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High Affinity Agonist Binding to Cloned 5-Hydroxytryptamine$_2$ Receptors Is not Sensitive to GTP Analogs

FRANCIS G. SZELE and DOLAN B. PRITCHETT

Neuroscience Research, Children's Seashore House (D.B.P.), and Departments of Pediatrics and Pharmacology (F.G.S., D.B.P.), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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SUMMARY

Agonists for GTP-binding protein (G protein)-coupled receptors are thought to bind with high affinity to the complex of receptor and G protein. Nonhydrolyzable GTP analogs have been shown to disrupt this complex and reduce the binding affinity for many agonists. Antagonists are thought to bind to the receptor whether or not it is coupled to the G protein, and therefore binding remains unchanged in the presence of GTP analogs. The binding of the serotonin 5-hydroxytryptamine (5-HT)$_2$ receptor agonists serotonin (5-HT) and 4-bromo-2,5-dimethoxyphenylisopropylamine is not affected by the presence of GTP analogs when the cloned 5-HT$_2$ receptor is expressed in the 293 human embryonic kidney cell line. The same receptor expressed in mouse NIH3T3 cells is partially sensitive to GTP analogs. Both cell lines have similar proportions of agonist and antagonist binding sites, and agonist stimulation of both cell lines leads to a robust increase in phosphoinositide hydrolysis. Differences in GTP metabolism in 293 cells is not likely to be the cause of the observed difference in inhibition of agonist binding, because the cloned 5-HT$_{1A}$ serotonin receptor expressed in these cells is sensitive to GTP analogs. The GTP-insensitive agonist binding is best explained by the existence of a G protein-receptor complex in 293 cells that is not sensitive to GTP analogs. Such a G protein-receptor complex may explain the fraction of agonist binding in the brain that is not sensitive to GTP analogs.

Receptors for many neurotransmitters and hormones are related in structure and mechanism of action. The transmembrane receptor binds the ligand extracellulary and undergoes a structural transformation that allows the receptor to interact with a G protein complex on the inside of the cell (1). The interaction of the receptor and the G protein leads to the dissociation of the G protein α subunit from the $\alpha\beta\gamma$ trimeric complex (2). The dissociated α subunit of the G protein then stimulates intracellular enzymes that control levels of second messages such as cAMP and PI (1). The general pattern of this mechanism is understandable in light of the structural conservation of the receptors revealed by molecular cloning experiments. Receptors for ligands as diverse as glycoprotein hormones and amino acid metabolites share a common structural motif of seven hydrophobic segments. This motif is seen in rhodopsins, proteins in which these seven hydrophobic segments have been shown to span the membrane. Therefore, a similar seven-transmembrane region structure has been proposed for all receptors containing the seven hydrophobic segments (3).

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Agonist ligands for these receptors are thought to bind with high affinity to the protein complex of receptor and G protein (2). Two types of experiments support this hypothesis. First, agonists in many systems compete for antagonist binding with Hill slopes less than 1. This suggests at least two affinities of the agonist ligand for the receptor. The addition of nonhydrolyzable GTP analogs such as Gpp(NH)p and GTPγS changes the Hill slope to 1 by eliminating the high affinity binding component. Second, radioligands with high affinity are available for many receptors, and the binding of these radiolabeled agonists is blocked in a concentration-dependent manner by GTP analogs. This inhibition often reaches a plateau before all the specific agonist binding is displaced. Such GTP-displaceable binding has been demonstrated for several serotonin receptors in brain tissue (4, 5).

Many serotonin receptor subtypes have been described pharmacologically (6), and many of these subtypes have been cloned (7). These structurally related receptors are members of the G protein-coupled receptor family. The 5-HT$_2$ and the 5-HT$_{1C}$ receptors have similar (70%) amino acid sequences (8, 9), whereas the 5-HT$_{1A}$ and 5-HT$_{1D}$ receptors are more distantly related (10, 11). This is consistent with the second messenger-coupling profile of these receptors. The 5-HT$_2$ and 5-HT$_{1C}$

ABBREVIATIONS: G protein, GTP-binding protein; 5-HT, 5-hydroxytryptamine; DOB, (±)-4-bromo-2,5-dimethoxyphenylisopropylamine; 8-OH-DPAT, 8-hydroxydipropylaminotetralin; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; PI, phosphoinositide(s).

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receptors are both coupled to PI metabolism (12, 13), whereas the 5-HT_{1A} and 5-HT_{1D} receptors both inhibit cAMP production (11, 14). [^{3}H]DOB is a high affinity agonist for the 5-HT_2 receptor (4), and [^{3}H]8-OH-DPAT is a high affinity agonist for the 5-HT_{1A} receptor (5). A wide variety of compounds are available that distinguish the receptor subtypes (15). It has also been hypothesized that there are subtypes of 5-HT_2 receptors. This hypothesis was based on the observation of differences between the levels of agonist and antagonist binding in rat and bovine brain (16, 17). Others have questioned the existence of such subtypes because they have shown that different levels of agonist and antagonist binding are observed when only a single receptor subtype is expressed in transfected cells (18, 19).

The 5-HT_2 receptor was cloned on the basis of its homology to the 5-HT_{1C} receptor and was shown to have a pharmacological profile similar to that of the native receptor when expressed in transfected human kidney 293 cells (8) or mouse NIH3T3 cells (20). Like the native receptor, the cloned receptor was also shown to efficiently couple to the PI second messenger pathway (8). Others have subsequently shown that radiolabeled agonists bind to the receptor and that this binding is sensitive to inhibition by GTP analogs, although not to the same degree observed using brain membranes (18). Less than 50% of the agonist binding sites can be displaced by GTP analogs when cloned 5-HT_2 receptors are expressed in mouse L, NIH3T3, or COS cells (18, 19). Our original results showed little if any high affinity serotonin binding sites in 293 cells (8). Muscarinic acetylcholine receptors have also been expressed in 293 cells and have been shown to be functionally coupled to the PI pathway, but no effect of GTP analogs on agonist carbachol displacement of *N*-[^{3}H]methylscopolamine antagonist binding was seen (21).

Recently a G protein α subunit has been purified based on its relative insensitivity to nonhydrolyzable GTP analogs. This subunit can be purified from other G protein α subunits with a $\beta\gamma$ subunit affinity column (22). The other α subunits elute from such a column after the addition of GTPγS, and the G_q protein is retained. In reconstitution experiments the G_q subunit has recently been shown to stimulate the activity of the phospholipase C β1 isozyme (23, 24) and to couple the muscarinic M1 receptor to phospholipase C (25). This suggests that G_q or closely related α subunits such as G_{11} are the G proteins that are responsible for stimulation of the PI pathway. It has been suggested that *in vivo* the low level of GTP hydrolysis of G_q is stimulated by some other protein (24). However, no such protein has yet been described.

Materials and Methods

Pharmacological agents were obtained from Janssen Pharmaceutica (Beerse, Belgium) or Research Biochemicals International (Bethesda, MD). Radioligands were purchased from NEN DuPont (Wilmington, DE). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Merck (Rahway, NJ).

Human embryonic kidney 293 cells (American Type Culture Collection) were grown in minimum essential medium supplemented with 10% fetal calf serum. Cells were plated at 1–3 × 10^6 cells/10-cm plate and were used for transfection when 50% confluent. The entire coding region of the cloned 5-HT_2 receptor (8), including the extended amino terminus (20), was cloned into the *Hpa*I restriction site of an expression vector plasmid (8). DNA of this construct was prepared and purified by equilibrium centrifugation banding on a cesium chloride gradient. The purified DNA was used to transfect exponentially growing 293 cells, using the calcium phosphate method as modified by Chen and Okayama (26). The precipitate was washed off after 20–24 hr and fresh growth medium was added. Cells were harvested the next day for transient expression experiments. Stable cell lines expressing 5-HT_2 receptors were selected by coexpression of a neomycin resistance gene. Resistant colonies were picked and grown as stable lines. Lines were tested for expression of ketanserin binding sites.

Cells were harvested by centrifugation after aspiration in cold phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. The cell pellet was frozen at −70°. Membranes were prepared by washing twice with cold 10 mM potassium phosphate buffer, pH 7.2. Briefly, cells were homogenized at medium speed with a Brinkmann homogenizer, centrifuged for 10 min at 37,500 × *g*, and resuspended in either 10 mM Tris, pH 7.5 (for [^{3}H] ketanserin binding), or 10 mM Tris, pH 7.5, containing 0.5 mM EDTA, 10 mM $MgCl_2$, 0.1% ascorbate, and 1 × 10^{-5} M pargyline (for [^{3}H]DOB and [^{3}H]8–0H-DPAT binding). Final protein concentrations ranged from 291 to 368 μg/assay tube for [^{3}H]ketanserin binding and from 26 to 141 μg/assay tube for [^{3}H]DOB binding. The assay volume was 1.0 ml and contained 0.1 ml of [^{3}H]DOB (0.03–6.0 nM), [^{3}H]ketanserin (0.02–6.0 nM), or [^{3}H]8–0H-DPAT (0.02–6.0 nM) and 0.1 ml of 1 × 10^{-6} M spiperone or 1 × 10^{-3} M 5-HT, or assay buffer. After a 45-min incubation at room temperature the duplicate samples were suction-filtered rapidly over Schleicher and Schuell no. 32 filters and washed three times with cold 10 mM potassium phosphate buffer, pH 7.2. The radioactivity in the samples was determined and the appropriate data were transformed by the method of Scatchard (27).

Stimulation of PI metabolism was measured as described by Littman *et al.* (28). Briefly, cells were incubated overnight with *myo*-[^{3}H]inositol. Unincorporated *myo*-[^{3}H]inositol was washed out by changing the cell growth medium. Lithium chloride was added to block degradation of inositol monophosphate into free inositol and phosphate. Cells were stimulated with the agonists at 37° for 30 min, after which perchloric acid was added to stop further metabolism. After neutralization with KOH the inositol phosphates were separated from *myo*-[^{3}H]inositol by ion exchange chromatography. The inositol phosphates inositol monophosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-triphosphate have been shown, using radiolabeled standards, to elute in the last wash in this procedure. Radioactivity in samples was determined by scintillation counting. The results were normalized to the amount of incorporated *myo*-[^{3}H]inositol.

Results

To investigate agonist binding to 5-HT_2 receptors expressed in human embryonic kidney 293 cells, the cells were stably transfected with a full length clone for the receptor expressed under the control of the cytomegalovirus promoter. Transient expression of receptors in these cells produced approximately 1 pmol of antagonist binding sites/mg of protein. Several stably transfected cell lines produce similar numbers of receptors (data not shown). One line was chosen for further study. Equilibrium binding studies (Fig. 1) with this cell line showed saturable binding of the 5-HT_2 antagonist [^{3}H]ketanserin. Scatchard transformation of the data showed that the cells produced 657 ± 80 fmol of binding sites/mg of protein. The affinity of [^{3}H]ketanserin for the receptors was 0.4 nM, similar to the reports of others using transfected cell membranes or brain membranes (4). Similar equilibrium binding experiments were performed using the serotonin agonist [^{3}H]DOB (Fig. 1B). These experiments detected far fewer agonist binding sites. Scatchard transformation of the data showed that 77 ± 22 fmol of binding sites/mg of membrane protein were present in the same membrane preparation used above for ketanserin binding. The 0.5 nM affinity of this binding is similar to that reported by others (4). The average of multiple membrane preparations

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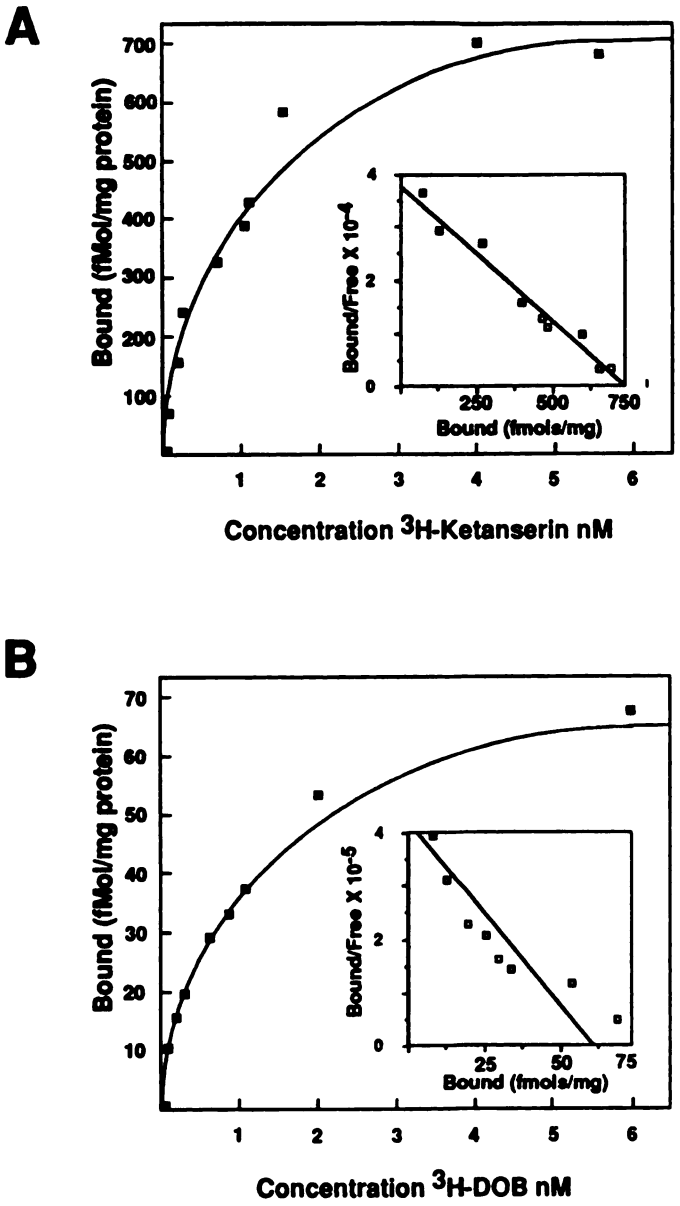

Fig. 1. Scatchard analyses of [^{3}H]ketanserin-labeled (A) and [^{3}H]DOB-labeled (B) 5-HT$_2$ receptors in transiently transfected 293 cell homogenates. Cinanserin (1 μM) was used to define nonspecific binding. The Scatchard plots shown are representative of more than five experiments. B_{max} values differed depending on the efficiency of transfection, so comparisons between [^{3}H]DOB and [^{3}H]ketanserin binding were done using cell membranes from the same transfection. *Points* are averages of triplicate determinations, and the standard error was <10%.

showed that agonist sites were consistently present at 14 ± 3% of the level of antagonist binding sites. This ratio of agonist to antagonist binding sites is higher than that observed in the rat brain membrane preparations (4). Those studies reported that levels of agonist sites were 5% of those of antagonist sites. Similar or higher ratios have been reported in other 5-HT$_2$ receptor-transfected cell lines (18, 19).

To test whether these agonist binding sites were sensitive to GTP and its analogs, Gpp(NH)p or GTPγS was added to the DOB binding assay at concentrations from 1 nM to 100 mM. At a concentration of DOB equivalent to the K_d value, <5% of the agonist binding was inhibited and there was no concentration dependence of the effect (Fig. 2). Similar experiments using brain membranes prepared in exactly the same manner as the cell membranes showed a GTPγS concentration-dependent decrease in [^{3}H]DOB binding (Fig. 2). In agreement with the results of others (18), membranes prepared from NIH3T3 cells expressing the same receptor clone displayed DOB binding, 30% of which could be displaced in a dose-dependent manner by Gpp(NH)p (data not shown). Lower concentrations of [^{3}H]DOB in the binding assay would produce a greater percentage of binding inhibition by Gpp(NH)p. Using concentrations of DOB up to 4-fold below the K_d still produced <5% Gpp(NH)p inhibition of DOB binding (Fig. 3).

There is evidence that DOB is not a full agonist for the 5-HT$_2$ receptor. Although this would not explain the differences described above, it was of interest to examine the native agonist, serotonin, to ensure that the findings were not limited to partial agonists. To test whether Gpp(NH)p could influence serotonin displacement of antagonist binding, a competition binding ex-

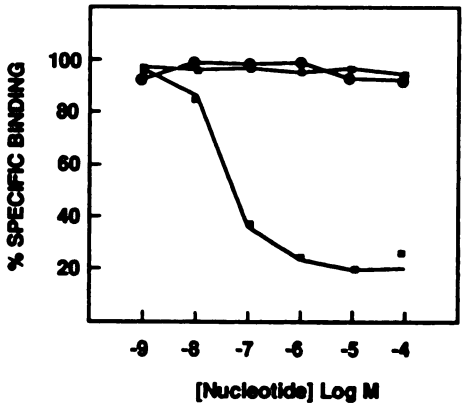

Fig. 2. Effect of GTP and GTPγS on [^{3}H]DOB binding to membrane homogenates from transfected 293 cells and rat brain. Membranes from transfected cells were incubated with the indicated concentration of GTP (●) or GTPγS (■) and rat membranes were incubated with GTPγS (□). Membrane preparation and binding assays were exactly the same for each tissue. [^{3}H]DOB was used at a concentration of 0.5 nM, approximately the K_d value. Cinanserin (1 μM) was used to define nonspecific binding. *Points* are averages of triplicate determinations, and the standard error was <10%.

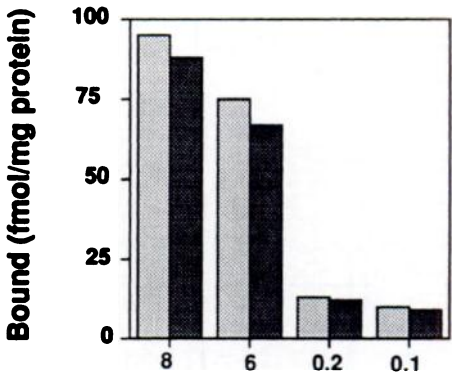

Fig. 3. Lack of a dependence of Gpp(NH)p effects on [^{3}H]DOB concentration. Gpp(NH)p (■) at a concentration of 10^{-4} M was included in all samples containing the indicated concentrations of [^{3}H]DOB. The *bars* are averages of triplicate determinations, and the standard error was <10%.

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periment was performed in the presence and absence of Gpp(NH)p. No difference in the displacement of ketanserin by serotonin was detected (Fig. 4A). This is different from the effect seen using brain membranes (Fig. 4B) (29) and reports of others using transfected mouse L cells (18). It is also important to note that serotonin inhibited the binding of [^{3}H]DOB at a lower concentration than that at which it inhibited [^{3}H] ketanserin binding (Fig. 5). The IC_{50} for inhibition of [^{3}H]DOB binding was 20 nM, whereas the IC_{50} for inhibition of [^{3}H] ketanserin binding was 560 nM. These results are similar to those obtained using rat frontal cortex (4) and further demonstrate that the serotonin binding in these cells is to an agonist-binding conformation of the receptor similar to that measured in brain tissue.

The previous results could be explained as a difference in the metabolism of GTP analogs by 293 cells. The GTP analogs could be inactivated by some modification or sequestered such that they are not able to compete with GDP bound to the G protein-receptor subunit complex. To test this hypothesis, 293 cells were transfected with 5-HT_{1A} receptor DNA together with 5-HT_2 receptor DNA. Membranes from these transfected cells were prepared as were those used in the [^{3}H]DOB binding experiments. The 5-HT_{1A} agonist [^{3}H]8-OH-DPAT was incubated with the membranes in the presence and absence of varying concentrations of Gpp(NH)p. A clear concentration-dependent decrease in [^{3}H]8-OH-DPAT was observed (Fig. 6). Such decreases have been well documented for [^{3}H]8-OH-DPAT binding in rat brain membranes (5). The effect of Gpp(NH)p on [^{3}H]8–OH-DPAT binding suggests that Gpp(NH)p is not inactivated or sequestered by 293 cells and that this GTP analog can displace GDP from a G protein α subunit, presumably G_i in this case. No effect of Gpp(NH)p on [^{3}H]DOB binding to the 5-HT_2 receptors expressed in these same membranes was seen (Fig. 6). ATP concentrations as high as 10^{-3} M had no effect on [^{3}H]DOB binding to either brain membranes or transfected cell membranes.

The functional coupling of 5-HT_2 receptors expressed in 293 cells has been described previously; however, serotonin was the only agonist used (8). To confirm that DOB could produce stimulation of the PI pathway in transfected 293 cells, various concentrations of DOB were added to cells that had been previously metabolically labeled with *myo*-[^{3}H]inositol. Like serotonin, DOB produced a robust stimulation of PI turnover in the transfected cells (Fig. 7). The results indicate that the magnitude of the response is close to that produced by serotonin.

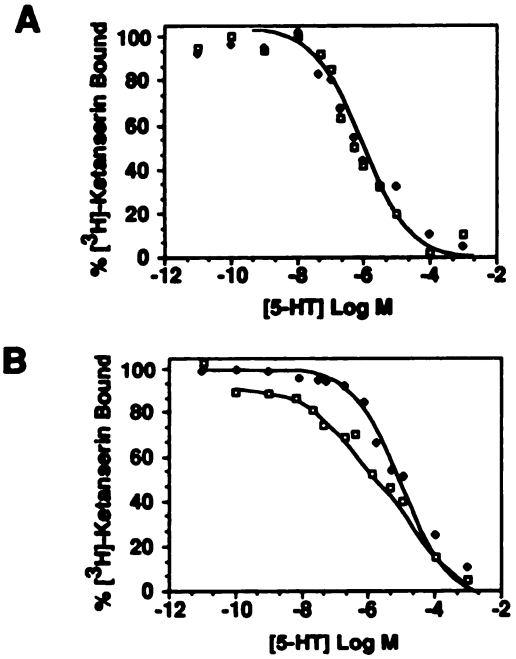

Fig. 4. Effects of GTP analogs on serotonin binding. Serotonin displacement of [^{3}H]ketanserin binding to transfected 293 cell membranes was not affected by the addition of Gpp(NH)p at 10^{-4} M (A). Brain membranes prepared in exactly the same way were sensitive to the effects of Gpp(NH)p (B). *Points* are averages of triplicate determinations, and the standard error was <10%.

Discussion

The major finding of this study is that 5-HT_2 receptor agonist binding sites are not sensitive to GTP analogs when the receptors are expressed in 293 cells. Control experiments show this phenomenon to be specific for this particular receptor in this

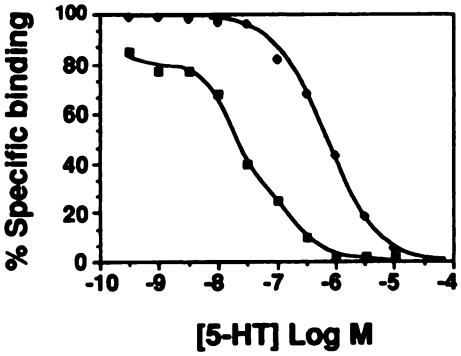

Fig. 5. Serotonin displacement of [^{3}H]ketanserin and [^{3}H]DOB binding to transfected 293 cell membranes. Serotonin displaced [^{3}H]DOB (0.5 nM) binding at a lower concentration (⊡) than that at which it displaced [^{3}H] ketanserin (0.5 nM) binding (♦). The *curve* shown for [^{3}H]ketanserin binding is a theoretical curve generated assuming a single binding site. The *curve* for [^{3}H]DOB is a computer-generated fit to the points, not constrained to 100% binding. *Points* are averages of triplicate determinations, and the standard error was <10%.

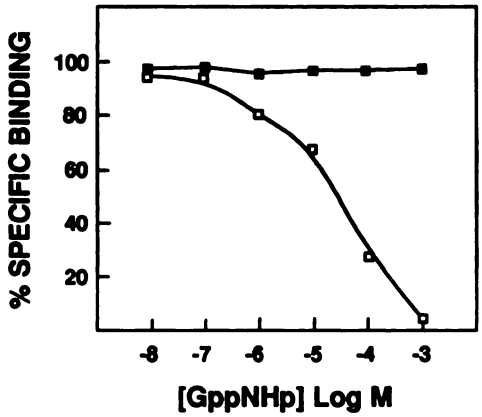

Fig. 6. Effect of Gpp(NH)p on [^{3}H]8-OH-DPAT and [^{3}H]DOB binding. Binding to membranes prepared from 293 cells transfected with 5-HT_{1A} together with 5-HT_2 DNA was measured. [^{3}H]8-OH-DPAT (□) or [^{3}H] DOB (■) was present at a concentration of 0.5 nM and Gpp(NH)p was included at the concentrations shown. *Points* are averages of triplicate determinations, and the standard error was <10%.

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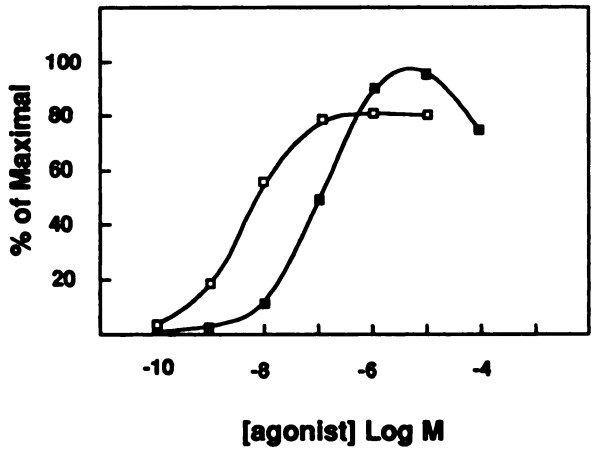

Fig. 7. Stimulation of inositol phosphate production by serotonin (■) and DOB (□). The levels of radiolabeled inositol monophosphate, inositol bisphosphate, and inositol trisphosphate were determined by scintillation counting after separation from *myo*-inositol by column chromatography. After maximal stimulation inositol monophosphate levels were 30% of the incorporated *myo*-inositol levels, and this was defined as 100% stimulation. *Points* are averages of triplicate determinations, and the standard error was <15%.

cell type. Other serotonin receptors expressed in these cells are sensitive to GTP analogs. Agonist binding to the same cloned receptors expressed in other cells is also sensitive to GTP analogs. These observations can be explained by the presence of a G protein in 293 cells that couples to the 5-HT_2 receptor and produces agonist binding. This postulated G protein must be insensitive to GTP because the addition of GTP analogs fails to disrupt the interaction between the receptor and the G protein. The failure to disrupt this complex allows a portion of the receptors to remain in the agonist-binding conformation. Such GTP-insensitive G proteins are not unexpected, based on work isolating G protein α subunits that have been shown to couple to PI metabolism.

The observation that the maximum number of binding sites for agonists is substantially less than the number of antagonist binding sites suggests that only a fraction of the receptors are in a conformation that can bind agonist. There are several possible explanations for this result. First, the receptor could contain multiple antagonist binding sites and only one agonist binding site. This is unlikely, given the varying ratios of agonist to antagonist binding seen in different systems. One would expect the ratio to be fixed for a particular receptor. Another possible explanation is that only a fraction of the receptors are processed or assembled such that they can bind agonists. It seems unlikely that 95% of the receptors in the brain would be incapable of binding agonist due to improper synthesis. It also seems unlikely that far higher percentages of receptors would be correctly processed in heterologous cell expression systems such as the one described here. It is also possible that only a fraction of the receptors are in a subcellular environment that can be accessed by agonists. This is also unlikely, given that Gpp(NH)p inhibition of agonist binding occurs in solubilized membranes. Possibly agonists can bind to receptors in the absence of G proteins. This idea is supported by experiments in which serotonin receptors are expressed in bacteria, which are not thought to express G proteins capable of interacting with mammalian receptors. Agonist binding has been detected in such systems (30). If such binding can indeed take place, it then becomes unclear why GTP analogs disrupt agonist binding in systems with G proteins. Although any of these possibilities could explain the data presented here, the simplest explanation is that agonists bind only to the fraction of receptors that are bound to a G protein and that some G proteins are insensitive to GTP analogs.

An important finding shown here is that the fraction of agonist binding sites is higher in the 293 cell expression system than in the brain. This suggests that 293 cells do not lack the correct G proteins to couple to the receptor. In fact, our results would suggest that a higher fraction of receptors are coupled to a G protein in the 293 cells than in brain tissue. Other transfected cell lines have even higher fractions of receptors coupled to G proteins (18, 19). There have been suggestions that cells expressing lower numbers of receptors have higher fractions of receptors coupled to G proteins, as expected. However, the brain has been reported to have the lowest percentage of coupled receptors, as measured by agonist DOB binding. Therefore, efforts to achieve higher levels of coupled receptors may be directed toward a less physiologically relevant situation.

The most surprising result presented here is that the levels of agonist binding sites in transfected 293 cells are not reduced by nonhydrolyzable GTP analogs. If, as presented above, the agonist binds to a receptor-G protein complex, why is this complex not disrupted by GTP analogs? There are several possible technical explanations for our findings. These can be ruled out based on the data shown here. Both Gpp(NH)p and GTPγS are active in membranes prepared either from brain or from other transfected cells. Because the membranes were prepared at the same time and by the same procedure, it seems very unlikely that the differences observed are artifacts of our compounds or membrane preparation. Differences in the total numbers of binding sites in each preparation are not a possible explanation for the differences because the NIH3T3 cells and 293 cells produce similar numbers of antagonist binding sites. It is conceivable that the 293 cells may inactivate or metabolize GTP analogs. However, effects using two different nonhydrolyzable GTP analogs, Gpp(NH)p and GTPγS, were observed. Moreover, the strongest argument against the 293 cells having a way to inactivate GTP analogs is that agonist binding to 5-HT_{1A} receptors expressed in the same cell line is inhibited by GTP analogs. In fact, Gpp(NH)p inhibits [^{3}H]8-OH-DPAT binding and not [^{3}H]DOB binding in membranes prepared from cells expressing both receptor types after transient cotransfection. This essentially eliminates inactivation of GTP analogs by 293 cells as a possible explanation.

The data presented here, compared with those of others, demonstrate that there is no correlation between the fraction of agonist binding and sensitivity to inhibition by GTP analogs. In the brain, where levels of agonist binding sites are 5% of those of antagonist binding sites, a maximum of 70% of those agonist binding sites can be displaced (4). In transfected COS-7 cells, agonist binds to 50% of the receptors measured by antagonist binding and 35% of those sites are displaced by GTP analogs. In 293 cells, the levels of agonist binding sites are 14% of the levels of antagonist binding sites and <5% of those sites can be inhibited by GTP analogs.

The best explanation for the data presented here is the paradoxical one that there is a G protein that interacts with the serotonin receptor in these cells that is not sensitive to GTP. There is evidence for a G protein α subunit that is

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relatively insensitive to GTP analogs due to the slow rate of GDP-GTP exchange. Interestingly, this subunit, G_q, has been shown to be capable of stimulating phospholipase C, thereby increasing the production of inositol phosphates (22). This is the same pathway stimulated by the 5-HT_2 receptors in 293 cells. This would suggest that G_q or a G protein like G_q interacts with the 5-HT_2 receptors in 293 cells.

It has been suggested that the slow rate of GDP-GTP exchange by G_q observed *in vitro* would be stimulated *in vivo* by a GTPase-activating protein analogous to the GTPase-activating protein that stimulates the GDP-GTP exchange activity of the oncogene *ras*. It may be that such activity is low in 293 cells. However, this activity would have to be specific for the G protein interacting with 5-HT_2 receptors, because 5-HT_{1A} receptor agonist binding is GTP sensitive. Because there is no direct evidence for the existence of such a GDP-GTP exchange-stimulating protein, it may be that there are proteins like G_q that are structurally similar to G proteins but are not sensitive to GTP as classically measured. These proteins may exchange GDP and GTP at a very slow rate *in vivo*. The existence of such proteins is not likely to be limited solely to transformed cell lines. It seems likely that the component of agonist binding remaining in brain tissue after maximal inhibition by GTP is a result of receptor bound to G proteins that are not sensitive to GTP under the conditions normally used.

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Send reprint requests to: Dolan B. Pritchett, Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

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