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PARTIAL AGONIST PROPERTIES OF RAUWOLSCINE AND YOHIMBINE FOR THE INHIBITION OF ADENYLYL CYCLASE BY RECOMBINANT HUMAN 5-HT_{1A} RECEPTORS

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Abstract—Previous studies by another group have suggested that the α_2-adrenergic receptor antagonist rauwolscine may function as an agonist at the serotonin$_{1A}$ (5-HT_{1A}) receptor expressed in human brain. To directly test that hypothesis, we transfected the human 5-HT_{1A} receptor cDNA into CHO cells and examined the ability of rauwolscine and its isomer, yohimbine, to inhibit ligand binding of [^{3}H]-(±)-8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) and the activity of adenylyl cyclase in membranes derived from a single transformant that stably expresses ≈225 fmol of 5-HT_{1A} receptor/mg of membrane protein. Both ligands competitively antagonized the binding of [^{3}H]8-OH-DPAT (K_i = 158 ± 69 nM for rauwolscine and 690 ± 223 nM for yohimbine), yielding shallow displacement curves consistent with agonist activity (Hill values = 0.69 ± 0.2 for rauwolscine and 0.63 ± 0.06 for yohimbine). Both ligands also inhibited forskolin-stimulated adenylyl cyclase activity in membranes derived from transfected (but not nontransfected) cells. For rauwolscine, the IC_{50} was 1.5 ± 0.2 μM, and for yohimbine 4.6 ± 1.0 μM, with activity ratios of 0.70 and 0.59, respectively, when compared to the full agonist serotonin. These studies demonstrated that rauwolscine and yohimbine are partial agonists for the human 5-HT_{1A} receptor.

In brain tissue, the serotonin$_{1A}$ (5-HT_{1A}‖) receptor prototypically inhibits the enzyme adenylyl cyclase [1, 2], although coupling to many other signaling pathways has been described [3–5]. Initially, there were difficulties in assigning these multiple signaling pathways to a single receptor subtype due to the homogeneous nature of the tissues and membrane preparations used and the potential pharmacologic overlap between various receptor subtypes. The molecular cloning of the human [6, 7] and rat [8] 5-HT_{1A} receptor has allowed the development of heterologous expression systems that were used to confirm that the multiple signaling pathways resulted from activation of a single receptor subtype [8–18].

De Vos *et al.* [19] recently suggested that the α_2-adrenergic receptor antagonist rauwolscine might be an agonist at 5-HT_{1A} receptors expressed in human brain. This hypothesis was based primarily on the loss of a high-affinity [^{3}H]rauwolscine binding site in brain membranes produced by GTP in the presence of a relatively specific α-adrenergic receptor antagonist. Because of the presence of α_2-adrenergic receptors in their preparation, the definite assignment of the binding site to the 5-HT_{1A} receptor was difficult. They based their arguments in part on previous radioligand binding studies that suggested that rauwolscine interacts with high affinity with 5-HT_{1A} receptors [20–22]. However, because the previous studies did not measure second messenger activation, the functional significance of the binding of rauwolscine to the 5-HT_{1A} receptor is unclear. A cell model transfected to stably express a physiologic amount of human 5-HT_{1A} receptor would be ideal to confirm the hypothesis introduced by De Vos *et al.* [19] and to measure the effects of receptor binding. Thus, for the current studies we used a Chinese Hamster Ovary (CHO) cell line that stably expresses 225 fmol of human 5-HT_{1A} receptor per mg of membrane protein, and measured the effects of rauwolscine and yohimbine on receptor-modulated adenylyl cyclase activity.

MATERIALS AND METHODS

Materials. ATP, cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate, phospho(enol)pyruvate, myokinase (adenylate kinase), and yohimbine were obtained from the Sigma Chemical Co. (St. Louis, MO). Pyruvate kinase was obtained from Calbiochem (La Jolla, CA), and forskolin was from Hoechst–Roussel (Somerville, NJ). [^{3}H]-(±)-8-Hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) (210 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and [α-^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear (Boston, MA). Medium, sera, and other tissue culture reagents were obtained from Gibco Laboratories (Grand Island, NY). Rauwolscine and NAN-190 were

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‖ Abbreviations: 5-HT_{1A}, serotonin$_{1A}$; [^{3}H]8-OH-DPAT, [^{3}H]-(±)-8-hydroxy-2-(di-*n*-propylamino)tetralin; and CHO, Chinese Hamster Ovary.

obtained from Research Biochemicals Inc. (Natick, MA). All other materials were obtained from commercial sources and were of the highest quality commercially available.

Receptor expression and cell culture. CHO cells were grown in monolayers at 37° in 95% air/5% CO_2 in Ham's F12 Medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Stable clones were obtained by dilution cloning and selection in the presence of G-418 as previously described [17, 23]. A single clone that stably expresses ≈225 fmol of 5-HT_{1A} receptor per mg of protein was chosen for further studies. Cells were passaged by trypsinization and split 1:5. Confluence was usually reached in 3–5 days.

Radioligand binding assay. Membranes were prepared from CHO cells by hypotonic lysis in lysis buffer (5 mM Tris–HCl, pH 7.4, 5 mM EDTA) supplemented with benzamidine, leupeptin and soybean trypsin inhibitor (each at 5 μg/mL). The suspensions were homogenized on ice using a glass on glass dounce, and then were centrifuged at 37,000 *g* for 20 min. Pellets were resuspended to a concentration of 1–3 mg protein/mL in binding buffer (50 mM Tris–HCl, pH 7.4, 2 mM EDTA), and used fresh. Radioligand binding was performed as previously described [6, 17, 24]. Competition curves with 10 or 12.5 nM [^{3}H]8-OH-DPAT were modelled by non-linear regression using the InPlot program (GraphPAD, San Diego, CA). Inhibitory constants for rauwolscine and yohimbine (K_i values) were determined from the calculated IC_{50} values as described in the legend to Fig. 1. The affinity constant (K_D = 3.0 nM) for 8-OH-DPAT in these membranes was determined from saturation binding isotherms (not shown).

Adenylyl cyclase assay. Adenylyl cyclase activities were measured in crude membrane suspensions prepared fresh from CHO cells as described above. The resulting pellets were then resuspended in adenylyl cyclase buffer (75 mM Tris–HCl, pH 7.2, 5 mM $MgCl_2$, 3 mM EDTA) and used immediately. Adenylyl cyclase activity in membranes was measured by the method of Salomon *et al.* [25] with modifications as previously described [23]. Assay mixtures contained 20 μL membranes, 30 mM Tris–HCl, pH 7.2, 2 mM $MgCl_2$, 0.8 mM EDTA, 120 μM ATP with 1–2 μCi [α-^{32}P]ATP/tube, 53 μM GTP, 100 μM cAMP, 2.7 mM phospho(enol)pyruvate, 0.2 IU pyruvate kinase, 1 IU myokinase, 0.02% ascorbate, and various concentrations of each drug in an assay volume of 50 μL. Assays, performed in triplicate, were incubated at 37° for 30 min and were terminated by the addition of 1 mL of 400 μM ATP, 300 μM cAMP and ~25,000 cpm of [^{3}H]cAMP. cAMP was isolated from ATP by chromatography over 1 mL Dowex and 1 mL alumina columns. Double-labeled samples were counted by liquid scintillation counting, and the amount of [^{32}P]cAMP produced was calculated on a personal computer after correcting for recovery. Drug stocks were made up fresh at 1 mM concentrations in water (rauwolscine and yohimbine) or 10% dimethyl sulfoxide (NAN-190), and then diluted to the appropriate concentrations with assay buffer. All incubations were adjusted with equal amounts of stock solvent, which never exceeded a final concentration of 0.1%.

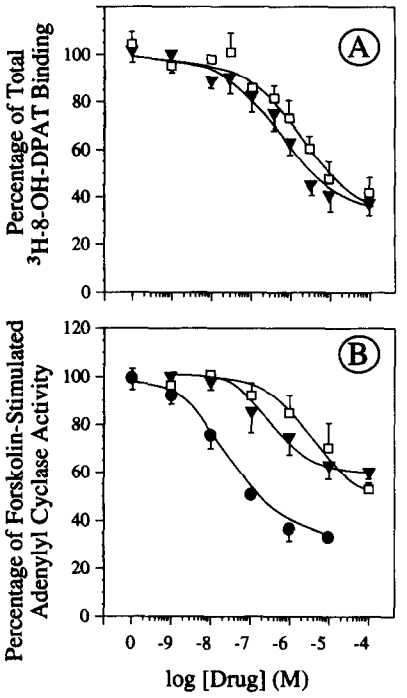

Fig. 1. (A) Competition curves for rauwolscine (▼) and yohimbine (□) vs 10–12 nM [^{3}H]8-OH-DPAT. The IC_{50} values were determined as described in Materials and Methods. K_i values were calculated from the IC_{50} values using the method of Cheng and Prussoff [27] (rauwolscine = 158 ± 69 nM; yohimbine = 690 ± 223 nM). Data presented are pooled means ± SEM of four separate experiments performed in triplicate or quadruplicate for each compound. Total binding was 3450 ± 500 cpm and non-specific binding was 1090 ± 100 cpm. Displaceable binding was equivalent for 5-HT, yohimbine and rauwolscine. (B) Concentration–response curves of adenylyl cyclase assays for 5-HT (●), rauwolscine (▼) and yohimbine (□) vs 100 μM forskolin. The IC_{50} values (rauwolscine = 1.5 ± 0.2 μM; yohimbine = 4.6 ± 10.0 μM) were determined as described in Materials and Methods. Curves presented are representative examples of five to nine experiments performed in triplicate for each compound. Absolute values for cAMP levels were 486 ± 37 pmol/mg protein/30 min in the basal state, and 8120 ± 830 pmol/mg protein/30 min when stimulated by forskolin (100 μM).

Protein assay. Membrane protein concentrations in the radioligand binding assay and adenylyl cyclase assay were determined by the method of Bradford [26] using bovine serum albumin as a standard.

RESULTS

Figure 1A shows that both rauwolscine and yohimbine displaced [^{3}H]8-OH-DPAT binding to CHO cell membranes bearing transfected human 5-HT_{1A} receptor, with a shallow slope. The Hill coefficients of 0.69 ± 0.2 for rauwolscine and 0.63 ± 0.06 for yohimbine suggest that these ligands are agonists at the 5-HT_{1A} receptor. Figure 1B

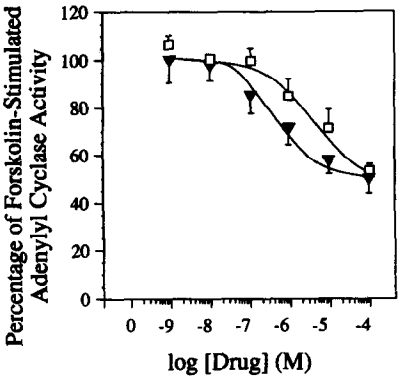

Fig. 2. Concentration–response curves of adenylyl cyclase assays for rauwolscine vs 100 μM forskolin in the absence (▼) and presence (□) of the specific 5-HT$_{1A}$ receptor antagonist NAN-190 (1 μM). The IC$_{50}$ values (rauwolscine alone = 1.3 ± 0.3 μM; rauwolscione + 1 μM NAN-190 = 13.5 ± 3.1 μM; N = 3) were calculated as described in Materials and Methods. As expected, the parallel shift to the right for the concentration–response curve suggests that NAN-190 is a competitive antagonist of rauwolscine at the 5-HT$_{1A}$ receptor. The curves shown are composites derived from the mean ± SEM of the data derived from three separate paired experiments. See legend of Fig. 1B for control values.

presents direct evidence that yohimbine and rauwolscine are partial agonists for the inhibition of adenylyl cyclase in CHO cells that express the human 5-HT$_{1A}$ receptor. Rauwolscine inhibited forskolin-stimulated adenylyl cyclase 46.7 ± 7.4%, with an IC$_{50}$ of 1.5 ± 0.2 μM (N = 6), and yohimbine inhibited adenylyl cyclase by 38.9 ± 4.3%, with an IC$_{50}$ of 4.6 ± 1.0 μM (N = 9). These compare with a 66.2 ± 3.4% inhibition by the full agonist serotonin, with an IC$_{50}$ of 146 ± 12 nM (N = 5). The calculated activity ratios for yohimbine and rauwolscine versus the full agonist serotonin were 0.59 and 0.70, respectively. The identity of the receptor activated by rauwolscine and yohimbine as the 5-HT$_{1A}$ receptor was confirmed by their lack of effect on non-transfected cells (not shown; N = 3), and by the ability of NAN-190, a highly specific antagonist at the 5-HT$_{1A}$ receptor, to function as a competitive antagonist by shifting the concentration–response curve to the right in a parallel fashion (Fig. 2).

DISCUSSION

Previous studies have shown that both yohimbine and rauwolscine behave as partial agonists for the inhibition of adenylyl cyclase in calf substantia nigra membranes through 5-HT$_{1D}$ receptors [28]. In fact, the partial agonist property of yohimbine was used recently to confirm that a 5-HT$_{1D}$ receptor mediated inhibition of [^{3}H]5-HT release in human neocortical slices [29]. The current studies clearly and unequivocally demonstrate that yohimbine and rauwolscine can serve as partial agonists at the human 5-HT$_{1A}$ receptor, and highlight the intricate overlap of pharmacologic properties between receptors that recognize distinct physiologic ligands. The overlap between D_2 dopamine receptor and 5-HT$_{1A}$ receptor agonist ligands [24], and between classic β-adrenergic receptor antagonists and the 5-HT$_{1A}$ receptor, has been noted previously [6, 30, 31]. This pharmacological overlap is particularly interesting in light of recent studies that conclusively show that a mutation of a single residue of the 5-HT$_{1A}$ receptor can abolish its ability to efficiently bind to classic β-receptor antagonists [31].

Our studies confirm and expand upon the hypothesis of De Vos *et al.* [19] that rauwolscine is an agonist ligand when interacting with human 5-HT$_{1A}$ receptors. They also confirm the suspicion that yohimbine may also be a 5-HT$_{1A}$ receptor agonist, elucidated recently by Winter and Rabin [32] in drug discrimination studies. These findings have both pharmacologic and clinical implications, because 5-HT$_{1A}$ receptors and α_2-adrenergic receptors are co-expressed in the central nervous system and vasculature, and may constitute distinct but overlapping components of various central and peripheral regulatory mechanisms. Therefore, effects of rauwolscine and yohimbine that have been attributed to α_2-adrenergic receptors may, in fact, be mediated through 5-HT$_{1A}$ receptors. For example, yohimbine has been popularized recently as an erectile stimulant in human males, but its exact mechanism of action is not at all clear. A similar pharmacological overlap between the α_1-adrenergic receptor antagonist urapidil and the 5-HT$_{1A}$ receptor has already been shown to be clinically important in its mechanism as an antihypertensive medication [33–36].

The current studies suggest that rauwolscine is a more potent agonist than yohimbine for the inhibition of adenylyl cyclase by the 5-HT$_{1A}$ receptor, and rauwolscine trended towards a slightly higher efficacy (statistically insignificant) (Fig. 1B). The potency ratio of 3.0:1 for rauwolscine and yohimbine in the adenylyl cyclase assays correlates very well with the ratio of K_i values obtained from the ligand binding studies (Fig. 1B) (158 nM for rauwolscine and 690 nM for yohimbine; ratio = 4.4). Because agonist activities of the 5-HT$_{1A}$ receptor are mediated through guanine nucleotide binding regulatory G proteins, it is tempting to speculate on the mechanism of the partial agonist behavior of rauwolscine and yohimbine. We have shown recently that both 5-HT and 8-OH-DPAT can induce the 5-HT$_{1A}$ receptor to couple physically to both $G_i\alpha 2$ and $G_i\alpha 3$ in CHO cells, and that both of those G proteins may be important in mediating the inhibition of adenylyl cyclase.* An interesting hypothesis to explain how these multiple intracellular signals are integrated is that rauwolscine and yohimbine are capable of inducing the 5-HT$_{1A}$ receptor only to couple to one or the other of those G proteins.

The current studies provide direct evidence that yohimbine and rauwolscine not only bind to the 5-HT$_{1A}$ receptor, but also function as partial agonists for the human 5-HT$_{1A}$ receptors expressed in CHO host cells. These findings have potential pharmacologic, physiologic and clinical ramifications.

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