cells and 85% for the stable cell lines at 1 nM [^{3}H]ketanserin. All experiments were run in triplicate.

In order to determine the equilibrium binding constants, membranes were incubated with [^{3}H]ketanserin over the concentration range of 0.01–10 nM. Incubations were allowed to proceed for 60 min to ensure that equilibrium was achieved at the lowest concentrations of radioligand. Nonspecific binding was determined by addition of 1 μM mianserin. Data were analyzed by computer-assisted curve fitting (ACCUFIT and ACCUCOMP; Lundon Softward, Chagrin Falls, OH).

[^{3}H]DOB binding. Incubations with the agonist radioligand [^{3}H]DOB (20.8 Ci/mmol; Dupont-NEN, Wilmington, DE) were performed as described for [^{3}H]ketanserin. The radioligand concentrations used for saturation analysis spanned the range of 0.1–20 nM. In order to obtain an adequate signal, the protein concentration used was increased to 50–80 μg/well. These experiments were run in parallel with the ketanserin binding studies on the same day, using the same cell preparation. Specific binding was defined using 1 μM mianserin and was 70% of total binding at a 2 nM concentration of [^{3}H]DOB. Competition studies were run at a radioligand concentration of 2 nM [^{3}H]DOB.

Drugs. (+)-Butaclamol, (−)-butaclamol, (±)-DOI, 5-methoxytryptamine, 8-OH-DPAT bromide, carboxyamidotryptamine maleate, clonidine hydrochloride, cyproheptidine, dopamine hydrochloride, histamine dihydrochloride, isoproteronol bitartrate, mianserin hydrochloride, NECA, norepinephrine bitartrate, quipazine maleate, pindolol, ritanserin, spiperone, tryptamine hydrochloride, and zacopride bromide were purchased from Research Biochemicals, Inc. (Natick, MA). Atropine sulfate, Gpp(NH)p, pargyline hydrochloride, prazosin hydrochloride, and serotonin hydrochloride were purchased from the Sigma Chemical Company (St. Louis, MO).

Results

Both [^{3}H]ketanserin and [^{3}H]DOB exhibited saturable specific binding to transiently transfected monkey kidney cells (Cos-7) expressing the human 5-HT$_2$ receptor. The average density of [^{3}H]ketanserin sites was 1.29 pmol/mg of protein. [^{3}H]DOB also exhibited specific binding to these preparations but with a lower site density, averaging 28% of the [^{3}H]ketanserin binding sites.

To facilitate further characterization, stable cell lines were prepared by transfection of the human 5-HT$_2$ receptor into mouse fibroblast L-M(TK$^-$) cells, along with a selectable marker. Approximately 18% of the cell lines isolated by G-418 resistance displayed significant specific binding of the radioligands. In all clonally selected colonies that exhibited binding, both [^{3}H]ketanserin and [^{3}H]DOB binding sites were observed. For the three cell lines that expressed the 5-HT$_2$ receptor, those lines that displayed the lowest density of 5-HT$_2$ receptors had the highest apparent ratio of [^{3}H]DOB/[^{3}H]ketanserin binding sites. However, this could not be investigated systematically because the receptor expression levels were very low (0.02 pmol/mg of protein or less) in cell lines expressing the highest ratios. Control cells did not exhibit detectable [^{3}H]DOB or [^{3}H]ketanserin binding in either Cos-7 or L-M(TK$^-$) cells.

Pharmacological characterization of [^{3}H]ketanserin and [^{3}H]DOB binding was obtained from competition analysis of binding data on a single stable cell line (S-5). The affinity constants measured in cells containing the cloned human 5-HT$_2$ gene were very similar to values reported in the literature for native membranes (Table 1). The affinities of antagonists for the [^{3}H]ketanserin binding site are similar to their affinities at the [^{3}H]DOB site. However, as has been noted in native membranes (1, 2, 4), agonist competition of [^{3}H]ketanserin binding in the

TABLE 1

Competition experiments for the agonist- and antagonist-labeled human 5-HT$_2$ receptor stably transfected into L-M(TK$^-$) cells as compared with published values for the native rat 5-HT$_2$ receptor (2)

Apparent inhibition constants (K_i values) are reported and Hill slopes are listed parenthetically below each entry. Each value is the mean ± standard error for three independent trials run as paired controls in triplicate. Eleven concentrations of each competing drug were used and values for K_i were obtained by computer-assisted analysis (ACCUCOMP; Lundon Software). For antagonists, Hill coefficients were close to unity for both ^{3}H-agonist and ^{3}H-antagonist binding to the cloned human 5-HT$_2$ receptor. For agonists, the Hill coefficients for ^{3}H-agonist binding were significantly less than unity. Values are reported for the forced one-site model.

Displacer	[^{3}H]Ketanserin binding K_i (n_H)		[^{3}H]DOB binding K_i (n_H)	
	Human clone	Rat cortex[a]	Human clone	Rat cortex[a]
	nM		nM	
(±)DOI	38 ± 7.2 (0.67 ± 0.03)	41 ± 5[b] (0.85 ± 0.04)	1.58 ± 0.11 (1.1 ± 0.08)	0.79 ± 0.01 (0.9 ± 0.2)
5-HT	598 ± 52 (0.59 ± 0.02)	928 ± 67 (0.70 ± 0.02)	12 ± 1.7 (1.0 ± 0.05)	7.8 ± 0.8 (0.88 ± 0.1)
Tryptamine	3,134 ± 481 (0.83 ± 0.09)	2,005 ± 116 (0.81 ± 0.04)	90 ± 13 (0.93 ± 0.03)	48 ± 5 (1.09 ± 0.07)
(+)-Butaclamol	2.3 ± 0.38 (0.94 ± 0.14)	2.4 ± 0.05 (0.88 ± 0.01)	11 ± 1.4 (0.95 ± 0.08)	13 ± 2 (0.88 ± 0.03)
Spiperone	1.1 ± 0.07 (0.85 ± 0.08)	0.42 ± 0.04 (0.81 ± 0.05)	50 ± 5 (1.1 ± 0.21)	0.8 ± 0.04 (1.12 ± 0.5)
Isoproterenol	>10,000		>10,000	
NECA	>10,000		>10,000	
Prazosin	>10,000		>10,000	

[a] Ref. 2.
[b] A value for (±)DOB is presented; (±)DOI was not tested in this study.

stable cell line displays shallow competition curves with Hill numbers less than unity (Table 1).

Guanine nucleotide effects were assayed by measurement of agonist competition for [^{3}H]ketanserin binding in the presence and absence of a nonhydrolyzable analog of GTP, Gpp(NH)p (0.1 mM) (see Fig. 1 and Tables 2 and 3). In the absence of Gpp(NH)p, approximately 40% of the sites were determined to be in the high affinity state (Table 3). Addition of Gpp(NH)p resulted in a steepening of the slope and a rightward shift of the competition curves. After addition of this GTP analog, no high affinity sites were detected and the data were best fit by a one-site model with a Hill number close to unity. Furthermore, in the presence of Gpp(NH)p the number of low affinity sites was approximately equal to the sum of the agonist high affinity

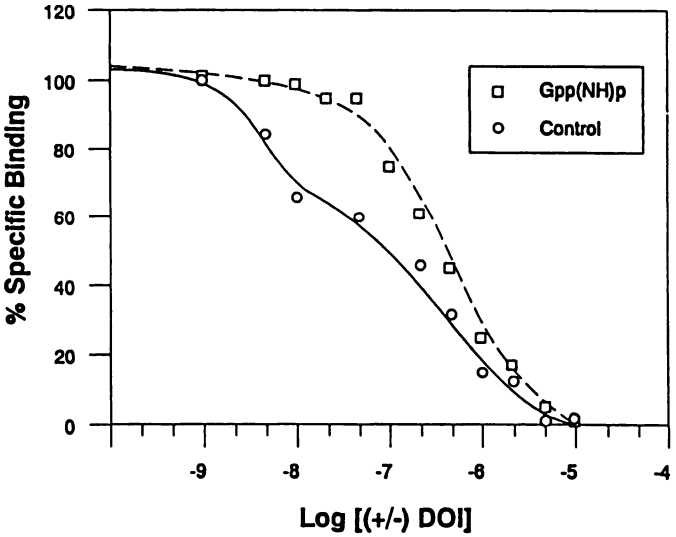


Fig. 1. Effect of Gpp(NH)p on DOI competition for [^{3}H]ketanserin binding to the cloned human 5-HT$_2$ receptor expressed in L-M(TK$^-$) cells. Before the addition of 100 μM Gpp(NH)p, the competition curve for DOI is biphasic. After addition, the slope of the competition curve is shifted to a lower affinity but steepened, consistent with a single population of noninteracting sites.

TABLE 2

Agonist competition experiments for the [^{3}H]ketanserin-labeled human 5-HT$_2$ receptor in L-M(TK$^-$) cells

Values were determined in the presence and absence of 100 μM Gpp(NH)p. Values are reported for the mean of three paired experiments run in triplicate. Eleven concentrations of competing agonist were used to construct competition curves. K_i values and standard errors are reported along with the computed Hill coefficients. For all three agonists, a statistically significant increase in Hill slope was observed after addition of Gpp(NH)p ($p < 0.01$).

Agonist	Control		plus Gpp(NH)p	
	K_i	n_H	K_i	n_H
	nM		nM	
(±)-DOI	38 ± 7.2	0.67 ± 0.3	72 ± 0.92	0.94 ± 0.2
Serotonin	598 ± 52	0.59 ± 0.02	1498 ± 244	0.93 ± 0.16
Tryptamine	3134 ± 481	0.83 ± 0.09	4992 ± 259	0.93 ± 0.09

TABLE 3

Effect of Gpp(NH)p (100 μM) on the number of sites in the high and low affinity state for each agonist

Experimental conditions were the same as described for Table 2. In the absence of Gpp(NH)p, a two-site model best fit the data; after addition, only the low affinity site was detected.

Agonist	Control		plus Gpp(NH)p, low affinity
	High affinity	Low affinity	
	fmol/mg of protein		
(±)-DOI	98 ± 23	134 ± 16	292 ± 59
Serotonin	90 ± 5.7	147 ± 11	222 ± 29
Tryptamine	110 ± 1.4	169 ± 82	236 ± 62

and agonist low affinity sites that existed in the absence of Gpp(NH)p (Table 3). Thus, it appears that Gpp(NH)p induces conversion of agonist high affinity to agonist low affinity states. Variations were seen in the degree of Gpp(NH)p-induced interconversion, but not in the ratios of high affinity to low affinity states, in different cell culture harvests.

Saturation binding studies were also performed to estimate the equilibrium dissociation constant and site density for each radioligand in the presence and absence of Gpp(NH)p. Experiments were run in parallel for the two radioligands so that an accurate estimate of the ratio of [^{3}H]DOB/[^{3}H]ketanserin sites

could be obtained. As shown in Table 4 and Fig. 2, the measured dissociation constant for [^{3}H]ketanserin binding was 0.31 nM. This value was not affected significantly by the addition of Gpp(NH)p. For [^{3}H]DOB, the measured dissociation constant was 1.95 nM. Those high affinity [^{3}H]DOB sites remaining after addition of Gpp(NH)p exhibited the same dissociation constant as that measured before guanine nucleotide addition. In the absence of Gpp(NH)p, the measured site density (B_{max}) for [^{3}H]ketanserin binding was 295 fmol/mg of protein and for [^{3}H]DOB binding was 131 fmol/mg of protein. Thus, the [^{3}H] DOB binding site represented approximately 50% of the [^{3}H] ketanserin binding sites. Addition of Gpp(NH)p caused a slight increase in [^{3}H]ketanserin binding site density (Table 4). In contrast, addition of Gpp(NH)p had a large effect on the density of [^{3}H]DOB binding sites, reducing them to 50% of their initial level. Thus, approximately half of the high affinity ^{3}H agonist binding sites of the 5-HT$_2$ receptor are sensitive to modulation by guanine nucleotides (Table 4).

Discussion

The exact nature of the DOB binding site in the brain has been controversial. Evidence has been presented that supports the assignment of the DOB site as the agonist high affinity state of the 5-HT$_2$ receptor (1, 2), in parallel with the model of GTP modulation of agonist binding to other G protein-coupled receptors (12–16). Contrasting evidence supports the view that [^{3}H]DOB labels a receptor subtype (5-HT$_{2A}$) that is related to the antagonist high affinity, agonist low affinity 5-HT$_2$ receptor (termed 5-HT$_{2B}$) but is a distinct new receptor protein (4, 5). Both models ("two state" and "two subtype") propose that [^{3}H] ketanserin can label both forms of the 5-HT$_2$ receptor, whereas [^{3}H]DOB labels only one.

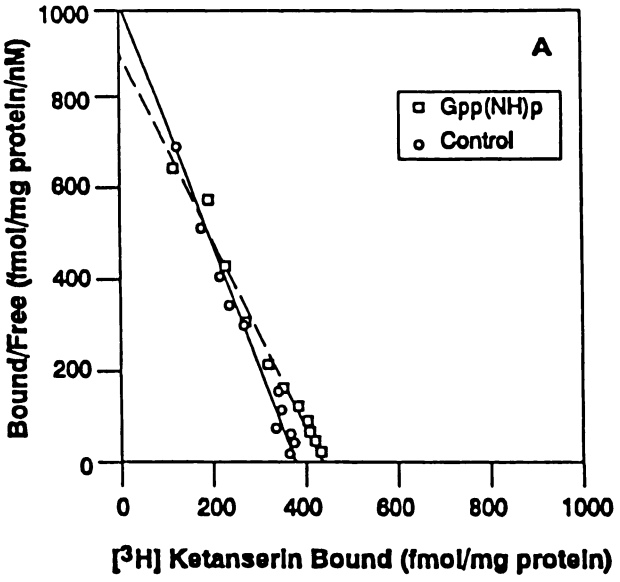
In order to achieve a clear resolution to this controversy, we decided to study the agonist and antagonist binding properties of a human 5-HT$_2$ receptor cDNA (6, 7) expressed in a mammalian cell line lacking other serotonin receptor subtypes. Because a cDNA clone containing a single open reading frame was used for these studies, only one primary transcript could

TABLE 4

Saturation binding experiments using [^{3}H]ketanserin and [^{3}H]DOB to label the transfected human 5-HT$_2$ receptor in L-M(TK$^-$) cells

Values for the dissociation constant (K_d) and site density (B_{max}) are reported with standard errors for each set of paired experiments. For each trial, membranes were derived from a pooled collection of stable transfectants that were harvested and assayed on the same day. Four saturation curves, each containing 12 different concentrations of radioligand, were run in parallel and each radioligand was tested in the presence and absence of Gpp(NH)p (100 μM). Every point was assayed in triplicate. Parameters were calculated by computer-assisted nonlinear regression analysis (ACCUFIT; London Software).

	[^{3}H]Ketanserin binding			
	K_d		B_{max}	
	Control	+Gpp(NH)p	Control	+Gpp(NH)p
	nM		fmol/mg	
Trial 1	0.24 ± 0.02	0.26 ± 0.06	230 ± 5	280 ± 15
Trial 2	0.38 ± 0.04	0.52 ± 0.06	360 ± 9	430 ± 11
	[^{3}H]DOB binding			
	K_d		B_{max}	
	Control	+Gpp(NH)p	Control	+Gpp(NH)p
	nM		fmol/mg	
Trial 1	2.0 ± 0.3	2.5 ± 0.2	113 ± 5	68 ± 2
Trial 2	1.9 ± 0.4	1.5 ± 0.1	150 ± 12	72 ± 5



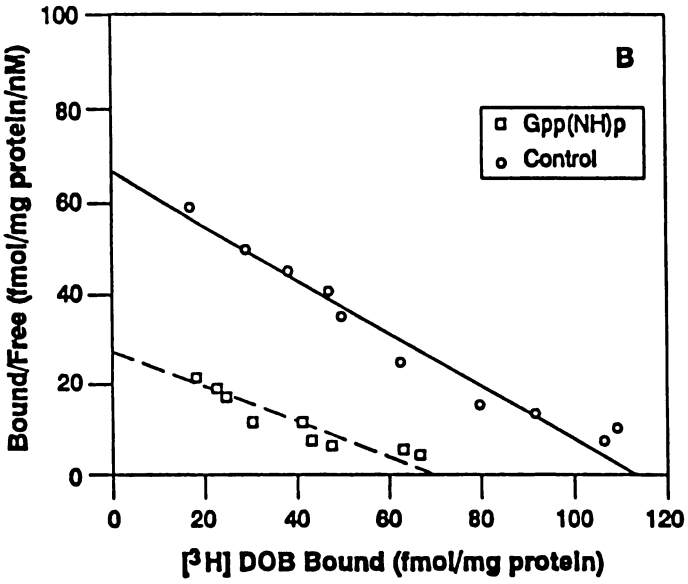


Fig. 2. Scatchard analyses of the binding of [^{3}H]ketanserin (A) and [^{3}H] DOB (B) to the cloned human 5-HT$_2$ receptor expressed in L-M(TK$^-$) cells. For each radioligand, full saturation curves were run in the presence and absence of 100 μM Gpp(NH)p as paired controls. Mianserin (1 μM) was used to define nonspecific binding. Al data points were run in triplicate.

be synthesized by the host cell (i.e., alternative splicing could not occur). Therefore, any binding properties measured in such a heterologous expression system must reside in the single protein product of the cDNA.

Transfection of the human 5-HT$_2$ cDNA into both Cos-7 (transient transfections) and L-M(TK$^-$) cells (stable transfections) produced a protein that was capable of binding both agonist and antagonist radioligands. The binding characteristics of the transfected human 5-HT$_2$ receptor for both [^{3}H]

DOB and [^{3}H]ketanserin were similar to those determined in native rat brain membranes (see Table 1), with certain exceptions that appear to be species specific (6). The discrepancy in the inhibition constant for spiperone in [^{3}H]DOB competition experiments (Table 1) may reflect such a species difference.

In native rat brain membranes, GTP and related analogs have been shown to inhibit high affinity [^{3}H]DOB binding (2). In addition, agonist competition of [^{3}H]ketanserin binding to rat brain membranes often exhibits shallow slopes with Hill coefficients significantly less than unity. In our transfected cells, which contain only one subtype of 5-HT$_2$ receptor, we have also observed shallow competition curves for agonist (but not antagonist) competition for the binding of [^{3}H]ketanserin. Addition of Gpp(NH)p, a nonhydrolyzable analog of GTP, caused these high affinity agonist binding sites to convert into low affinity sites. Besides demonstrating the interconversion of agonist binding states, these data indicate that [^{3}H]ketanserin is able to bind to both high and low affinity states of the cloned 5-HT$_2$ receptor. Gpp(NH)p was also shown to reduce the density of high affinity [^{3}H]DOB sites in our transfected cells. It is likely that Gpp(NH)p did not destroy [^{3}H]DOB binding sites but rather reduced their affinity to a value too low to measure. Thus, both 5-HT and DOB show a consistent reduction in high affinity agonist binding sites upon addition of Gpp(NH)p, which was shown in the case of 5-HT to result in a proportionate increase in the number of low affinity 5-HT binding sites. This conversion of high affinity agonist sites to low affinity sites fits the classical model for G protein modulation of agonist binding, in which guanine nucleotide binding uncouples the G protein from the high affinity state of the receptor, thus reducing its affinity for agonists such as 5-HT. Paradoxically, although apparently complete conversion of the high affinity [^{3}H]ketanserin binding site to the low affinity state was achieved with Gpp(NH)p, only 50% of the total [^{3}H]DOB binding sites were converted to the low affinity state with this reagent. We can only speculate that the presence of an antagonist in the assay (as the radioligand) may cause us to measure a different G protein coupling state than that which is seen in pure ^{3}H agonist binding assays.

The stable cell line chosen for this study (S-5) possessed approximately the same receptor density as that found in native rat brain membranes. However, the ratio of high affinity [^{3}H]DOB binding sites to total [^{3}H]ketanserin binding sites was found to be much higher than reported in rat brain (2). We observed that approximately 30% of transfected 5-HT$_2$ sites (labeled by [^{3}H]ketanserin) are in the agonist-labeled ([^{3}H]DOB) population, as compared with 5% in rat brain as reported by Lyon *et al.* (2) or 20% as reported by McKenna and Peroutka (5) for [^{125}I]DOI binding. It appears that the heterologous expression system used in the present study possesses a high degree of receptor-G protein coupling, as compared with native brain tissue. Nonetheless, not all of the [^{3}H]DOB binding sites are sensitive to the effects of guanine nucleotides. The average proportion of this "sensitive" population was 50% (range, 30–75%), which is much higher than the average sensitive population in normal rat brain tissue but lower than has been seen in some other transfected receptor assays (17). This implies that a certain fraction of binding sites for [^{3}H]DOB may be incapable of coupling to their G proteins in our transfection systems, due either to damage during the processing of these cells for the binding assays or to factors related to the proteins found in these cells.

Recent attempts have been made to explore the effect of receptor density on affinity states and function in cell lines containing cloned receptor genes. Using the M1 muscarinic receptor, for example, Mei *et al.* (17) have observed that the percentage of high affinity sites for the agonist carbachol decreased as the receptor density increased over a 22-fold range. Further, their observations were consistent with the hypothesis that the high affinity state of the receptor acted as the functional state. In contrast, Fargin *et al.* (18) failed to find a correlation between the 5-HT$_{1A}$ receptor density and the magnitude of the functional response. It is possible, however, that the lowest receptor density studied had already achieved a maximal response, perhaps because the G protein concentration might have been rate limiting. In the case of the 5-HT$_2$ receptor, we have observed an apparent inverse relationship between receptor density and proportion of receptors in the high affinity state. Furthermore, the rat 5-HT$_2$ receptor, when expressed at significantly higher levels than those reported here, displays an extremely low ratio of high to low affinity states.[1] The functional consequences of changes in both receptor density and the ratio of high to low affinity states on second messenger responses remain to be determined.

It is unclear how the agonist high affinity state relates to the functionally active form of G protein-coupled receptors. In a functional response system using smooth muscle contraction as a final index of receptor function, Kaumann (19) has proposed that the 5-HT$_2$ receptor exists in two interconvertible affinity states. In biochemical assays of both the 5-HT$_{1C}$ and 5-HT$_2$ receptors, the midpoint concentration for activation of phosphoinositide hydrolysis (EC_{50}) is similar to the midpoint concentration for agonist inhibition of antagonist binding (20). In the case of the 5-HT$_{1A}$ receptor, however, agonist binding exhibits a much higher apparent affinity than is observed in adenylate cyclase response experiments (21). In both response and radioligand binding studies, multiple binding states may interact in complex ways to yield the apparent midpoint concentration. Use of heterologous expression systems containing a single receptor subtype should facilitate the understanding of these complexities in future experiments.

In summary, we have shown that transfection of a single human 5-HT$_2$ receptor cDNA into two different host cell lines produces both [^{3}H]DOB and [^{3}H]ketanserin binding sites with properties similar to those of native mammalian brain membranes. These sites are capable of partial interconversion upon addition of Gpp(NH)p. Thus, these data indicate that both [^{3}H]DOB and [^{3}H]ketanserin bind to the same gene product and they strongly support the assignment of high affinity [^{3}H]DOB sites as the agonist high affinity binding state of the serotonin 5-HT$_2$ receptor. Although we cannot rule out the possibility that posttranslational modification may generate multiple products in our system, this seems unlikely to be able to explain the present data, because Gpp(NH)p interconversion of the agonist binding states has been shown. This study also serves to demonstrate the problems inherent in using agonist binding to classify serotonin receptor subtypes. An alternative classification scheme based on serotonin receptor genes provides an unambiguous alternative to pharmacological schemes

[1] Titeler, personal communication.

based on complex, and poorly understood, native animal tissues (22). Finally, it should be noted that the present data do not rule out the possibility that agonist and antagonist binding sites may represent partially overlapping or completely separate binding domains on the same receptor protein. Future site-directed mutagenesis studies, in conjunction with detailed response characterizations, will provide a much clearer view of these interactions.

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