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Characterization of a Novel and Potent 5-Hydroxytryptamine$_{1A}$ Receptor Antagonist

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LIAU, L. M., A. J. SLEIGHT, J. PITHA AND S. J. PEROUTKA. *Characterization of a novel and potent 5-hydroxytryptamine$_{1A}$ receptor antagonist.* PHARMACOL BIOCHEM BEHAV **38**(3) 555–559, 1991.—A series of pindolol derivatives (n = 7) was analyzed in radioligand binding, biochemical and behavioral studies. Three of these drugs (Compounds A, B, and C) are extremely potent (i.e., K_i values <1.0 nM) at 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) sites labeled by [^{3}H] 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT). Moreover, these drugs are selective in that they are approximately an order of magnitude less potent at beta-adrenergic receptors labeled by ^{3}H-dihydroalprenolol (DHA). Compound A (N^1-(bromoacetyl)-N^8-[3-(4-indolyloxy)-2-hydroxypropyl]-(Z)-1,8-diamino-p-methane) is also significantly less potent at 10 other neurotransmitter receptor sites analyzed. In addition, Compound A (10^{-10} M to 10^{-3} M) has no effect on baseline forskolin-stimulated adenylate cyclase activity in rat hippocampus. By contrast, nanomolar concentrations of the drug significantly ($p<0.01$) reverse 8-OH-DPAT-induced inhibition of forskolin-stimulated activity. In behavioral studies, Compound A (0.5 mg/kg) alone has no effect on baseline measures of reciprocal forepaw treading in the rat. Pretreatment with Compound A, however, significantly ($p<0.05$) inhibits the reciprocal forepaw treading induced by 8-OH-DPAT. These data suggest that Compound A is a potent and selective antagonist of 5-HT$_{1A}$ receptors in the CNS.

5-HT$_{1A}$ receptors Adenylate cyclase 5-HT behavioral syndrome

DURING the past few years, significant progress has been made in the molecular, pharmacological, biochemical and behavioral analysis of 5-HT$_{1A}$ receptors. For example, the amino acid structure of the 5-HT$_{1A}$ receptor was first reported in 1988 (8). At the biochemical level, the 5-HT$_{1A}$ receptor modulates adenylate cyclase activity (3,5). The modulation of cyclase activity by 5-HT$_{1A}$ receptors is specifically mediated via a pertussis-sensitive G protein (1,11). Behaviorally, the 5-HT$_{1A}$ receptor appears to mediate certain aspects of the 5-HT behavioral syndrome, such as reciprocal forepaw treading, head weaving, flat body posture and tremor (25). Clinically, a role for the 5-HT$_{1A}$ receptor in the pathophysiology of anxiety, depression, and hallucinogenic behavior has been proposed (18,24).

Pharmacologically, a large number of potent and selective 5-HT$_{1A}$ agents have now been described. Drugs such as 5-HT and 8-OH-DPAT are full agonists at this receptor, whereas drugs such as buspirone, ipsapirone, and BMY-7378 act as partial agonists (3,9). By contrast, only a limited number of 5-HT$_{1A}$ antagonists, such as (−)pindolol, (−)propranolol, and more recently NAN-190 and spiroxatrine have been described (9, 15, 16, 25). However, such antagonists tend to lack selectivity, as they are nearly equipotent at 5-HT$_{1A}$ and 5-HT$_{1B}$ sites. Because of the potential scientific and clinical usefulness of selective 5-HT$_{1A}$ antagonists, we have analyzed a series of recently developed agents (21) for their ability to interact with 5-HT$_{1A}$ and other neurotransmitter receptor binding sites.

METHOD

Radioligand Binding Assay

Radioligand binding studies were performed as described previously (19). Briefly, frozen rat and pig brains were purchased from Pel-Freez (Rogers, AR) and cow brains from Ferrara Meats (San Jose, CA). On the day of study, the samples were thawed in Tris-HCl buffer. Cortices were obtained from a dissection of whole rat brains by removing the noncortical tissues. Tissues were homogenized in 20 volumes of Tris-HCl buffer (pH 7.7 at 25°C), using a Brinkman Polytron and then centrifuged in an IEC B20A centrifuge at 49,000 × g for 10 minutes. The supernatant was discarded and the pellet was resuspended in the same volume of Tris-HCl buffer and incubated at 37°C for 10 minutes prior to a second centrifugation at 49,000 × g for 10 minutes. The final pellet was resuspended in 80 volumes of Tris-HCl buffer containing 10 μM pargyline, 4 mM calcium chloride and 0.1% ascorbic

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acid. The suspensions were immediately used in the binding assay. Radioligand binding studies consisted of 0.1 ml of ^{3}H-radioligand, 0.1 ml buffer or displacing drug, and 0.8 ml tissue suspension. Following incubation at 25°C for 30 minutes, the assays were rapidly filtered under vacuum through No. 32 glass fiber filters (Schleicher and Schuell; Keene, NH) with two 5 ml washes using 50 mM Tris-HCl buffer. Radioactivity was measured by liquid scintillation spectroscopy in 5 ml of 3a70 Counting Cocktail (Research Products International; Mt. Prospect, IL) at 54% efficiency.

All radioligand assays were performed using rat cortical membranes, except as noted below. Specific binding was defined as the excess over blanks taken in the presence of 0.1 ml ^{3}H-radioligand: 10^{-5} M 5-HT for 5-HT_{1A} sites labeled by 0.25 nM ^{3}H-8-OH-DPAT, 10^{-5} M 5-HT for 5-HT_{1B} sites labeled by 1.4 nM ^{3}H-5-HT (in the presence of 10^{-7} M 8-OH-DPAT and 10^{-6} M mianserin), 10^{-5} M 5-HT for 5-HT_{1C} sites labeled by 0.80 nM ^{3}H-mesulergine in pig cortex, 10^{-5} M 5-HT for 5-HT_{1D} sites labeled by 1.4 nM ^{3}H-5-HT in bovine caudate (in the presence of 10^{-7} M 8-OH-DPAT and 10^{-7} M mesulergine), 10^{-6} M cinanserin for 5-HT_2 sites labeled by 0.30 nM ^{3}H-spiperone, 10^{-7} M ICS 205-930 for 5-HT_3 sites labeled by 0.80 nM ^{3}H-quipazine, 10^{-6} M prazosin for $alpha_1$-adrenergic sites labeled by 0.90 nM ^{3}H-WB-4101, 10^{-4} M yohimbine for $alpha_2$-adrenergic sites labeled by 0.90 nM ^{3}H-rauwolscine, 10^{-6} M propranolol for beta-adrenergic receptors labeled by 0.20 nM ^{3}H-dihydroalprenolol (^{3}H-DHA), 10^{-6} M (+)butaclamol for $dopamine_1$ sites labeled by 0.30 nM ^{3}H-SCH 23390 in rat striatum, 10^{-6} M (+)butaclamol for $dopamine_2$ sites labeled by 0.30 nM ^{3}H-spiperone in rat striatum, and 10^{-6} M scopolamine for muscarinic cholinergic sites labeled by 0.10 nM ^{3}H-QNB. Drugs were dissolved and diluted in ethanol and assay buffer. These conditions were consistent with methods described previously (14).

IC_{50} values were determined by log-logit analysis of drug competition studies. K_i values were determined by the equation $K_i = IC_{50}/(1 + [I]/K_D)$, where K_D was derived from the literature for each receptor binding site. Each experiment was performed in triplicate and repeated 3–6 times.

Adenylate Cyclase Assay

Male Sprague-Dawley rats (180–200 g) were killed by decapitation, and the hippocampi were removed. Tissue medium was prepared daily (pH 7.4 at room temperature) and contained 300 mM sucrose, 20 mM Tris-HCl, 1 mM ethylene glycol bis-(beta-aminoethylether)-N,N′-tetraacidic acid (EGTA), 5 mM Na_2-EDTA, and 5 mM dithiothreitol (DTT). The hippocampi from each rat were homogenized by hand in 4 ml of ice-cold tissue medium. The homogenate was diluted 1:8 with medium and centrifuged at 39,000 × g for 10 minutes at 4°C. Pellets were resuspended in the same volume used for homogenization. This particulate fraction was put on ice and assayed within an hour (5).

Adenylate cyclase activity was determined by measuring the conversion of [alpha-^{32}P]ATP to [^{32}P]cAMP. The reaction was initiated with 50 μl (50–80 μg) of the membrane preparation. The final assay mixture (200 μl) consisted of 25 mM Tris-HCl (pH 7.4), 0.2 mM ATP, 5 mM magnesium acetate, 10 μM GTP, 10 μM GTP, 10 μM pargyline, 0.6 mM ascorbate, 4 mM theophylline, 50 μM cAMP, 50 μg of creatine phosphokinase, 5 mM creatine phosphate, 1.5 μCi of [alpha-^{32}P]ATP, 60 mM sucrose, 0.2 mM EGTA, 1 mM Na_2-EDTA, 1 mM dithiothreitol and various concentrations of drugs. The incubation was carried out at 30°C for 10 minutes. Assays were stopped by the addition of 100 μl of a solution containing 2% sodium lauryl sulfate, 45 mM ATP, and 1.3 mM cAMP in Tris-HCl buffer (pH 7.4). After addition of [^{3}H]cAMP, 0.53–2.1 pmole (15,000 cpm) to monitor recovery, the samples were boiled for 3 minutes and cooled to room temperature. Labeled cAMP was isolated by sequential chromatography on a Dowex 50 cation exchanger and on a neutral alumina column (22). Adenylate cyclase activity was expressed as pmole of cAMP/min/mg of protein, as measured by the technique of Lowry et al. (13).

Behavioral Observations

The methods for behavioral studies were modified from those described by Tricklebank et al. (25). Male Wistar rats (200–300 g) were fed a standard laboratory diet and tap water. They were housed in groups of 3 for at least 2 to 4 days before use, under a 12-h light–12-h dark cycle. Experiments were carried out between 1200 and 1600 hours. Animals were placed singly in clear plastic cages, 430 × 290 × 160 mm high, 15 minutes before injection with drug. The experiments were blind in that the observer did not know which injection each rat received. Observation sessions of 2 minutes duration began 10 minutes later and were repeated every 6 minutes over a period of 30 minutes. Reciprocal forepaw treading, head weaving, flat body posture, and straub tail were scored using a ranked intensity scale from 0 to 3: 0 = absent, 1 = equivocal, 2 = present, and 3 = intense/constant.

After 30 minutes of pretreatment with either Compound A (0.5 mg/kg subcutaneous), (+)pindolol (1 mg/kg subcutaneous), or saline, an injection of 8-OH-DPAT (0.032, 0.16, 0.25, 0.50 or 1.0 mg/kg) was given. Observation sessions of 2 minutes duration were again repeated 10 minutes later, using the same time intervals and scoring scales as described above. Each score was summed over the 6 observation periods and averaged for 6 different animals. Statistical analyses were performed using both the Mann-Whitney U-test and analysis of variance. A probability of $p<0.05$ was regarded as significant (25).

Drugs

Drug sources were as follows: ^{3}H-radioligands (Dupont-New England Nuclear; Boston, MA); 5-HT, GTP, ATP (disodium salt), cyclic AMP, creatine phosphate, creatine phosphokinase, EGTA, theophylline and pargyline (Sigma Chemical Company; St. Louis, MO); (−) and (+)pindolol (Sandoz, Inc.; East Hanover, NJ); and [^{3}H]8-OH-DPAT (100 Ci/mmol) and [^{32}P]ATP (54 Ci/mmol) (Dupont-New England Nuclear; Boston, MA). Drugs were dissolved in ethanol to 10^{-2} M and then diluted in assay buffer. Synthesis and preparation of the evaluated compounds A through G were previously described by Pitha et al. (21).

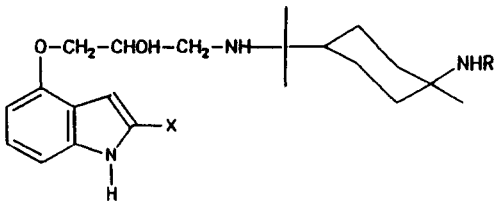
RESULTS

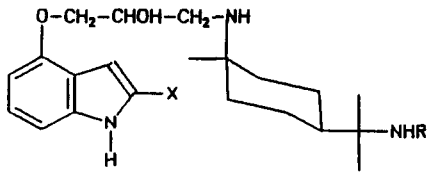
Drug Interactions With 5-HT_{1A} and Beta-Adrenergic Receptors

A series of 7 agents (Fig. 1), as well as (−) and (+)pindolol, were analyzed in radioligand binding studies. As shown in Table 1, (−)pindolol is essentially equipotent at 5-HT_{1A} and beta-adrenergic receptor binding sites, while (+)pindolol is a significantly more selective beta-adrenergic agent. By contrast, three of the novel compounds (Compounds A, B, and C) are unique in that they are extremely potent (i.e., K_i values <1.0 nM) and relatively selective for the 5-HT_{1A} binding site. These agents are approximately an order of magnitude less potent at beta-adrenergic receptors labeled by ^{3}H-DHA (Table 1). Compounds D, E, F, and G, like (−) and (+)pindolol, are more selective for beta-adrenergic receptors. These agents display a similar affinity for 5-HT_{1A} binding sites, and they are slightly less potent than Compounds A–C.

Compound A Interactions With CNS Neurotransmitter Binding Sites

Drug competition studies were performed in order to deter





COMPOUND A: X=H, R=-CO-CH_2Br

COMPOUND B: X=H, R=-CO

COMPOUND C: X=H, R=-CO-CH
HC-$COOCH_3$

COMPOUND D: X=CN, R=-CO-CH_2Br

COMPOUND E: X=H, R=-CO-CH_2Br

COMPOUND F: X=H, R=-CO

COMPOUND G: X=H, R=-CO-CH
HC-$COOCH_3$

FIG. 1. Structure of agents analyzed in the present study.

mine the inhibitory concentration$_{50}$ (IC_{50}) of Compound A at 10 other neurotransmitter receptor binding sites. K_i values were then determined by the Cheng-Prusoff equation. Results indicate that Compound A is most potent at 5-HT$_{1A}$ binding sites labeled by ^{3}H-8-OH-DPAT in rat cortex. As noted above, the drug is 10-fold less potent at beta-adrenergic sites labeled by ^{3}H-DHA (Table 2). By contrast, Compound A is approximately three orders of magnitude less potent at 5-HT$_{1D}$ and alpha$_2$-adrenergic sites, and is essentially inactive at the other sites tested (i.e., K_i >1000 nM). These data indicate that Compound A interacts primarily with 5-HT$_{1A}$ receptors, moderately with beta-adrenergic receptors, and minimally with the 10 other neurotransmitter receptor sites examined.

TABLE 1

DRUG POTENCIES AT 5-HT$_{1A}$ AND BETA-ADRENERGIC RECEPTORS IN RAT CORTEX

	Drug Potency (K_i, nM)		
Drug	5-HT$_{1A}$ (^{3}H-8-OH-DPAT)	Beta-adrenergic (^{3}H-DHA)	Ratio (beta/5-HT$_{1A}$)
(−)Pindolol	6.4 ± 3	4.8 ± 2	0.8
(+)Pindolol	1,400 ± 400	60 ± 1	0.04
A	0.71 ± 0.1	6.7 ± 0.7	9
B	0.86 ± 0.1	18 ± 9	20
C	0.96 ± 0.3	8 ± 5	8
D	4.3 ± 2	0.9 ± 0.02	0.2
E	5.1 ± 2	1.1 ± 0.2	0.2
F	20 ± 9	3.6 ± 3	0.2
G	26 ± 8	2.3 ± 0.7	0.1

Radioligand binding studies were performed as described in the Method section. Data shown are the mean ± standard error of 3–4 experiments, each performed in triplicate on individual brain samples.

8-OH-DPAT and Compound A Effects on Forskolin-Stimulated Adenylate Cyclase Activity

In the rat hippocampal membrane preparation, basal adenylate cyclase activity is approximately 15 pmol/mg protein/min. Forskolin (10^{-5} M) causes an approximately 8-fold stimulation of basal adenylate cyclase activity. As shown in Fig. 2, the forskolin-induced stimulation is inhibited by 8-OH-DPAT at concentrations above 10^{-9} M. At a concentration of 10^{-6} M 8-OH-DPAT, the amount of forskolin-stimulated adenylate cyclase activity is reduced to 70 ± 6% of initial levels, with only minimal additional effects with 8-OH-DPAT concentrations up to 10^{-3} M. By contrast, Compound A (10^{-10} M to 10^{-3} M) shows no significant alteration of forskolin-stimulated cyclase activity in rat hippocampal membranes (Fig. 2).

The ability of Compound A to modulate 8-OH-DPAT inhibition of forskolin-stimulated adenylate cyclase activity was determined under the experimental conditions described in the Method section. A concentration of 10^{-6} M 8-OH-DPAT was used in all the assays, and increasing concentrations of Compound A were

TABLE 2

POTENCIES OF *COMPOUND A* AT NEUROTRANSMITTER RECEPTOR SITES

Receptor	Radioligand	Drug Potency (K_i, nM)
Serotonergic		
5-HT_{1A}	^{3}H-8-OH-DPAT	0.71 ± 0.01
5-HT_{1B}	^{3}H-5-HT	1300 ± 100
5-HT_{1C}	^{3}H-Mesulergine	33000 ± 3000
5-HT_{1D}	^{3}H-5-HT	300 ± 100
5-HT_2	^{3}H-Spiperone	9400 ± 100
5-HT_3	^{3}H-Quipazine	4300 ± 200
Adrenergic		
$Alpha_1$	^{3}H-WB-4101	6700 ± 500
$Alpha_2$	^{3}H-Rauwolscine	460 ± 200
Beta	^{3}H-DHA	6.7 ± 0.7
Dopaminergic		
$Dopamine_1$	^{3}H-SCH 23390	83000 ± 5000
$Dopamine_2$	^{3}H-Spiperone	3700 ± 900
Cholinergic		
Muscarinic	^{3}H-QNB	>100,000

Radioligand binding assays were performed using rat membranes as described in the Method section. Values shown are the means ± standard errors of 3–5 experiments, each performed in triplicate.

tested. As shown in Fig. 3, nanomolar concentrations of Compound A reverse the inhibition of the forskolin-induced stimulation caused by 8-OH-DPAT. The initial effects of such reversal are shown at concentrations as low as 10^{-9} M of Compound A. In the presence of micromolar amounts of Compound A, the inhibition produced by 8-OH-DPAT is completely reversed.

Behavioral Effects of Compound A

There is no observable behavioral change in the rat following subcutaneous (SC) injection of either saline or Compound A (0.5 mg/kg). Both control and experimental animals received total scores of 0 to 1 in all of the 4 behavioral effects that were investigated (i.e., reciprocal forepaw treading, head weaving, straub tail, and adoption of a flat body posture). A separate set of rats were then injected with either saline (control group), Compound A (0.5 mg/kg SC), or (+)pindolol (1 mg/kg SC) prior to injection of various doses of 8-OH-DPAT. At all doses of 8-OH-DPAT tested, the reciprocal forepaw treading scores of the control rats are significantly greater ($p<0.05$) than those of rats pretreated with Compound A (Fig. 4). Since Compound A also has a high affinity for beta-adrenergic receptors, the effect of (+)pindolol (1 mg/kg SC) on the behaviors induced by 8-OH-DPAT was also quantitated. As shown in Fig. 4, (+)pindolol has no significant effect on the forepaw treading induced by 8-OH-DPAT.

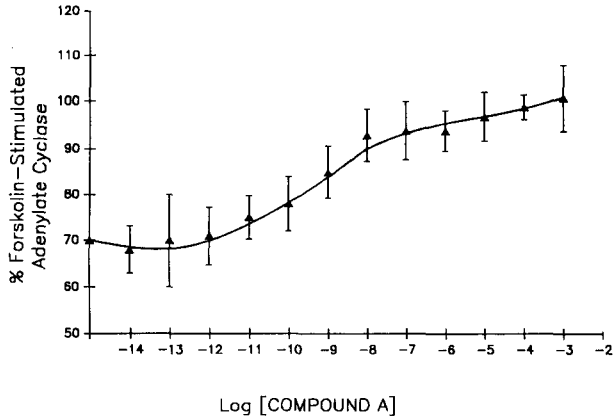


FIG. 3. Reversal of the effects of 8-OH-DPAT on forskolin-stimulated adenylate cyclase activity by Compound A. Adenylate cyclase activity was determined as described in the Method section. In all assays 10^{-6} M 8-OH-DPAT was present. Increasing concentrations of Compound A were analyzed. The data points represent the mean of 6 assay experiments, each performed in duplicate; vertical bars represent ± 1 S.E.M.

DISCUSSION

The major finding of the present study is that Compound A is a potent and relatively selective 5-HT_{1A} receptor antagonist. In addition, Compounds A–C are extremely potent at 5-HT_{1A} bind-

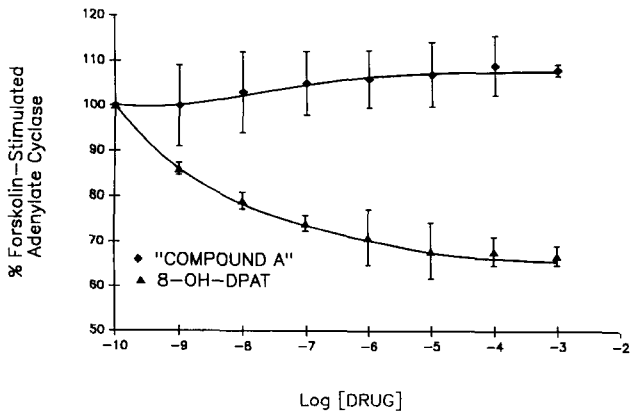


FIG. 2. 8-OH-DPAT and Compound A effects on forskolin-stimulated adenylate cyclase activity. Adenylate cyclase activity was determined as described in the Method section. Various concentrations of 8-OH-DPAT and Compound A were used to determine drug effects on forskolin-stimulated adenylate cyclase activity. The curves were normalized to 100% for each experiment. Data shown are the mean results of 6 experiments, each performed in duplicate; vertical bars represent ± 1 S.E.M.

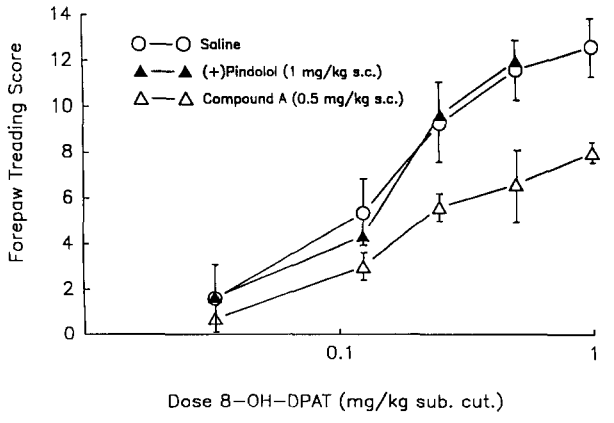


FIG. 4. Behavioral response to 8-OH-DPAT in rats pretreated with Compound A, (+)pindolol, or saline. The intensity of reciprocal forepaw treading was scored 10–40 minutes after various doses of 8-OH-DPAT. Scores were summed over six observation periods lasting 2 minutes each. Data were analyzed using analysis of variance and the Mann-Whitney U-test. Points on the graph represent the mean scores from 6 rats at each dose; vertical bars represent ± 1 S.E.M.

ing sites. Indeed, they represent some of the most potent agents that have been described to date at this 5-HT receptor subtype. Moreover, these compounds display 10- to 20-fold selectivity for the 5-HT$_{1A}$ site versus beta-adrenergic binding sites. This pharmacological profile is unique since 5-HT$_{1A}$ antagonists such as (−)pindolol and (−)propranolol are either equipotent or more potent at beta-adrenergic receptors (7,17).

Of the series of drugs evaluated, Compound A was the most readily synthesized. Therefore, it was chosen for further evaluation using radioligand binding, biochemical and behavioral models of 5-HT$_{1A}$ receptor activation. In the adenylate cyclase assay system, the conversion of radioactive ATP to cAMP was measured. Compound A has no direct significant effect on forskolin-stimulated cyclase activity. However, it reverses the inhibitory effect of the selective 5-HT$_{1A}$ agonist 8-OH-DPAT on forskolin-induced stimulation. Therefore, the data in the present study indicates that Compound A is a potent antagonist of 5-HT$_{1A}$ receptor-mediated effects on adenylate cyclase activity in rat hippocampal membranes.

Behavioral studies on rats also support the antagonist effects of Compound A at 5-HT$_{1A}$ receptors. When tested alone, the drug has no significant effect on the induction of the 5-HT behavioral syndrome of reciprocal forepaw treading, head weaving, and flat body posture. However, Compound A potently inhibits the agonist effects of 8-OH-DPAT in this animal model. These studies further indicate that Compound A is a 5-HT$_{1A}$ receptor antagonist. Moreover, the data suggest that the antagonism of 8-OH-DPAT-induced forepaw treading is mediated directly by the 5-HT$_{1A}$ receptor, since pretreatment with the beta-adrenergic agent (+)pindolol has virtually no effect on the intensity of the behavioral syndrome.

Although great progress has been made in the development of potent 5-HT$_{1A}$ agents, the development of a potent and selective 5-HT$_{1A}$ antagonist is still needed. Currently, it is unresolved as to whether the putative anxiolytic effects of 5-HT$_{1A}$-selective agents like buspirone are due to their potency, selectivity, and/or efficacy at the 5-HT$_{1A}$ receptor. Conceivably, a drug which is an antagonist at this site (e.g., Compound A) may be a more optimal anxiolytic agent. Alternatively, the partial agonist properties of buspirone and related agents may be the crucial determinants of their anxiolytic effects. In order to clarify this issue, the development of a potent and selective 5-HT$_{1A}$ antagonist is required. Thus the significance of Compound A may lie in its potential to help elucidate the clinical consequences of activation and/or inhibition of 5-HT$_{1A}$ receptors.

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