0026-895X/90/050594-05$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 38:594-598

MOL PHARM

ACCELERATED COMMUNICATION

4-[^{125}I]Iodo-(2,5-dimethoxy)phenylisopropylamine and [^{3}H]Ketanserin Labeling of 5-Hydroxytryptamine$_2$ (5HT$_2$) Receptors in Mammalian Cells Transfected with a Rat 5HT$_2$ cDNA: Evidence for Multiple States and Not Multiple 5HT$_2$ Receptor Subtypes

MILT TEITLER,[1] SIGRUN LEONHARDT, ELLEN L. WEISBERG, and BETH J. HOFFMAN

Department of Pharmacology and Toxicology, Albany Medical College, Albany, New York 12208 (M.T., S.L., E.L.W.), and Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland (B.J.H.)

Received May 1, 1990; Accepted August 6, 1990

SUMMARY

Evidence has accumulated indicating that the radioactive hallucinogens 4-bromo-[^{3}H](2,5-dimethoxy)phenylisopropylamine ([^{3}H]DOB) and 4-[^{125}I]iodo-(2,5-dimethoxy)phenylisopropylamine ([^{125}I]DOI) label an agonist high affinity state of the 5-hydroxytryptamine$_2$ (5HT$_2$) receptor and [^{3}H]ketanserin labels both agonist high and low affinity states. Recently, an alternative hypothesis has been put forward proposing that the radioactive hallucinogens are labeling a 5HT$_2$ receptor subtype distinct from the receptor labeled by [^{3}H]ketanserin. In order to provide definitive evidence as to which of these hypotheses is correct, the rat 5HT$_2$ receptor gene was transfected into NIH-3T3 (mammalian fibroblast) cells and COS (green monkey kidney) cells. Neither nontransfected cell type expresses 5HT$_2$ receptors; the transfected cells expressed high affinity binding sites for both [^{125}I] DOI (K_D = 0.8 nM and B_{max} = 363 fmol/mg in NIH-3T3 cells; K_D = 0.2 nM and B_{max} = 26 fmol/mg in COS cells) and [^{3}H]ketanserin (K_D = 0.4 nM and B_{max} = 5034 fmol/mg in NIH-3T3 cells; K_D = 1.0 nM and B_{max} = 432 fmol/mg in COS cells). The affinities of agonists and antagonists for the [^{125}I]DOI-labeled receptor were significantly higher than for the [^{3}H]ketanserin-labeled receptor. The affinities of agonists and antagonists for these binding sites were essentially identical to their affinities for the sites radiolabeled by these radioligands in mammalian brain homogenates. The [^{125}I]DOI binding was guanyl nucleotide sensitive, indicating a coupling to a GTP-binding protein. These data indicate that the 5HT$_2$ receptor gene product contains both the guanyl nucleotide-sensitive [^{125}I]DOI binding site and the [^{3}H]ketanserin binding site. Therefore, these data indicate that the 5HT$_2$ receptor gene product can produce a high affinity binding site for the phenylisopropylamine hallucinogen agonists as well as for the 5HT$_2$ receptor antagonists. These results strongly support the two-state hypothesis for the 5HT$_2$ receptor and do not support the multiple 5HT$_2$ receptor subtype hypothesis.

5HT$_2$ receptors mediate many of the central and peripheral physiological functions of serotonin (5HT) and serotonergic drugs. Cardiovascular effects include contraction of blood vessels (1) and shape change in platelets (2); central nervous system effects include neuronal sensitization to tactile stimuli (3) and mediation of the hallucinogenic effects of lysergic acid diethylamide and related phenylisopropylamine hallucinogens (4). The molecular properties of 5HT$_2$ receptors have been studied extensively using radioligand binding technology; [^{3}H] spiperone and [^{3}H]ketanserin, antagonist radioligands, have been used extensively to radiolabel brain 5HT$_2$ receptors (5, 6). Detailed studies in which the effects of agonists on [^{3}H]ketanserin-labeled 5HT$_2$ receptors were observed revealed evidence that the 5HT$_2$ receptor is a two-state receptor (7). This evidence included the observation that agonists competed with high and low affinity phases, whereas antagonists competed in a manner indicative of a homogeneous population of sites. Further evidence included the observation that guanyl nucleotides eliminated the high affinity phase of agonist competition curves. In order to selectively label the agonist high affinity state of the 5HT$_2$ receptor, two hallucinogenic 5HT$_2$ receptor agonists, DOB and DOI, were radiolabeled with tritium and ^{125}I, respectively (8, 9). Consistent with the 5HT$_2$ receptor two-state hypothesis, these radioligands apparently labeled a subset of the 5HT$_2$ receptor population demonstrating high affinity for both agonists and antagonists. Furthermore, guanyl nucleotides

This work was supported by United States Public Health Service Grant MH-40716.

[1] Formerly spelled Titeler.

ABBREVIATIONS: 5HT, 5-hydroxytryptamine; DOB, 4-bromo-(2,5-dimethoxy)phenylisopropylamine; DOI, 4-iodo-(2,5-dimethoxy) phenylisopropylamine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

potently inhibited the specific binding of these two radioligands.

Recently, an alternative hypothesis has appeared in the literature. Peroutka and co-workers (10–12) have used [^{77}Br]DOB, [^{3}H]DOB, and [^{125}I]DOI to label $5HT_2$ receptors in cortical homogenates. Although their data have not differed significantly from the data previously published, they have offered a very different interpretation. Peroutka and co-workers have proposed that the site labeled by the agonist radioligands is a $5HT_2$ receptor subtype distinct from the $5HT_2$ receptor labeled by [^{3}H]ketanserin. They proposed that the binding site labeled by [^{77}Br]DOB, [^{3}H]DOB, and [^{125}I]DOI represents a $5HT_2$ receptor ($5HT_{2A}$) possessing high affinity for agonists and antagonists. The site labeled by [^{3}H]ketanserin ($5HT_{2B}$) was proposed to possess low affinity for agonists and high affinity for antagonists.

In order to provide more compelling evidence in favor of the two-state hypothesis or the two-receptor hypothesis, we decided to utilize the recently cloned rat $5HT_2$ receptor gene (13). Murine fibroblast (NIH-3T3) cells have been stably transfected with this gene and express a functional $5HT_2$ receptor (14). Also, green monkey kidney (COS) cells have been successfully transfected with the cloned rat $5HT_2$ receptor gene in one of our laboratories. We hypothesized that if the two-state receptor hypothesis was correct then membranes from these transfected cells should contain both the [^{125}I]DOI and the [^{3}H]ketanserin binding sites and agonists would display higher affinities for the [^{125}I]DOI-labeled receptor than for the [^{3}H]ketanserin-labeled receptor; if the two-receptor hypothesis was correct then [^{125}I]DOI and [^{3}H]ketanserin binding would be present if the "$5HT_{2A}$ receptor" gene was being transfected; however, agonists would be expected to have similar affinities for the receptor regardless of whether [^{125}I]DOI or [^{3}H]ketanserin was the radioligand. If the "$5HT_{2B}$ receptor" was being transfected, only [^{3}H]ketanserin binding would be expected to be detected.

Materials and Methods

NIH-3T3 cells, stably expressing $5HT_2$ receptors (14), were cultured and generously donated by Dr. David Julius, University of California, San Francisco. To produce transfected COS cells, a 5.3-kilobase rat $5HT_2$ receptor cDNA was isolated from a rat cortex cDNA library[2] in the expression vector pCD1 (15). This plasmid was introduced into COS cells by the calcium phosphate method of Chen and Okayama (16). Forty-eight hours after transfection, medium was removed and cells were washed with phosphate-buffered saline. Membranes were collected in 10 mM Tris·HCl, pH 7.6 (at 23°), 5 mM $MgCl_2$, homogenized using a Tekmar Tissumizer (50% of maximum speed, 30 sec), and centrifuged at 20,000 × g for 20 min. The membrane pellet was washed again by homogenization and centrifugation and then resuspended in 50 mM Tris·HCl, pH 7.6 (at 23°). Aliquots were frozen and stored in liquid nitrogen.

[^{125}I]DOI (2200 Ci/mmol) and [^{3}H]ketanserin (64 Ci/mmol) were synthesized by New England Nuclear. GTPγS was obtained from Boehringer. Membranes were prepared from transfected NIH-3T3 and COS cells by homogenization in 50 mM Tris·HCl, 0.5 mM EDTA, 10 mM $MgCl_2$, pH 7.4 (1:10 w/v), and centrifuged at 30,000 × g for 15 min. The pellet was resuspended in buffer, incubated at 37° for 15 min, and then centrifuged at 30,000 × g for 10 min twice (with a resuspension between centrifugations). The final pellet was resuspended in 50 mM Tris·HCl, 0.5 mM EDTA, 10 mM $MgCl_2$, 0.1% ascorbate, 10^{-5} M pargyline.

[2] B. J. Hoffman and M. J. Brownstein, unpublished observations.

Assays were performed in triplicate 1.0-ml volumes containing 10 μg of protein (which was added last). Competition assays were conducted using three log units of radioligand concentration, using 1 μM cinanserin to define nonspecific binding. Competition experiments were performed using 1×10^{-10} M [^{125}I]DOI or 4×10^{-10} M [^{3}H]ketanserin. At this concentration, 70% of the total binding was specific for both radioligands. Tubes were incubated for 15 min at 37°, filtered on Schleicher and Schuell (Keene, NH) glass fiber filters (presoaked in 0.1% polyethyleneimine), and washed with 10 ml of ice-cold buffer. Preliminary experiments indicated higher levels of [^{125}I]DOI binding at room temperature; however, because our results were to be compared with brain tissue experiments, which had all been performed at 37°, we decided to perform all the experiments at this higher temperature. The filters were counted on a LKB γ-counter (efficiency = 85%) for [^{125}I]DOI experiments. The filters were immersed in liquid scintillation fluid and counted at an efficiency of 50% for [^{3}H]ketanserin experiments. Saturation and competition experiments were analyzed using an updated version of the program EBDA (17) to obtain equilibrium dissociation constants (K_I), B_{max} values, and IC_{50} values. K_I values for competition experiments were obtained using the equation $K_I = IC_{50}/1 + (D^*/K_{D^*})$, where IC_{50} is the experimentally observed concentration of competing drug that inhibits 50% of the specific binding, K_{D^*} is the equilibrium dissociation constant determined in saturation studies, and D^* is the concentration of radioactive ligand used in the competition assays. This equation was applied to competition curves

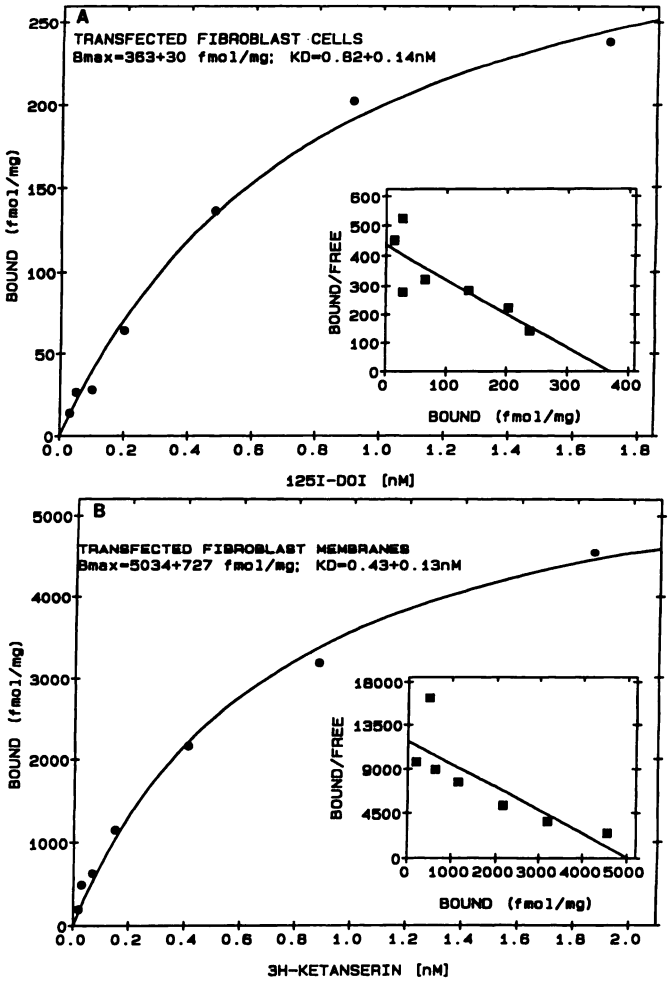

Fig. 1. Saturable specific binding of [^{125}I]DOI (A) and [^{3}H]ketanserin (B) to membranes from fibroblast (NIH-3T3) cells transfected with the rat $5HT_2$ receptor gene. *Insets*, Scatchard transformation of the saturable binding data. The results are the means of two experiments, each performed with duplicate data points.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

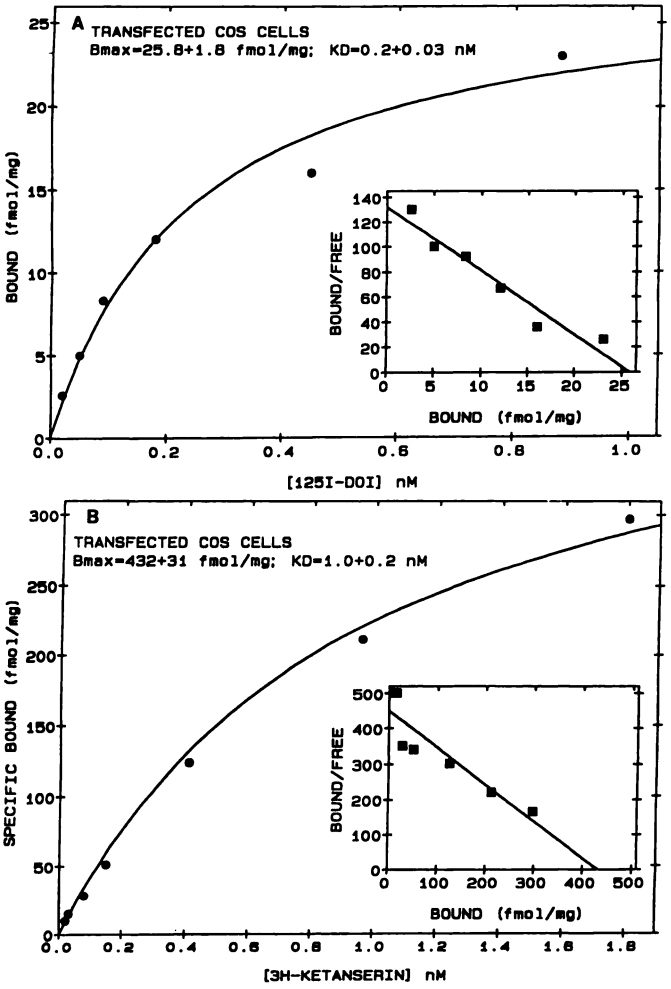

Fig. 2. Saturable specific binding of [^{125}I]DOI (A) and [^{3}H]ketanserin (B) to membranes from green monkey kidney (COS) cells transfected with the rat 5HT$_2$ receptor gene. *Insets*, Scatchard transformation of the saturable binding data. The results are the means of two experiments, each performed with duplicate data points.

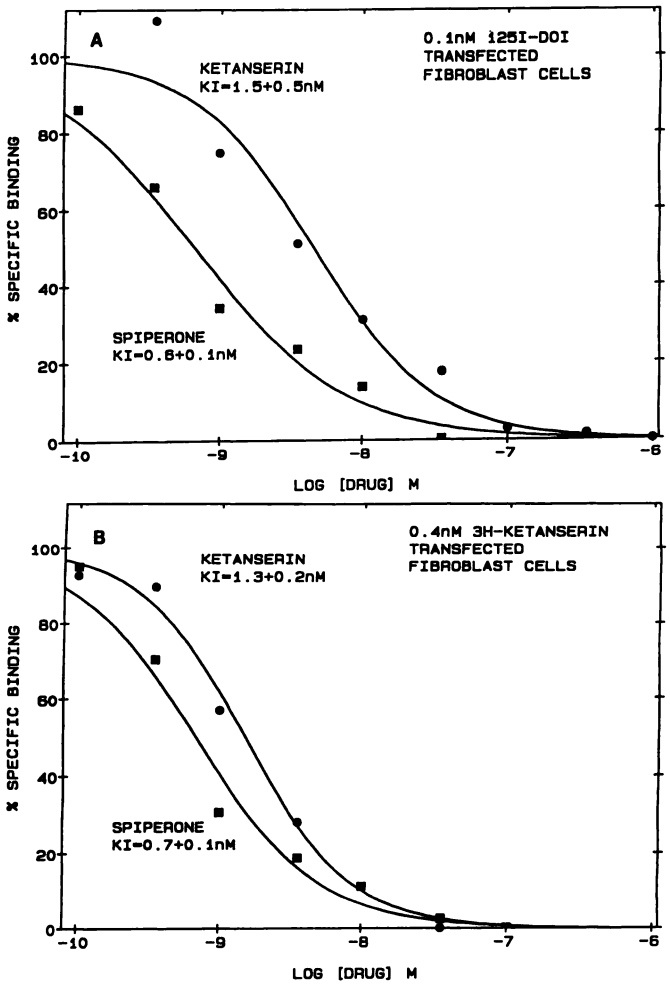

Fig. 3. Representative competition curves generated by competition of varying concentrations of spiperone and ketanserin for [^{125}I]DOI (A) and [^{3}H]ketanserin (B) specific binding to fibroblast (NIH-3T3) cell membranes. Standard errors were less than 10% for all data points.

producing Hill coefficients less than unity. Drugs used in competition studies were purchased from Sigma or Research Biochemicals, Inc.

Results

As shown in Figs. 1 and 2, membranes from NIH-3T3 cells and COS cells (both transfected with the rat 5HT$_2$ receptor gene) displayed saturable [^{125}I]DOI and [^{3}H]ketanserin specific binding. The K_D values of 0.8 and 0.4 nM for [^{125}I]DOI and [^{3}H] ketanserin in transfected COS cells and 0.2 and 1.0 nM for [^{125}I] DOI and [^{3}H]ketanserin in transfected COS cells are close to the values observed in mammalian brain (7, 8). The B_{max} values in the NIH-3T3 cells are similar to the values reported previously in cells transfected with the rat 5HT$_2$ receptor gene (14). The affinities of drugs in competing for the [^{125}I]DOI- and [^{3}H] ketanserin-labeled 5HT$_2$ receptors (Figs. 3 and 4 and Table 1) were also very similar to values observed in mammalian brain tissue (7, 8); the mode of competition of the antagonists spiperone and ketanserin, as revealed by the Hill coefficients of the competition curves (Figs. 3 and 4), were also very similar to the results observed in mammalian brain tissue. The nonhydrolyzable GTP derivative GTPγS inhibited specific [^{125}I] DOI binding in the COS cells; at 10^{-5} M GTPγS, 30% of specific [^{125}I]DOI binding was inhibited (data not shown).

Discussion

Many receptors coupled to GTP-binding proteins have been radiolabeled with agonist radioligands and antagonist radioligands (18). These studies have revealed that receptors that stimulate GTP-binding proteins exist in two detectable states, the free receptor and a receptor-GTP-binding protein complex. GTP and nonhydrolyzable derivatives of GTP [Gpp(NH)p and GTPγS] dissociate the complex. The presence of these two states can be detected with radioligand binding methodology. Antagonists appear to bind to both states with equal affinity; agonists appear to have much higher affinity for the receptor-GTP-binding protein complex than for the free receptor. Antagonist radioligands, therefore, label the total population of sites, whereas agonist radioligands only label the receptor-GTP-binding protein complex. Guanyl nucleotides inhibit agonist radioligand binding without affecting the levels of antagonist radioligand binding.

Radioligand binding studies of 5HT$_2$ receptors have been consistent with the hypothesis that the 5HT$_2$ receptor is a two-state receptor (7, 8). Nevertheless, it was recently proposed

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

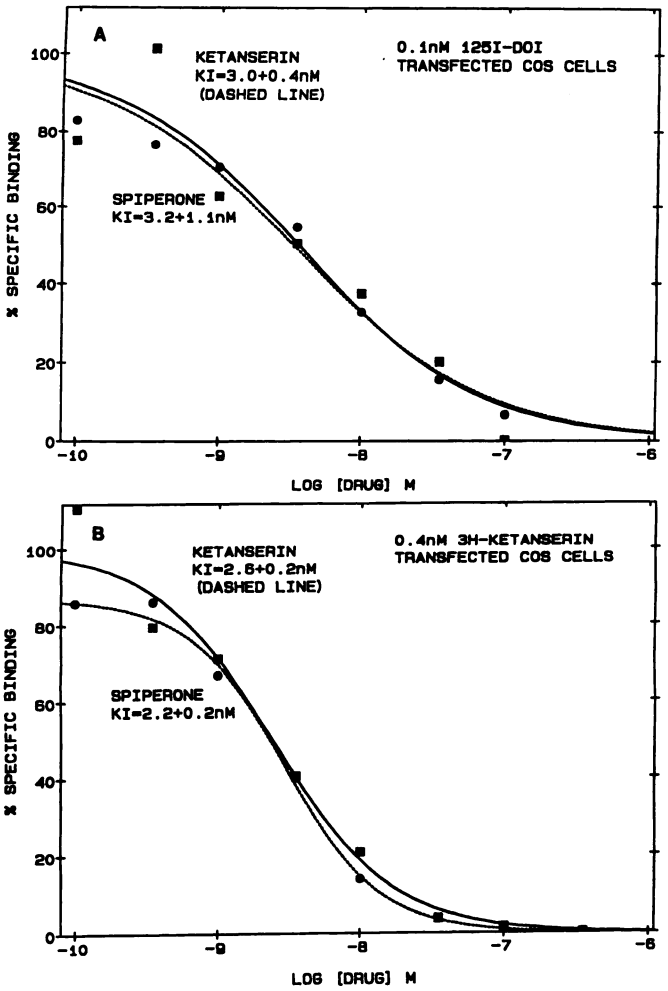

Fig. 4. Representative competition curves generated by competition of varying concentrations of spiperone and ketanserin for [^{125}I]DOI (A) and [^{3}H]ketanserin (B) specific binding to green monkey kidney (COS) cell membranes. Standard errors were less than 10% for all data points.

TABLE 1

K_i values of drugs for radiolabeled 5HT$_2$ receptors on transfected cell membranes

Reported values are the mean ± standard error of three independent experiments performed in duplicate. Eleven concentrations of competing drug were used.

Drug	K_i			
	NIH-3T3 cells		COS cells	
	[^{3}H]Ketanserin	[^{125}I]DOI	[^{3}H]Ketanserin	[^{125}I]DOI
	nM			
DOI	57 ± 0.6	2.5 ± 0.2	87 ± 12	0.8 ± 0.5
Serotonin	479 ± 2	5.7 ± 1.1	703 ± 169	2 ± 0.4
Cinanserin	ND*	ND	6 ± 1.4	6 ± 0.7
Spiperone	0.4 ± 0.1	0.6 ± 0.1	2 ± 0.1	4 ± 0.1
Ketanserin	1.0 ± 0.2	5.0 ± 2.1	2.0 ± 0.2	4 ± 0.4

* Not done.

that the agonist radioligands [^{3}H]DOB, [^{125}I]DOI, and [^{77}Br]DOB label a 5HT$_2$ receptor subtype distinct from the 5HT$_2$ receptor subtype labeled by [^{3}H]ketanserin (10–12). The agonist high affinity site was designated the "5HT$_{2A}$" receptor and the receptor labeled by [^{3}H]ketanserin was designated the "5HT$_{2B}$" receptor. The data produced by Peroutka and coworkers were essentially identical to the data presented previously; thus, the discrepancy resided in interpretation of the

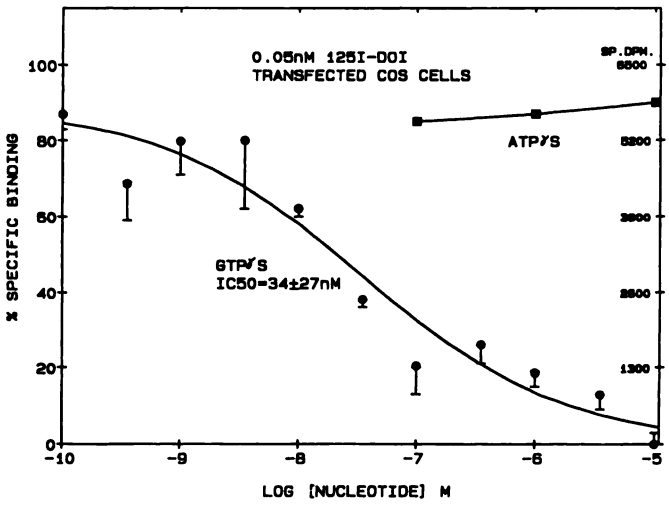

Fig. 5. Representative inhibition curve of guanyl nucleotide-sensitive specific [^{125}I]DOI binding to transfected COS cells by GTPγS. ATPγS was inactive. GTPγS had no effect on specific [^{3}H]ketanserin binding to transfected COS cells (data not shown). Data points are the means and standard errors of triplicate determinations. Seventy percent of total specific [^{125}I]DOI binding was guanyl nucleotide sensitive.

data. Therefore, we designed an experiment, using the recently cloned 5HT$_2$ receptor gene, that would provide novel data differentiating multiple 5HT$_2$ receptor states from multiple 5HT$_2$ receptor subtypes.

The transfected cells present an opportunity to test the conflicting hypotheses concerning the two-state 5HT$_2$ receptor hypothesis versus the two-5HT$_2$ receptor subtype hypothesis. If the two-state hypothesis is correct, then the one gene should produce both [^{125}I]DOI and [^{3}H]ketanserin binding and agonist affinities for [^{125}I]DOI binding should be significantly higher than agonist affinities for [^{3}H]ketanserin binding. If the two-receptor hypothesis is correct, then one of two situations should arise in the transfected cells. If the transfected gene is the 5HT$_{2A}$ receptor gene, then both [^{125}I]DOI and [^{3}H]ketanserin binding should be present and agonist affinities should be the same (high affinity) regardless of the radioligand used. If the transfected gene is the 5HT$_{2B}$ receptor gene, then only [^{3}H]ketanserin binding should be present.

The results presented herein conclusively demonstrate both [^{125}I]DOI and [^{3}H]ketanserin binding in the membranes of the transfected cells (Figs. 1 and 2). Furthermore, the affinities of some representative 5HT$_2$ drugs for the sites labeled by the ligands are essentially identical to those for the sites labeled in mammalian cortical tissue homogenates (Figs. 3 and 4 and Table 1). [^{125}I]DOI binding to 5HT$_2$ receptors in COS cell membranes was modulated by the guanyl nucleotide GTPγS, indicating that the 5HT$_2$ receptor expressed in the transfected cell is functionally coupled to a GTP-binding protein. The affinities of the agonists DOI and serotonin are 10–25-fold higher for the [^{125}I]DOI-labeled receptor than for the [^{3}H]ketanserin-labeled receptor. Taken together, these results do not correspond to the results expected if a distinct 5HT$_{2A}$ or 5HT$_{2B}$ receptor gene had been transfected. The level of [^{125}I]DOI binding is significantly lower in both cell lines (Figs. 1 and 2) than the level of [^{3}H]ketanserin binding, which is again consistent with the observations from mammalian brain membranes (8). The GTP-binding protein interacting with the 5HT$_2$ receptor is undoubtedly a constituent component of the host

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012

cell's membrane, because receptor-mediated stimulation of phospholipase C activity (a GTP-binding protein-mediated response) has been observed in these cells (14).

In summary, the results presented herein demonstrate that the $5HT_2$ receptor gene codes for a receptor that is radiolabeled by both the agonist radioligand [^{125}I]DOI and the antagonist radioligand [^{3}H]ketanserin and that agonists display higher affinities for the [^{125}I]DOI-labeled receptor than for the [^{3}H]ketanserin-labeled receptor. These data indicate that the proposed $5HT_{2A}$ and $5HT_{2B}$ receptor subtypes are actually states of one $5HT_2$ receptor.

Acknowledgments

The transfected NIH-3T3 cells were generously donated by Dr. David Julius, University of California, San Francisco. We thank Dr. Michael Brownstein for his critical evaluation of the manuscript.

References

1. Houston, D. S., and P. M. Vanhoutte. Comparison of serotonergic receptor subtypes on the smooth muscle and endothelium of the canine coronary artery. *J. Pharmacol. Exp. Ther.* **244**:1–10 (1988).
2. De Clerck, F., B. Xhonneux, J. Leysen, and P. A. J. Janssen. Evidence for functional $5HT_2$ receptor sites on human blood platelets. *Biochem. Pharmacol.* **33**:2807–2811 (1984).
3. Rasmussen, K., and G. K. Aghajanian. Effect of hallucinogens on spontaneous and sensory-evoked locus coeruleus unit activity in the rat: reversal by selective $5HT_2$ antagonists. *Brain Res.* **385**:395–400 (1986).
4. Glennon, R. A., M. Titeler, and J. D. McKenney. Evidence for $5HT_2$ receptor involvement in the mechanism of action of hallucinogenic agents. *Life Sci.* **35**:2505–2511 (1984).
5. Leysen, J. E., and P. M. Laduron. A serotonergic component of neuroleptic receptors. *Arch. Int. Pharmacodyn. Ther.* **230**:337–339 (1977).
6. Leysen, J. E., C. J. E. Niemegeers, J. M. Van Nueten, and P. M. Laduron. ^{3}H-Ketanserin (R41468), a selective ^{3}H-ligand for $5HT_2$ receptor binding sites. *Mol. Pharmacol.* **21**:301–314 (1982).
7. Battaglia, G., M. Shannon, and M. Titeler. Guanyl nucleotide and divalent cation regulation of cortical $5HT_2$ serotonin receptors. *J. Neurochem.* **43**:1213–1219 (1984).
8. Lyon, R. A., K. H. Davis, and M. Titeler. ^{3}H-DOB labels a guanyl nucleotide-sensitive state of cortical $5HT_2$ receptors. *Mol. Pharmacol.* **31**:194–199 (1987).
9. Glennon, R. A., M. R. Seggel, W. Soine, K. H. Davis, R. A. Lyon, and M. Titeler. ^{125}I-2,5-Dimethoxy-4-iodophenyl-2-aminopropane (DOI): an iodinated radioligand that specifically labels the agonist high affinity state of the $5HT_2$ serotonin receptor. *J. Med. Chem.* **31**:5–7 (1988).
10. Wang, S. S. H., C. A. Mathis, and S. J. Peroutka. *R*(−)^{77}Br-2,5-Dimethoxy-4-bromoamphetamine (^{77}Br-DOB): a novel radioligand which labels a 5-hydroxytryptamine binding site subtype. *Psychopharmacology* **94**:431–432 (1988).
11. Pierce, P. A., and S. J. Peroutka. Evidence for distinct 5-hydroxytryptamine-2 binding site subtypes in cortical membrane preparations. *J. Neurochem.* **52**:656–658 (1989).
12. McKenna, D. J., and S. J. Peroutka. Differentiation of 5-hydroxytryptamine$_2$ receptor subtypes using ^{125}I-*R*-(−)2,5-dimethoxy-4-iodo-phenylisopropylamine and ^{3}H-ketanserin. *J. Neurosci.* **9**:3482–3490 (1989).
13. Pritchett, D. B., A. W. J. Bach, M. Wozny, O. Taleb, R. Dal Toso, J. C. Shih, and P. H. Seeburg. Structure and functional expression of cloned rat serotonin $5HT_2$ receptor. *EMBO J.* **7**:4135–4140 (1988).
14. Julius, D., K. N. Huang, T. J. Livelli, R. Axel, and T. M. Jessell. The $5HT_2$ receptor defines a family of structurally distinct but functionally conserved serotonin receptors. *Proc. Natl. Acad. Sci. USA* **87**:928–932 (1989).
15. Okayama, H., and P. Berg. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell. Biol.* **3**:280–289 (1983).
16. Chen, C., and H. Okayama. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
17. MacPherson, G. A. A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Programs Biomed.* **17**:107–114 (1983).
18. Lefkowitz, R. J. Clinical physiology of adrenergic receptor regulation. *Am. Physiol. Soc.* **243**:E43–47 (1982).

Send reprint requests to: Milt Teitler, Department of Pharmacology and Toxicology, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 4, 2012