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Multiple Conformations of Native and Recombinant Human 5-Hydroxytryptamine$_{2A}$ Receptors Are Labeled by Agonists and Discriminated by Antagonists

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ABSTRACT

We have expanded previous studies with the 5-hydroxytryptamine (5-HT)$_2$ receptor agonist (±)-1-(2,5-dimethoxy-4-[^{125}I]iodophenyl)-2-aminopropane [(±)-[^{125}I]DOI] in human brain that had shown biphasic competition curves for several 5-HT$_{2A}$ receptor antagonists by using new selective antagonists of 5-HT$_{2A}$ (MDL100,907) and 5-HT$_{2C}$ (SB242084) receptors together with ketanserin and mesulergine. Autoradiographic competition experiments were performed with these antagonists in human brain regions where (±)-[^{125}I]DOI labels almost exclusively 5-HT$_{2A}$ receptors (frontal cortex and striosomes). Furthermore, the effect of uncoupling receptor/G protein complexes on antagonist competition was studied with guanosine-5′-(β,γ-imido)triphosphate [Gpp(NH)p]. Competition experiments with (±)-[^{3}H]1-(4-bromo-2,5-dimethoxyphenil)-2-aminopropane [(±)-[^{3}H]DOB] were also performed in membranes from Chinese hamster ovary cells (CHOFA4) expressing cloned human 5-HT$_{2A}$ receptors. In both systems, ketanserin and MDL100,907 displayed biphasic competition profiles, whereas SB242084 and mesulergine competed monophasically. In absence of antagonist, 100 μM Gpp(NH)p decreased brain (±)-[^{125}I]DOI specific binding by 40 to 50% and (±)-[^{3}H]DOB specific binding to CHOFA4 cells by 30%. The remaining agonist-labeled uncoupled sites were still displaced biphasically by ketanserin and MDL100,907, with unaltered affinities. Saturation experiments were performed in CHOFA4 cells. (±)-[^{3}H]DOB labeled two sites (K_{d_h} = 0.8 nM, K_{d_l} = 31.22 nM). Addition of 100 μM Gpp(NH)p resulted in a single low-affinity (K_d = 24.44 nM) site with unchanged B_{max}. [^{3}H]5-HT showed no specific binding to 5-HT$_{2A}$ receptors. These results conform with the extended ternary complex model of receptor action that postulates the existence of partly activated receptor conformation(s) (R*) in equilibrium with the ground (R) and the activated G protein-coupled (R*G) conformations. Thus, both in human brain and CHOFA4 cells, the agonists possibly label all three conformations and ketanserin and MDL100,907 recognize with different affinities at least two of these conformations.

5-Hydroxytryptamine (5-HT)$_{2A}$ receptors belong to the 5-HT$_2$ receptor family, which comprises at present 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$ subtypes. These three subtypes share a high homology in their DNA sequence and they are all coupled via G protein to stimulation of phospholipase C (PLC) after agonist interaction (Hoyer et al., 1994; Barnes and Sharp, 1999). The first member of the family to be described was the 5-HT$_{2A}$ receptor by means of radioligand binding techniques (Peroutka and Snyder, 1979). Since then, several radiolabeled drugs have been used to label 5-HT$_2$ receptor subtypes, although the high homology in their primary structure has made difficult the pharmacological characterization of each subtype due to the lack of selective drugs (Baxter et al., 1995). Among the different radioligands used to label 5-HT$_2$ receptors, there are two phenylalkylamine agonists: (±)-[^{125}I]DOI and (±)-[^{3}H]DOB. (±)-[^{125}I]DOI was used to visualize 5-HT$_2$ receptors in rat brain by means of receptor autoradiography (McKenna et al., 1987; Appel et al., 1990), whereas (±)-[^{3}H]DOB failed to label 5-HT$_2$ receptors using this technique probably due to its low specific activity as radioligand (Glennon et al., 1988). Subsequent membrane binding studies using (±)-[^{125}I]DOI and [^{3}H]ketanserin as

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PLC, phospholipase C; (±)-DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; (±)-DOB, (±)-1-(4-bromo-2,5-dimethoxyphenil)-2-aminopropane; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; MDL100.907, (*R*)-(+)-4-[1-hydroxy-1(2,3-dimethoxyphenyl)methyl]*N*-2–4-fluorophenylethyl)piperidine; SB242084, 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-yl-carbamoyl]indoline; CHO, Chinese hamster ovary; FBS, fetal bovine serum; IP, inositol phosphate.

radioligands and brain homogenates from several mammalian species led to the postulated existence of different receptor subtypes labeled by these radioligands (McKenna and Peroutka, 1989; Pierce and Peroutka, 1989). Nevertheless, further studies carried out with cloned rat and human 5-HT$_{2A}$ receptors expressed in cell lines, and using (±)-[^{125}I]DOI and (±)-[^{3}H]DOB as radioligands, respectively, demonstrated that the different binding sites observed previously corresponded to multiple affinity states of the same receptor and not to different receptors. These multiple affinity states were dependent on the receptor/G protein-coupling status of the system (Branchek et al., 1990; Teitler et al., 1990).

Previous to these findings with cloned receptors, pharmacological studies in human brain described biphasic displacement curves for several antagonists when competing against radioligands that mainly labeled 5-HT$_{2A}$ receptors. The earliest report described the presence of two binding sites when spiperone and cinanserin competed against [^{3}H]ketanserin in human frontal cortex homogenates (Hoyer et al., 1986). The interpretation of these results was based on the possibility that [^{3}H]ketanserin labels other receptors different from 5-HT$_{2A}$ (i.e., histamine H$_1$ receptors and α_1-adrenoreceptors), because it had been demonstrated that ketanserin presented relatively high affinity for them (Leysen et al., 1981). More recently, receptor binding autoradiography revealed a similar biphasic pharmacological profile for ketanserin, spiperone, and cinanserin when displacing (±)-[^{125}I]DOI labeling from human striosomes, cortex, and claustrum (Waeber and Palacios, 1994). The possibility that this radioligand labels other neurotransmitter receptors was dismissed, due to the lack of affinity of (±)-[^{125}I]DOI for receptors other than 5-HT$_2$ subtypes (Appel et al., 1990). In this case, it was hypothesized that allosteric events could take place in human 5-HT$_{2A}$ receptors.

In recent years, new selective drugs have been described that discriminate between 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors, such as MDL100,907, a 5-HT$_{2A}$-selective antagonist (Kehne et al., 1996) and SB242084, a 5-HT$_{2C}$-selective antagonist (Kennett et al., 1997). The aims of the present work were to further investigate the nature of the heterogeneous binding sites observed when displacing (±)-[^{125}I]DOI from different areas of human brain by taking advantage of the availability of these new selective drugs and at the same time by examining the effects of guanosine-5′-(β,γ-imido)triphosphate [Gpp(NH)p], a nonhydrolizable GTP analog. For this purpose, (±)-[^{125}I]DOI competition experiments were carried out with human brain regions by means of receptor autoradiography and the results were compared with those obtained from (±)-[^{3}H]DOB membrane binding assays performed with cloned human 5-HT$_{2A}$ receptors expressed in a recombinant system.

Materials and Methods

Human Samples. Human brain tissues were obtained at autopsy by Dr. J. Pascual (Hospital de Valdecilla, Santander, Spain) and from the Neurological Tissue Bank (University of Barcelona, Hospital Clínic, Barcelona, Spain). Human brains were from subjects without clinical or histopathological evidence of neurological or psychiatric diseases (two men and three women, mean age 55 years, range 43–70 years). The brains were dissected, frozen in dry ice, and kept at −20°C. Tissue sections, 14 μm thick, were cut using a microtome-cryostat (HM 500 M; Microm, Walldorf, Germany), thaw mounted onto 3-aminopropyltriethoxylane-coated slides (Sigma, St. Louis, MO), and kept at −20°C until use.

Drugs. (±)-[^{125}I]DOI [(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] (2200 Ci/mmol) and (±)-[^{3}H]DOB [(±)-1-(4-bromo-2,5-dimethoxyphenil)-2-aminopropane] (23.1Ci/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). [^{3}H]Myoinositol (20 Ci/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO). [^{3}H]5-HT (111 Ci/mmol) was purchased from Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Ritanserin, mianserin, ketanserin, mesulergine, and (±)-DOI were purchased from Sigma/RBI (Natick, MA). Gpp(NH)p and 5-HT were purchased from Sigma. MDL100,907 [(*R*)-(+)-4-[1-hydroxy-1(2,3-dimethoxyphenyl)methyl]*N*-2–4-fluorophenylethyl)piperidine] was a generous gift from Dr. M. Galvan (Marion Merrell Dow, Strasbourg, France). SB242084 [6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-yl-carbamoyl]indoline] was kindly provided by GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK).

Receptor Autoradiography in Human Brain. Human brain tissue samples from striatum (caudate and putamen) and frontal cortex were chosen for (±)-[^{125}I]DOI competition binding studies. Experimental procedures were basically as described previously (McKenna et al., 1989). Briefly, cryostat tissue sections were air dried, preincubated for 30 min at room temperature in 50 mM Tris-HCl, pH 7.4, 0.1% ascorbic acid, and 4 mM $CaCl_2$, and incubated in the presence of radioligand (100–150 pM) in the same buffer for 60 min at room temperature. Tissues were washed twice for 10 min each in the same ice-cold buffer. Nonspecific binding was defined as that remaining in the presence of 1 μM ritanserin. After incubation, washing, and drying under cold air, tissue sections were exposed to tritium-sensitive film (Hyperfilm-^{3}H; Amersham Pharmacia Biotech UK, Ltd.) together with plastic standards (^{125}I-microscales; Amersham Pharmacia Biotech UK, Ltd.) for 42 to 72 h. Quantitative analysis of the autoradiograms obtained was done with a computerized image analysis system (MCID, M4; St. Catharines, ON, Canada). Three specific human brain areas were selected to analyze the radioligand signal: the patchy pattern distribution of (±)-[^{125}I]DOI autoradiographic signal observed in caudate and putamen corresponding to striosomes (Waeber and Palacios, 1994), and lamina V of the frontal cortex.

Cell Culture. Chinese hamster ovary cells that stably express the cloned human 5-HT$_{2A}$ serotonin receptors (CHOFA4 cells) were kindly provided by Drs. Kelly Berg and William P. Clarke (University of Texas Health Science Center, San Antonio, TX). Cells were maintained in standard tissue culture plates (150 mm in diameter) in α-minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 300 μg/ml hygromycin (Invitrogen).

Receptor Binding Studies with CHOFA4 Cells Expressing 5-HT$_{2A}$ Receptors. Prior to harvesting, stably transfected cells were grown for 24 h in α-minimal essential medium supplemented with 10% dialyzed FBS (Invitrogen), without hygromycin. Culture medium was aspirated, cells were washed twice with ice-cold phosphate-buffered saline, scraped off the culture plate in phosphate-buffered saline, and pelleted by centrifugation at 1000*g*, 10 min, 4°C. Cell pellets were homogenized with a Polytron homogenizer in 50 mM Tris-HCl, pH 7.5, at 4°C. The homogenate was centrifuged at 24,000*g* for 30 min at 4°C and the resulting pellet was resuspended in assay buffer (50 mM Tris-HCl, 0.5 mM EDTA, 10 mM $MgSO_4$, 0.1% ascorbic acid, and 10 μM pargyline, pH 7.5). Protein concentration was determined by the Bradford method with Bio-Rad Protein Assay (Bio-Rad, München, Germany) with bovine serum albumin as standard. Homogenates were kept on ice at all stages and binding assays were run on the same day of cell collection and membrane preparation, because it was observed that (±)-[^{3}H]DOB binding decreased with freezing of membranes, in agreement with previous observations (Branchek et al., 1990).

Binding assays were performed in duplicate in a 0.5-ml total volume. Competition experiments were performed using 5 nM (±)-[^{3}H]DOB. For saturation analysis, the radioligand concentration varied from 0 to 30 nM diluted in assay buffer. Nonspecific binding was defined by the addition of 10 μM mianserin. Incubations were initiated by the addition of membrane preparation (200–250 μg of protein) and were conducted at 37°C for 20 min. Binding reactions were terminated by rapid filtration under reduced pressure using a Brandel cell harvester (Brandel Laboratories, Gaithersburg, MD), fitted with Whatman GF/B filters (Whatman Inc., Clifton, NJ) presoaked in 0.3% polyethylenimine (Sigma) in ice-cold assay buffer. The filters were then washed rapidly four times with 4 ml of ice-cold washing buffer (50 mM Tris-HCl, pH 7.5) and counted in a Wallac 1414 liquid scintillation counter (Wallac, Turku, Finland). The experimental procedure for [^{3}H]5-HT saturation binding assays was similar except for the composition of assay buffer (50 mM Tris-HCl, 4 mM $CaCl_2$, 0.1% ascorbate, and 10 μM pargyline, pH 7.5) and the incubation time (30 min). Saturation studies with [^{3}H]5-HT were performed within two radioligand concentration ranges (0–30 and 0–100 nM)

[^{3}H]IP Accumulation Measurement in CHOFA4 Expressing 5-HT$_{2A}$ Receptors. Cells were seeded in 12-well plates. After 24 h, medium was replaced by medium containing dialyzed FBS and 1 μCi/ml [^{3}H]myoinositol. Twenty-four hours later, cells were washed three times with Hanks' balanced salt solution containing calcium and magnesium, 20 mM HEPES, and 20 mM LiCl (incubation medium). After washing, cells were incubated for 10 min in 1 ml of incubation medium at 37°C, containing vehicle or the indicated drug concentrations. Antagonists, when used, were coincubated with the agonist. After the incubation time the medium was quickly removed, 2 ml of 10 mM formic acid (4°C) was added, and the plates were maintained at 4°C for 30 min to extract the accumulated [^{3}H]IPs (IP_1, IP_2, and IP_3, collectively referred to as IP). The released [^{3}H]IPs were separated by the anion exchange chromatography method of Berridge et al. (1982) and counted in a liquid scintillation counter (Beckman LS-6000 LL; Beckman, Fullerton, CA).

Data Analysis. Estimates of binding and stimulation response parameters (K_d, B_{max}, IC_{50}, Hill slope, E_{max}, and EC_{50}) were derived with Prism software (GraphPad Software, San Diego, CA) from the data obtained after quantification of the autoradiograms or from liquid scintillation counting. In agonist stimulation studies, antagonist potency was measured in terms of pA_2 (−logarithm of the concentration of antagonist required to maintain a constant response when the agonist concentration is doubled; MacKay, 1978), when the slopes of cumulative concentration-response curves did not differ significantly from 1.

Results

Receptor Autoradiography in Human Brain. To further investigate the biphasic curves previously described for several antagonists when displacing (±)-[^{125}I]DOI in human brain (Waeber and Palacios, 1994), four different antagonist compounds were assayed in competition experiments against (±)-[^{125}I]DOI. These compounds included two selective antagonists for human 5-HT$_{2A}$ receptors, and two selective antagonists for human 5-HT$_{2C}$ receptors. Each pair comprised a new selective drug together with a more classical, extensively described one: MDL100,907 and ketanserin, respectively, for 5-HT$_{2A}$ receptors and SB242084 and mesulergine for 5-HT$_{2C}$ receptors. The human brain regions selected for these studies were frontal cortex (lamina V) and caudate and putamen (patches corresponding to striosomes). The results obtained in these competition experiments are summarized in Table 1, and Fig. 1 shows the sigmoidal curves displayed for each competitor in one representative human brain region (frontal cortex). MDL100,907 and ketanserin differentiated two binding site populations when displacing (±)-[^{125}I]DOI in all three aforementioned regions, whereas SB242084 and mesulergine presented inhibition curves with a single low-affinity component. However, one exception was found in putamen patches, where the displacement of (±)-[^{125}I]DOI binding by SB242084 was best fitted by

TABLE 1

Competition parameters of several compounds for (±)-[^{125}I]DOI-labeled receptors in different human brain regions

Values represent mean ± S.E.M. of five subjects.

	−LogIC$_{50}$high	−LogIC$_{50}$low	%High	n_H[a]
Frontal cortex				
MDL 100,907	8.99 ± 0.26	6.50 ± 0.52	61.8 ± 9.9	0.39 ± 0.01
MDL 100,907 plus 100μM Gpp(NH)p	8.76 ± 0.29	6.00 ± 0.26	49.3 ± 6.9	0.35 ± 0.01
Ketanserin	8.51 ± 0.32	6.07 ± 1.17	75.8 ± 13.9	0.53 ± 0.02
Ketanserin plus 100μM Gpp(NH)p	8.40 ± 0.16	5.53 ± 0.52	75.9 ± 6.6	0.52 ± 0.01
SB242084		7.14 ± 0.13		1.00
SB242084 plus 100μM Gpp(NH)p		7.15 ± 0.07		1.00
Mesulergine		7.23 ± 0.07		1.00
Mesulergine plus 100μM Gpp(NH)p		6.99 ± 0.10		1.00
Caudate patches				
MDL 100,907	8.55 ± 0.24	5.66 ± 0.39	61.9 ± 6.6	0.41 ± 0.01
MDL 100,907 plus 100μM Gpp(NH)p	8.69 ± 0.24	5.37 ± 0.16	48.8 ± 6.8	0.35 ± 0.01
Ketanserin	8.47 ± 0.19	6.26 ± 0.62	74.6 ± 8.7	0.51 ± 0.01
Ketanserin plus 100μM Gpp(NH)p	8.64 ± 0.39	5.79 ± 0.19	37.3 ± 0.06	0.35 ± 0.01
SB242084		7.02 ± 0.06		1.00
SB242084 plus 100μM Gpp(NH)p		7.05 ± 0.12		1.00
Mesulergine		7.18 ± 0.08		0.92 ± 0.01
Mesulergine plus 100μM Gpp(NH)p		7.00 ± 0.13		0.78 ± 0.01
Putamen patches				
MDL 100,907	7.91 ± 0.08	5.50 ± 0.22	67.9 ± 8.3	0.52 ± 0.01
MDL 100,907 plus 100μM Gpp(NH)p	8.69 ± 0.24	5.37 ± 0.15	41.6 ± 4.4	0.35 ± 0.01
Ketanserin	8.32 ± 0.34	6.22 ± 0.43	51.9 ± 11.6	0.45 ± 0.01
Ketanserin plus 100μM Gpp(NH)p	8.71 ± 0.63	5.92 ± 0.31	25.7 ± 8.2	0.55 ± 0.02
SB242084	9.23 ± 0.57	7.00 ± 0.12	18.7 ± 6.5	0.64 ± 0.02
SB242084 plus 100μM Gpp(NH)p	8.42 ± 0.57	6.80 ± 0.25	33.3 ± 17.1	0.66 ± 0.01
Mesulergine		7.20 ± 0.09		0.82 ± 0.01
Mesulergine plus 100μM Gpp(NH)p		6.60 ± 0.09		0.92 ± 0.06

[a] Hill coefficient.

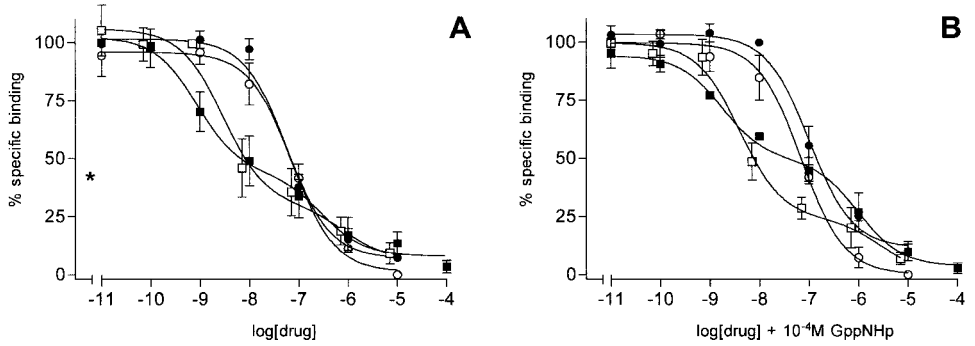

Fig. 1. Competition curves of (±)-[^{125}I]DOI binding in human frontal cortex (layer V) in absence (A) or presence (B) of 100 μM Gpp(NH)p by MDL100,907 (■), ketanserin (□), SB242082 (●), and mesulergine (○). Each point represents the mean ± S.E.M. of five subjects. In A, * indicates the remaining specific binding in presence of 100 μM Gpp(NH)p and in absence of competitor drug. This remaining binding is considered as 100% in B.

a two-site model and presented a higher affinity component in addition to the single site observed for this compound in caudate patches and frontal cortex (Table 1). Subsequently, similar experiments were carried out in presence of 100 μM GTP analog Gpp(NH)p to discern whether the two binding site populations observed with some of these antagonists were related to the degree of G protein coupling of the receptors labeled by (±)-[^{125}I]DOI. In the absence of competitor drug, addition of 100 μM Gpp(NH)p resulted in a reduction of 40 to 50% of specific (±)-[^{125}I]DOI binding in all three regions examined. Under these experimental conditions, the number of sites and affinities revealed by each of the four antagonists when displacing the remaining (±)-[^{125}I]DOI binding sites persisted essentially unaltered, except for ketanserin that presented a marked reduction in the proportion of high-affinity binding sites in caudate patches (Table 1).

Receptor Binding Studies in CHOFA4 Cells Expressing Human 5-HT$_{2A}$ Receptors. To ascertain whether the pharmacological profile obtained for the different antagonists when displacing (±)-[^{125}I]DOI binding in human brain could be ascribed entirely to 5-HT$_{2A}$ receptors, radioligand binding experiments were performed with (±)-[^{3}H]DOB in membrane homogenates from CHOFA4 cells permanently expressing human 5-HT$_{2A}$ receptors. Nonlinear regression analysis of saturation experiments revealed the existence of two saturable (±)-[^{3}H]DOB binding sites, one of high affinity and low capacity and a second one of lower affinity and higher capacity (Table 2; Fig. 2). Saturation experiments conducted in the presence of 100 μM Gpp(NH)p resulted in the observation of a single binding site, whose pharmacological parameters (K_d and B_{max}) were most similar to the ones shown by the low-affinity, high-capacity site observed when the GTP analog was not present in the incubation medium (Table 2; Fig. 2). This change induced by Gpp(NH)p is most obvious in the Scatchard representation of the saturation data (Fig. 2B). These results indicate that (±)-[^{3}H]DOB binds to two populations/states of human 5-HT$_{2A}$ receptors in CHO cells, and that these states are dependent on the coupling of these receptors to G proteins.

TABLE 2

Binding parameters of (±)-[^{3}H]DOB obtained from saturation experiments with CHOFA4 cells expressing human 5-HT$_{2A}$ receptors

Values represent mean ± S.E.M. of three independent experiments performed with duplicate points.

	K_d	B_{max}
	nM	*fmol/mg of protein*
Control	0.80 ± 0.16	62.85 ± 22.27
	31.22 ± 11.41	307.63 ± 54.84
Plus 100μM Gpp(NH)p	24.44 ± 7.34	346.20 ± 79.89

The subsequent experiments involved competition assays against (±)-[^{3}H]DOB using the same four antagonists that had been used in autoradiographic competition experiments against (±)-[^{125}I]DOI in human brain sections. In the experimental conditions established for (±)-[^{3}H]DOB competition assays (5 nM (±)-[^{3}H]DOB and 200–250 μg of protein/tube), specific binding was about 75% of total binding. In the presence of 100 μM Gpp(NH)p (but absence of competitor drugs) specific binding was reduced by approximately 30% (Fig. 3A). The pharmacological parameters obtained for the different antagonists are summarized in Table 3. In this heterologous system, MDL100,907 and ketanserin also differentiated two binding site populations when displacing (±)-[^{3}H]DOB binding from 5-HT$_{2A}$ receptors. Although the presence of Gpp(NH)p caused a decrease of the maximal (±)-[^{3}H]DOB specific binding, both antagonists still displaced the remaining (±)-[^{3}H]DOB binding in a biphasic manner, without alteration in the observed affinities and percentages of the two sites (Fig. 3, A and B). The other two antagonists, SB242084 and mesulergine, displaced (±)-[^{3}H]DOB binding with low affinity in a monophasic manner. After addition of Gpp(NH)p, they displaced the remaining binding with the same affinities and also monophasically (Table 3). Unexpectedly, SB242084 showed much lower affinity when competing for

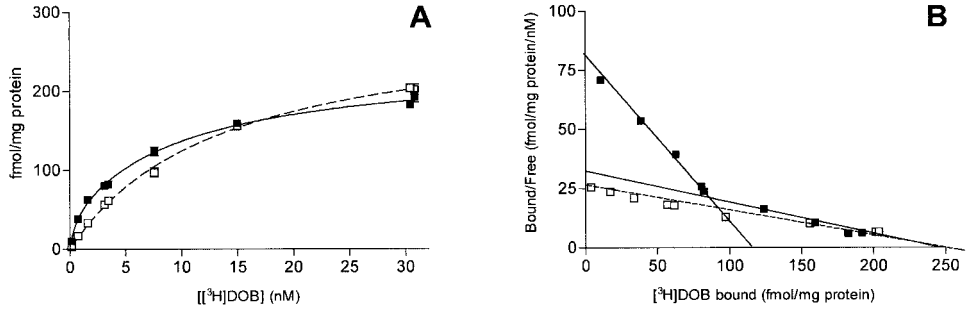

Fig. 2. A, saturation analysis of (±)-[^{3}H]DOB binding to CHOFA4 cells expressing human 5-HT$_{2A}$ receptors in absence (■) or presence (□) of 100 μM Gpp(NH)p. Each point represents the mean ± S.E.M. specific binding of duplicate points from one representative experiment. B, Scatchard plot drawing of the same points plotted in A.

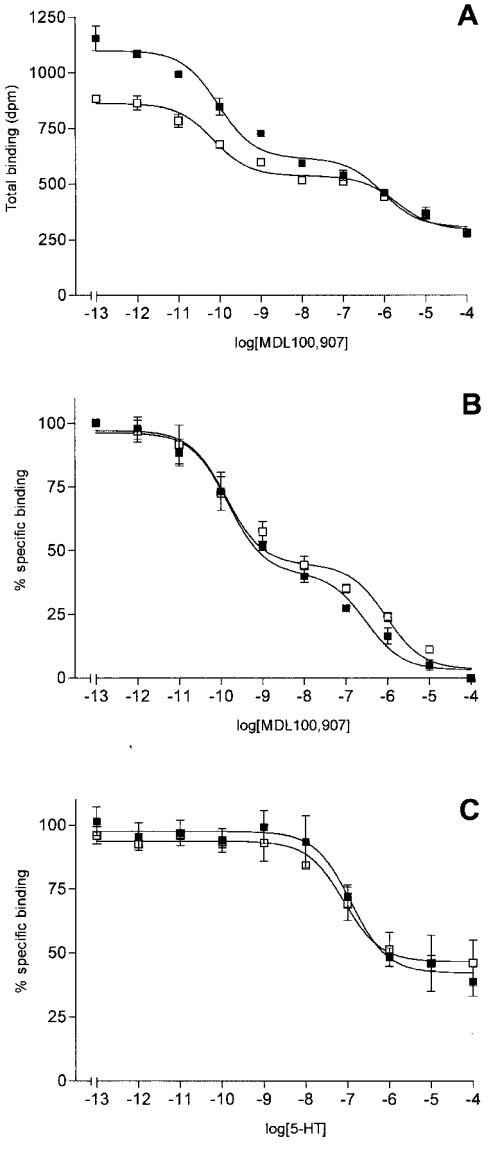

Fig. 3. Competition curves of (±)-[^{3}H]DOB binding in CHOFA4 cells expressing human 5-HT$_{2A}$ receptor in absence (■) or presence (□) of 100 μM Gpp(NH)p by MDL100,907 (A and B) and 5-HT (C). A, each point represents total binding (mean ± S.E.M.) of duplicate points from one representative experiment. B and C, each point represents percentage of specific binding (mean ± S.E.M.) of four independent experiments performed with duplicate points. For the curves with added Gpp(NH)p in B and C, the specific binding remaining in presence of Gpp(NH)p and absence of competing antagonist is taken as 100%.

(±)-[^{3}H]DOB binding in CHOFA4 cells than when competing for the binding of (±)-[^{125}I]DOI in brain regions. We have, at present no clear explanation for this discrepancy. Finally, displacement of (±)-[^{3}H]DOB by another agonist, 5-HT, resulted in monophasic curves presenting a low-affinity site both in absence and presence of Gpp(NH)p (Table 3). Unexpectedly, about 40 to 45% of specific (±)-[^{3}H]DOB binding remained at the maximal concentration of 5-HT tested, with a bottom plateau clearly defined by the last three concentrations: control curve bottom 42.2% ± 3.45 (n = 4 experiments with duplicate points); Gpp(NH)p curve bottom 46.6% ± 3.05 (n = 3 experiments with duplicate points) (Fig. 3C).

Saturation experiments with [^{3}H]5-HT were performed to analyze further the sites bound by serotonin in CHOFA4 cells expressing 5-HT$_{2A}$ receptors. Saturation assays were carried out within two concentration ranges (0–30 and 0–100 nM). Both ranges resulted in very low specific binding (Fig. 4, A and B) and even in the widest range, practically no difference was observed between total and nonspecific binding as determined with 10 μM mianserin (Fig. 3B). Nevertheless, when the first six concentration points of the shortest range were analyzed separately, the resulting specific binding was well fitted by a one-site hyperbolic curve (Fig. 4, C and D). The results of this saturation analysis were as follows: K_d = 1.03 ± 0.09 nM and B_{max} = 10.84 ± 0.35 fmol/mg of protein (n = 2 experiments with duplicate points).

Measurement of [^{3}H]IP Accumulation in CHOFA4 Cells Expressing 5-HT$_{2A}$ Receptors. [^{3}H]IP formation after agonist stimulation was determined in CHOFA4 cells expressing human 5-HT$_{2A}$ receptors. In preliminary experiments, [^{3}H]IP formation assays were carried out in native CHO cells, resulting in no significant [^{3}H]IP accumulation above basal levels after incubation with 10 μM 5-HT or 10 μM (±)-DOI, whereas 100 mM NaF, a direct activator of phospholipase C, increased IP formation about 350% above basal (data not shown). In CHOFA4 cells, 5-HT caused a concentration-dependent increase in PI hydrolysis with a pEC$_{50}$ (−log EC$_{50}$) value of 6.34 ± 0.18 and an E_{max} value of 1156 ± 73.02% over basal (Fig. 5), whereas (±)-DOI caused a stimulation of lower magnitude with pEC$_{50}$ value of 7.33 ± 0.15 and an E_{max} value of 512 ± 26.11% over basal (n = 3 experiments with triplicate points in both cases). The maximal (±)-DOI response represents 44.3% of the maximal response produced by 5-HT, revealing the partial agonist nature of (±)-DOI in this experimental system and corroborating previous results reported with the same cell line (Berg et al., 1994). The antagonist effects of MDL100,907, ketanserin, SB242084, and mesulergine against

TABLE 3

Competition parameters of several compounds for (±)-[^{3}H]DOB-labeled receptors in CHOFA4 cells expressing human 5-HT$_{2A}$ receptors

Values represent mean ± S.E.M. of at least three independent experiments performed with duplicate points.

	−LogIC$_{50}$high	−LogIC$_{50}$low	%High	n_H[a]
MDL 100,907	9.28 ± 0.15	6.51 ± 0.24	59.5 ± 3.9	0.32 ± 0.01
MDL 100,907 plus 100μM Gpp(NH)p	9.87 ± 0.16	6.03 ± 0.22	56.2 ± 3.9	0.29 ± 0.01
Ketanserin	8.03 ± 0.38	5.04 ± 0.18	24.6 ± 4.7	0.44 ± 0.02
Ketanserin plus 100μM Gpp(NH)p	7.66 ± 0.39	4.70 ± 0.27	30.3 ± 5.9	0.38 ± 0.01
SB242084		5.22 ± 0.11		1.00
SB242084 plus 100μM Gpp(NH)p		5.11 ± 0.09		1.00
Mesulergine		6.91 ± 0.12		1.00
Mesulergine plus 100μM Gpp(NH)p		6.57 ± 0.13		1.00
5-HT		6.92 ± 0.18		1.00
5-HT plus 100μM Gpp(NH)p		7.09 ± 0.19		1.00

[a] Hill coefficient.

the response induced by the two agonists are summarized as pA_2 in Table 4, and Fig. 6 displays one representative experiment for each agonist. For each antagonist, no marked differences were found in the pA_2 values obtained with both agonists (Table 4).

Discussion

The main finding of the present report is the detection by the antagonist compounds MDL100,907 and ketanserin of different states/conformations of agonist-labeled human 5-HT$_{2A}$ serotonin receptors. These different populations have been observed with two phenylisopropylamine agonists [(±)-[^{125}I]DOI and (±)-[^{3}H]DOB] and in two systems: a recombinant expression system (CHOFA4 cell line expressing human 5-HT$_{2A}$ receptors) and native intact tissue (different regions of human brain). This recognition of multiple states/conformations of the receptor is unexpected for alleged antagonist compounds such as MDL100,907 and ketanserin. Furthermore, in the same systems and experimental conditions, two other antagonists, mesulergine and SB242084, showed the expected monophasic curves.

Previous reports in human brain had shown that biphasic displacement by ketanserin of (±)-[^{125}I]DOI binding sites most probably corresponds to agonist-labeled 5-HT$_{2A}$ receptor binding sites (Waeber and Palacios, 1994). In the present study, not only ketanserin but also MDL100,907, a new and more selective 5-HT$_{2A}$ receptor antagonist, showed biphasic displacement of (±)-[^{125}I]DOI binding, suggesting that this is not a behavior exclusive of ketanserin. The selectivity of MDL100,907 for 5-HT$_{2A}$ receptors in human brain has been previously demonstrated using its radiolabeled form ([^{3}H]MDL100,907) (López-Giménez et al., 1998). It was also shown that the pattern of distribution of (±)-[^{125}I]DOI-labeled receptors in several human brain regions, in particular in caudate and putamen striosomes, was identical to the pattern revealed by [^{3}H]MDL100,907 (López-Giménez et al., 1999). Taken together, these results strongly support the predominant 5-HT$_{2A}$ receptor nature of the binding sites labeled by (±)-[^{125}I]DOI in the human brain regions considered in the present study. The possibility that the two sites detected by MDL100,907 and ketanserin when displacing (±)-[^{125}I]DOI were merely dependent on the receptor/G protein-coupling status could be discarded by the fact that both compounds still showed biphasic profiles even in the presence of 100 μM Gpp(NH)p, a concentration thought to uncouple receptor/G protein complexes. Nevertheless, the possibility that (±)-[^{125}I]DOI labels other sites in native tissue, different from 5-HT$_{2A}$ receptors and as yet undescribed, cannot be totally excluded. For this reason, similar radioligand binding experiments were performed with CHOFA4 cells permanently expressing human 5-HT$_{2A}$ receptors. In such a heterologous system, possible radioligand binding to other undesired sites is avoided. In this case the radioligand of choice was (±)-[^{3}H]DOB, an agonist compound with a chemical structure very similar to (±)-[^{125}I]DOI (phenylisopropylamines) and that presents the same affinity for human 5-HT$_{2A}$ receptor as its iodinated analog (Nelson et al., 1999).

Saturation experiments with CHOFA4 cells were performed before competition studies and they showed, as expected according to the ternary complex model for an agonist drug, that (±)-[^{3}H]DOB binds to a heterogeneous population of 5-HT$_{2A}$ receptors that contains two different affinity states/components, with the high-affinity one depending on receptor/G protein coupling. Previous studies where (±)-[^{3}H]DOB was used as radioligand to label human 5-HT$_{2A}$ receptors expressed permanently in cell lines, failed to detect two binding site populations in saturation experiments (Branchek et al., 1990; Sleight et al., 1996). Furthermore, in one of these studies (Branchek et al., 1990) the presence of 100 μM Gpp(NH)p in saturation assays caused a decrease of the maximal density of radiolabeled receptor (B_{max}) with no alteration of the radioligand affinity (K_d). This discrepancy with the present results is unlikely to have been caused by methodological issues, because we followed the same exper-

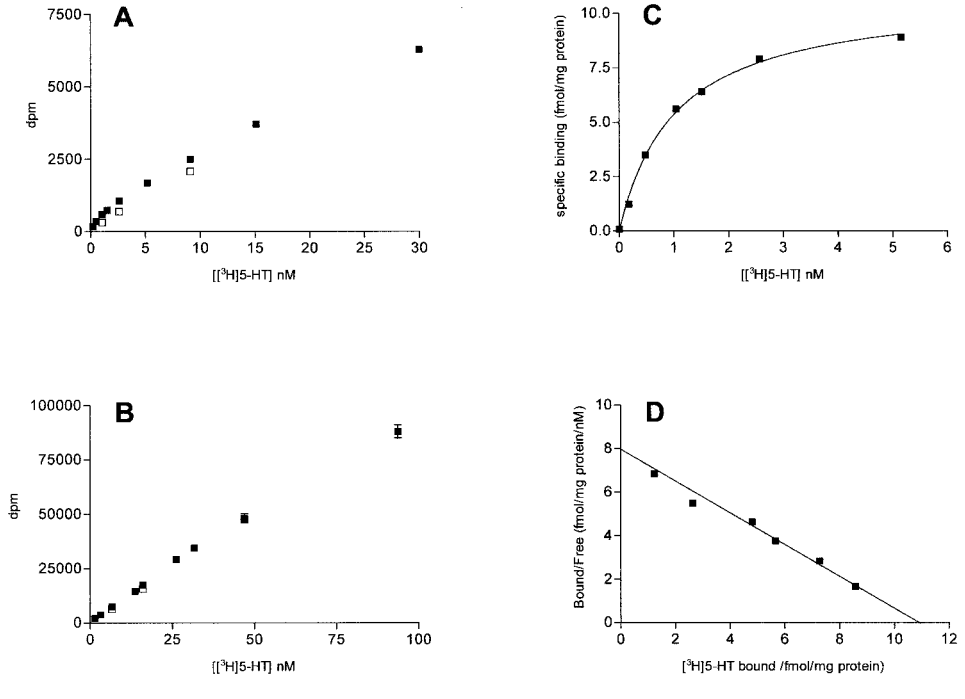

Fig. 4. Saturation analysis of [^{3}H]5-HT binding to membranes from CHOFA4 cells expressing human 5-HT$_{2A}$ receptors. Total (■) and nonspecific binding (□) of a narrow (A) and a wide (B) range of concentrations. Each point represents the mean ± S.E.M. of three independent experiments performed with duplicate points. C, amplification of specific binding corresponding to the points between 0 and 5 nM from A. D, Scatchard plot drawing of the same points represented in C.

imental protocol as Branchek and collaborators, even in those aspects concerning membrane preparation: in both cases, binding assays were run on the same day of cell harvesting and membrane preparation to avoid adverse effects of freezing. In addition, the B_{max} for (±)-[^{3}H]DOB described by these studies was of the same order of magnitude as the one observed in the present study (150 versus 300 fmol/mg of protein). Therefore, the differences observed between the studies can be attributed most probably to the different host cell line used in each case: murine fibroblast L-M(TK−) cells (Branchek et al., 1990) and CHO cells (present work). Differences in the pharmacology of a given recombinant receptor depending on the host expression system have been widely described (for an extensive review, see Kenakin, 1996). With regard to the work by Sleight et al. (1996), in addition to differences in experimental protocol and host cell line (NIH3T3 mouse fibroblast), the 30-fold higher maximal receptor density labeled by (±)-[^{3}H]DOB in their cell line could also explain the discrepancy between their data and our present data, possibly related to a less favorable receptor:G protein stoichiometry in their highly expressed system (Kenakin, 1997b).

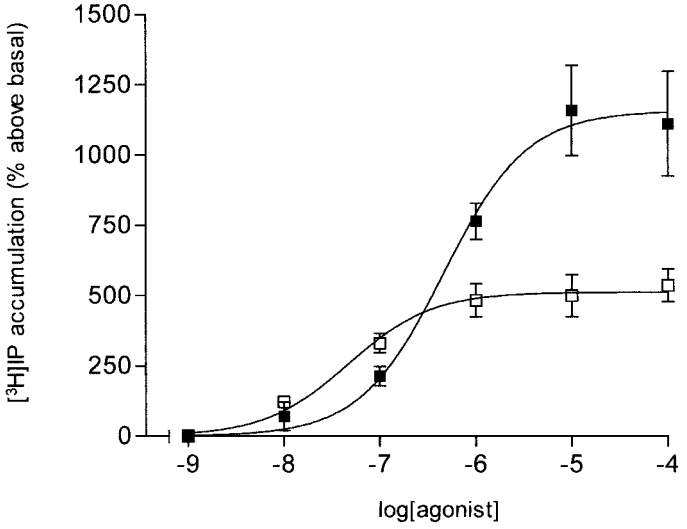

Fig. 5. Concentration-response curves of PI hydrolysis after stimulation with 5-HT (■) and (±)-DOI (□) in CHOFA4 cells expressing human 5-HT$_{2A}$ receptors. Data are expressed as percentage of increase above basal [^{3}H]IP accumulation and represent the mean ± S.E.M. of three independent experiments performed with triplicate points.

TABLE 4

Antagonist potency of several compounds against the response of 5-HT and (±)-DOI ([^{3}H]IP formation) in CHOFA4 cells expressing human 5-HT$_{2A}$ receptors

Values represent mean ± S.E.M. of at least three independent experiments performed with triplicate points.

	pA$_2$	
	5-HT	(±)-DOI
MDL 100,907	8.73 ± 0.46	9.34 ± 0.06
Ketanserin	8.45 ± 0.25	9.16 ± 0.30
SB242084	7.17 ± 0.33	7.47 ± 0.15
Mesulergine	7.27 ± 0.30	7.14 ± 0.30

The biphasic displacement profile that had been observed for MDL100,907 and ketanserin when displacing (±)-[^{125}I]DOI from human brain regions was observed as well when these compounds displaced (±)-[^{3}H]DOB binding from human 5-HT$_{2A}$ receptors expressed in CHOFA4 cells. Furthermore, these two sites were still observed in presence of 100 μM Gpp(NH)p (i.e., even when receptor/G protein coupling was impeded). It might be argued that the biphasic displacement curves observed in the presence of Gpp(NH)p may in fact reflect some level of residual coupling between receptor and G protein that has not been completely canceled in the present experimental conditions (Kenakin et al., 1995). However, evidence against this comes from the (±)-[^{3}H]DOB saturation experiments (performed in the same conditions as the competition experiments). Two clearly different sites were observed in presence of receptor/G protein coupling, whereas addition of 100 μM Gpp(NH)p resulted in complete loss of the high-affinity site (receptor coupled to G protein) with no alteration of the parameters (B_{max} and K_d) of the low-affinity site (receptor not coupled to G protein).

This atypical profile observed for MDL100,907 and ketanserin may be interpreted on the basis of the extended ternary complex model of receptor action (Lefkowitz et al., 1993). According to this model, a receptor can exist as an equilibrium of different conformational states that include the inactive or ground state (R), partly activated form(s) that have been called R*, and the activated form coupled to G proteins (R*G) (for review, see Strange, 1999). Certain drugs would present preferential affinity for some of these receptor conformations. Indeed, an agonist drug could induce and stabilize some of these receptor conformations, in particular R* and R*G (Kenakin, 1995, 1997a). According to this model, the stabilization of an intermediate state (R*) by agonist means that agonist binding in the absence of G protein coupling will not reflect the true ground state (R) of the receptor (Strange, 1998). Several experimental findings obtained from rat 5-HT$_{2A}$ receptors have already been explained in the light of

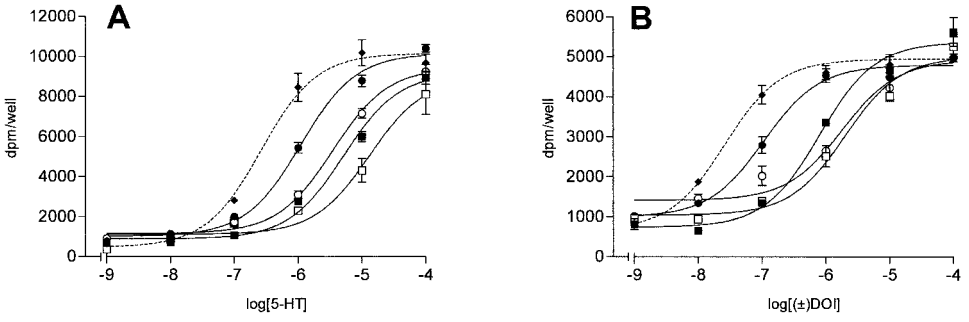

Fig. 6. Concentration-response curves of PI hydrolysis with 5-HT (A) and (±)-DOI (B) stimulation in the absence (◆, dotted line) and in the presence of several antagonists: ■, 30 nM MDL100,907; □, 100 nM ketanserin; ●, 1 μM SB242084; ○, 1 μM mesulergine. Results correspond to one representative experiment. Each point represents mean ± S.E.M. of triplicate determinations.

this model. Thus, there is evidence from mutated receptors (Roth et al., 1997) supporting the hypothesis that the ability of an agonist to promote the high-affinity state of the rat 5-HT$_{2A}$ receptor is not necessarily correlated with its ability to generate second messenger production, which is consistent with the existence of R*. Similarly, recent results comparing binding affinity and intrinsic efficacy of several agonists at rat 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors in heterologous systems were also best modeled by assuming the existence of R* (Egan et al., 2000). However, another recent report carried out with human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors in recombinant systems (Fitzgerald et al., 1999), where affinities and intrinsic activities of several agonists were compared as well, supported the classical two state model of the ternary receptor complex (R and R*G) rather than the three-state model (R, R*, and R*G). Our present results concerning MDL100,907 and ketanserin are in agreement with the extended ternary complex model (three states) because we observed that these compounds differentiated heterogeneous binding site populations even when 5-HT$_{2A}$ receptors are uncoupled from G proteins, both in a recombinant expression system (CHOFA4 cells) and in native intact tissue (human brain). Thus, it can be postulated that the radiolabeled agonists, in the absence of Gpp(NH)p, label R, R*, and R*G receptor conformations and the antagonists MDL100,907 and ketanserin are able to discriminate two of these three subpopulations of human 5-HT$_{2A}$ receptors, most likely R on one hand and (R* + R*G) on the other hand. In the presence of Gpp(NH)p, the R*G population uncouples, the remaining subpopulations reequilibrate, and the competitors recognize two populations (R and R*) with essentially unaltered affinities. According to the extended ternary complex model, however, true silent antagonists present the same affinity for the different receptor states/conformations and therefore should not recognize different receptor subpopulations. Thus, the present discrimination of receptor subpopulations by MDL100,907 and ketanserin is not consistent with the behavior expected for a true silent antagonist and may be taken as an indication that these two compounds may possess some degree of intrinsic efficacy (positive or negative). In fact, inverse agonist activity of ketanserin has been described in a mutated rat 5-HT$_{2A}$ receptor, which showed constitutive activity (Egan et al., 1998). Nevertheless, a definite ascription of some degree of intrinsic efficacy to these compounds can only be established in functional assays (see below) and the present results from radioligand binding studies can only be taken as an indirect suggestion in need of functional confirmation.

The observation of multiple agonist-labeled receptor states/conformations can be taken as an indication that the receptor:G protein stoichiometry of the systems studied is such that complete receptor/G protein complexation is not possible due to shortage of G protein (Kenakin, 1997b) or to restricted access of receptors to G proteins (Strange, 1999). Because receptor conformations can interconvert and agonists stabilize R* and ultimately R*G conformations (Strange, 1999), if enough G protein was available for complexation then the observed labeled conformation with an agonist radioligand (A) would be AR*G. Alternatively, the observation of multiple agonist-labeled receptor conformations can also be due to the partial agonist nature of (±)-[^{125}I]DOI and (±)-[^{3}H]DOB, which would be therefore unable to fully convert receptors to the AR*G conformation (Kenakin, 1997b).

Attempts were made to elucidate whether the heterogeneous populations of 5-HT$_{2A}$ receptors recognized by the antagonists MDL100,907 and ketanserin when competing against (±)-[^{125}I]DOI and (±)-[^{3}H]DOB were also detected when competing against the endogenous agonist 5-HT. For this purpose, saturation experiments were performed with [^{3}H]5-HT in CHOFA4 cells. It was anticipated that labeling of 5-HT$_{2A}$ receptors with [^{3}H]5-HT would be difficult to accomplish given the low affinity that 5-HT presents for human 5-HT$_{2A}$ receptors (e.g., IC$_{50}$ = 100 nM when displacing (±)-[^{3}H]DOB). Unexpectedly, however, in the present saturation experiments performed with [^{3}H]5-HT in CHOFA4 cells, a high-affinity site with a very low maximal density was detected when analyzing a low, narrow radioligand concentration range (0–5 nM). In fact, CHO native cells have been previously shown to express endogenous 5-HT$_{1B}$ receptors, which upon saturation experiments with [^{3}H]5-HT, result in small amounts of specific binding only at low concentrations of radioligand (Giles et al., 1996), very similar to the present results (compare our Fig. 4A with Fig. 6 in Giles et al., 1996). Furthermore, the authors that provided us with the CHOFA4 cell line also described expression of 5-HT$_{1B}$ receptors in these cells by means of functional assays (inhibition of forskolin-stimulated cAMP accumulation) (Berg et al., 1994). Thus, the data indicate that the high-affinity, low-capacity site observed in CHOFA4 cells with low concentrations of [^{3}H]5-HT corresponds to 5-HT$_{1B}$ receptors expressed natively in CHO cells. In experiments with (±)-[^{3}H]DOB, no interference of this small population of endogenous 5-HT$_{1B}$ receptors can be suspected because this radioligand shows extremely low affinity for 5-HT$_{1B}$ receptors (K_i = 831 nM; Titeler et al., 1987). Nevertheless, competition experiments were carried out with GR127935 (a 5-HT$_{1B/1D}$ receptor antagonist; Skingle et al., 1996; Doménech et al., 1997) against (±)-[^{3}H]DOB to completely rule out a possible labeling of 5-HT$_{1B}$ receptors by this radioligand in CHOFA4 cells. These experiments revealed the existence of a single site with pIC$_{50}$ value of 6.9 (J. F. López-Giménez, G. Mengod, M. T. Vilaró, unpublished observations), in agreement with the described low affinity of GR127935 for 5-HT$_{2A}$ receptors (pK_i = 7.8), and different from the expected high affinity of GR127935 for 5-HT$_{1B}$ receptors (pK_i = 9.0) (Price et al., 1997). A part from this 5-HT$_{1B}$ high-affinity component of [^{3}H]5-HT binding, no specific binding to 5-HT$_{2A}$ receptors in CHOFA4 cells could be detected even at concentrations as high as 100 nM, precluding the use of the radiolabeled endogenous agonist for competition experiments. In fact, only one report exists, to our knowledge, where direct labeling of 5-HT$_{2A}$ receptors with [^{3}H]5-HT has been achieved. In that study (Sleight et al., 1996), the cloned human 5-HT$_{2A}$ receptor was expressed in NIH3T3 cells at extremely high densities (B_{max} for [^{3}H]ketanserin = 27684 fmol/mg of protein, B_{max} for (±)-[^{3}H]DOB = 8332 fmol/mg of protein, K_d for (±)-[^{3}H]DOB = 0.8 nM). In this system, [^{3}H]5-HT labeled with high affinity (K_d = 1.3 nM) approximately 12% of the sites labeled by [^{3}H]ketanserin and 41% of the sites labeled by (±)-[^{3}H]DOB. Given that our heterologous system expresses much lower densities of 5-HT$_{2A}$ receptors [B_{max} of the high affinity (K_d = 0.8 nM) (±)-[^{3}H]DOB site = 62 fmol/mg of protein], the population of receptors in a high-affinity state/conformation for [^{3}H]5-HT

may be too small to be detected with the current methodology. However small, this population must be very efficiently coupled to the second messenger system studied in the present work, because 5-HT showed a very robust response in the [^{3}H]IP formation assays.

In competition experiments with 5-HT against (±)-[^{3}H]DOB in our heterologous expression system, there was 40 to 45% of (±)-[^{3}H]DOB specific binding that was not displaced even at very high concentrations of 5-HT and both in the presence and in the absence of Gpp(NH)p. This indicates that a fraction of the 5-HT$_{2A}$ receptors labeled by (±)-[^{3}H]DOB in this system is absolutely refractory to binding of 5-HT. This population can be reasonably assigned to the ground state (R) of the receptor if it is assumed that (±)-[^{3}H]DOB labels the R and R* conformations of 5-HT$_{2A}$ receptors in the presence of Gpp(NH)p and R, R*, and R*G conformations in the absence of the GTP analog.

Given the marked differences in the magnitude of the population recognized with high affinity by [^{3}H]5-HT and by (±)-[^{3}H]DOB (Sleight et al., 1996; this study), it was of interest to study the functional effects of these two agonists classes in the PLC pathway and the effects of the antagonists. The magnitude of the response elicited by 5-HT was much higher than that of (±)-DOI, suggesting that 5-HT may induce more efficient coupling with the effector system or, alternatively and more speculative, that the 5-HT- and (±)-DOI-elicited PLC stimulation are mediated by different receptor conformations.

In the experiments with the antagonists, no indication was observed of a behavior other than that expected for a neutral antagonist. Despite the much stronger response elicited by 5-HT, the potencies of each antagonist in the assays with the two agonists did not differ significantly in functional experiments, as would be expected in classical operational pharmacology for antagonists that compete at the same receptor. However, it has to be taken into account that we have no indication of the existence of constitutive receptor activity in our heterologous expression system. Therefore, if any of the supposedly antagonist compounds were in fact inverse agonists, our present functional assays would not allow detection of such inverse agonism and the compounds would display antagonist behavior. The fact that an apparently heterogeneous, multiple conformation receptor system (as detected in radioligand binding studies) results in a classical functional pharmacology (as shown in PLC stimulation experiments), stresses the importance of combining, whenever possible, both experimental approaches. Ideally, also, the availability of a system expressing constitutively active human 5-HT$_{2A}$ receptors would allow for the unequivocal detection of putative negative efficacy for those compounds whose receptor binding behavior is not consistent with true neutral antagonism.

In conclusion, the atypical pharmacological profile observed for some supposedly antagonist drugs when interacting with agonist-labeled human 5-HT$_{2A}$ receptors provides evidence for the existence of multiple conformations/states of these receptors in a recombinant system as well as in native intact human tissue. Further experiments involving other 5-HT$_{2A}$ compounds will improve the understanding of drug/5-HT$_{2A}$ receptor interaction and of the action of these drugs as therapeutic agents in neuropsychiatric disorders.

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