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Positive Allosteric Modulator of the Human 5-HT_{2C} Receptor

WHA BIN IM, CHRISTOPHER L. CHIO, GLEN L. ALBERTS, and DAC M. DINH

Biology II/Neurobiology (W.B.I., C.L.C., G.L.A.) and Research and Development Discovery Technologies (D.M.D.), Pharmacia, Kalamazoo, Michigan

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ABSTRACT

The human 5-hydroxytryptamine-2C (5-HT_{2C}) receptor has been the target of potential anxiolytics and antiobesity drugs, and its positive allosteric modulator was discovered to be L-*threo*-α-D-galacto-octopyranoside, methyl-7-chloro-6,7,8-trideoxy-6-[[(4-undecyl-2-piperidinyl)carbonyl]amino]-1-thio-monohydrochloride (2*S*-*cis*) (PNU-69176E). The drug at low micromolar concentrations (<25 μM) markedly enhanced [^{3}H]5-HT binding (more than 300%) by increasing its affinity for low-affinity sites but with no appreciable effect on antagonist ([^{3}H]mesulergine) binding. Functionally, PNU-69176E alone rendered receptors constitutively active, producing the phenotypes of 5-HT–activated receptors, as measured with mesulergine-sensitive guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding, transient inositol 1,4,5-triphosphate release, and [^{3}H]inositol phosphate accumulation. These actions of PNU-69176E were observed with the human 5-HT_{2C} receptor expressed in several mammalian cell lines (human embryonic kidney 293, NIH3T3, and SH-EP) at variable receptor densities (6 to 45 pmol/mg of protein), but not with analogous 5-HT and dopamine receptors (human 5-HT_{2A}, 5-HT_{2B}, 5-HT_6, 5-HT_7, and dopamine D2-long and D3 receptors). Structurally, PNU-69176E consists of a long alkyl chain and a polar moiety, including the α-D-galactopyranoside. Its analogs with shorter alkyl chains (methyl to *n*-hexyl instead of *n*-undecyl group) failed to enhance [^{3}H]5-HT binding, and also long alkyl amides are without allosteric modulation. We propose that PNU-69176E may represent a new class of membrane receptor modulators, which probably need a long alkyl chain as a membrane anchor and target a selective polar head group to receptor modulatory sites near the membrane surface.

The 5-HT_{2C} receptor is a G protein-coupled receptor primarily distributed in the brain, and it mediates the release of intracellular inositol 1,4,5-triphosphate (IP_3) via activation of $G_{q/11}$ and G_i subtypes of G proteins (Conn et al., 1979; Julius et al., 1988; Salzman et al., 1991; Stam et al., 1994; Kaufman et al., 1995; Xie et al., 1996; Alberts et al., 1999). Numerous studies implicated the receptor in a number of diseases such as anxiety, obesity, depression, schizophrenia, and affective disorders (Canton et al., 1990; Sanders-Bush and Breeding, 1991; Moreau et al., 1993; Dourish, 1995; Tecott et al., 1995; Cowen et al., 1996; Kennett et al., 1996; Epstein et al., 1997). Recently, the receptor has become a therapeutic target for potential anxiolytics and antiobesity drugs (Dourish, 1995; Cowen et al., 1996; Kennett et al., 1996). Much effort has been directed, so far, to the discovery of selective agonists for its serotonin binding site. Another potentially profitable approach could be a search of positive allosteric modulators, which could achieve greater receptor selectivity than the agents for 5-HT sites that are shared by many analogous 5-HT receptors and transporters. In this study, we searched for compounds that stimulate [^{3}H]5-HT binding, the seeming hallmark of positive allosteric modulators. We report the discovery and characterization of PNU-69176E as positive allosteric modulators that are highly selective for 5-HT_{2C} receptors.

Methods and Materials

Cloning of the cDNA for the human 5-HT_{2C} receptor into a mammalian expression vector, PCI-Neo (Promega, Madison, WI), has been described elsewhere (Alberts et al., 1999) and was cloned into the PCRscript vector via a blunt end ligation. The recombinant vector was used to transfect human embryonic kidney 293 cells (HEK293), NIH3T3, and a human epithelial cell line (SH-EP) using Ca^{2+} phosphate precipitation techniques. Stably transfected cells were selected in the presence of G418 (400 μg/ml). Cell membranes expressing the 5-HT_{2C} receptor were prepared with the use of procedures described elsewhere (Alberts et al., 1999).

For [^{3}H]5-HT binding, scintillation proximity binding assays were initially carried out using wheat germ agglutinin-coated beads saturated with membranes from HEK293-A cell line expressing human 5-HT_{2C} receptors. Assay mixtures contained [^{3}H]5-HT at 4 nM and test ligands from Pharmacia (Peapack, NJ) chemical library at 10 μM in medium that contained 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, and 20 mM HEPES/Tris, pH 7.4. Nonspecific binding was

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; IP_3, inositol 1,4,5-triphosphate; PNU-69176E, L-*threo*-α-D-galacto-octopyranoside, methyl-7-chloro-6,7,8-trideoxy-6-[[(4-undecyl-2-piperidinyl)carbonyl]amino]-1-thio-monohydrochloride (2*S*-*cis*); HEK, human embryonic kidney; IP, inositol phosphate; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate; Org 37684, (*S*)-3-[(2,3-dihydro-5-methoxy-1H-inden-4-yl)oxy]-pyrollidine HCl; PGE_2, prostaglandin E_2.

measured in the presence of mianserin at 5 μM. Hits from the high-throughput screening were further examined using regular filtration-binding techniques as described elsewhere (Alberts et al., 1999). Briefly, binding of [^{3}H]5-HT or [^{3}H]mesulergine to 5-HT$_{2C}$ membranes was measured in the above-described medium with use of the radioactive ligand at varying concentrations (0.1 to 20 nM for typical binding profiles) and 5 to 20 μg membrane protein in a total volume of 500 μl. Reaction mixtures were incubated at 23°C for 60 min and filtered over Whatman GF/B filters under vacuum (Whatman, Clifton, NJ), which were then washed three times with 4 ml of an ice-cold 50 mM Tris/HCl buffer, pH 7.4. Nonspecific binding was estimated in the presence of excess unlabeled clozapine (100 μM). Ligand stock solutions were prepared in 0.1% ascorbic acid. Displacement of [^{3}H]mesulergine (2 nM) by test compounds at various concentrations (competition assay) was carried out in the same manner.

[^{35}S]GTPγS binding was measured by following the procedure reported earlier (Chabert et al., 1994) in medium that contained 25 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.003% mM digitonin, 2 nM [^{35}S]GTPγS (3 to 5 $\times$ 10^5 cpm /assay), and approximately 10 μg of membrane protein in a volume of 120 μl. Membranes were preincubated with 100 μM 5′-adenylylimidodiphosphate for 30 min at room temperature and subsequently with 10 μM GDP for 10 min on ice. Test ligands were included at 10 μM, unless indicated otherwise. Reaction mixtures were incubated for 45 min at 30°C and were filtered over a Whatman GF/B filter under vacuum. Filters were washed three times with 4 ml of an ice-cold buffer that contained 100 mM NaCl, 20 mM Tris/HCl, pH 8.0, and 25 mM $MgCl_2$. Agonist-induced [^{35}S]GTPγS binding was obtained by subtracting that which was observed without agonists. Binding data were analyzed using a nonlinear regression method (Sigma plot).

The agonist-induced IP_3 release in intact cells was measured using the inositol-1,4,5-trisphophate ^{3}H radioreceptor assay kit from PerkinElmer Life Sciences (Boston, MA). Briefly, cells were grown in a 24-well plate to approximately 80% confluence and were treated with 5-HT or test ligands at indicated concentrations for 45 s (initially a time course from 0 to 1200 s). Each reaction was stopped with trichloroacetic acid (20% final concentration), and each was then extracted with 1,1,2-trichloro-1,2,2-trifluoroethane and trioctyl amine. An aliquot (300 μl) was analyzed for IP_3 using [^{3}H]IP_3/IP_3 receptor preparations from calf cerebellum by following the protocols provided by the vender. For each experiment, a dose-response profile for IP_3 was constructed by adding known amounts of exogenous IP_3 to trichloroacetic acid extracts of untreated cells.

5-HT– or PNU-69176E–induced [^{3}H]inositol phosphate (IP) accumulation was measured in cells that had been labeled with [*myo*-^{3}H]inositol for 24 h. Briefly, semiconfluent cell cultures in 24-well plates were washed and incubated with 0.5 μCi of [*myo*-^{3}H]inositol in 0.5 ml of Dulbecco's modified Eagle's medium without inositol for 24 h. After washing the cells, they were treated with 5-HT or PNU-69176E at 10 μM in the presence of Li (10 mM) and pargyline (10 μM) for 30 min. IPs were extracted from cells and isolated using an anion exchanger, AG1-X8 (formate form; Bio-Rad, Hercules, CA) column chromatography as described elsewhere (Berridge et al., 1982).

Results

To search for positive allosteric modulators, the Pharmacia chemical library was screened with use of the assay of [^{3}H]5-HT binding to the human 5-HT$_{2C}$ receptor. The receptor was heterologously expressed in HEK293 cells at a density of 45 $\pm$ 3 pmol/mg of protein (HEK293-A). From the screening, we discovered PNU-69176E (Fig. 1), which at 10 μM markedly stimulated [^{3}H]5-HT (2 nM) binding (~300%). The concentration-response profile for PNU-69176E was biphasic (Fig. 2A). At concentrations of less than 25 μM, the drug enhanced [^{3}H]5-HT binding, but at higher concentrations it decreased binding. Peak stimulation of [^{3}H]5-HT binding by PNU-69176E was observed at the drug concentration of 25 μM, with a net increase of 355 $\pm$ 37%, as normalized to that in the absence of the drug. As the drug concentration increased to levels greater than 25 μM, [^{3}H]5-HT binding decreased gradually and disappeared at the drug concentration of 200 μM. The latter phase is probably caused by disturbances of membrane structures by the amphipathic PNU-69176E, which contains both a hydrophobic long alkyl chain and a polar head group (Fig. 1). The biphasic profiles fitted to a two-site logistic equation. The stimulatory phase for PNU-69176E showed an EC_{50} value of 6.3 $\pm$ 1 μM and a slope of 2.3 $\pm$ 0.5, and the inhibitory phase showed an IC_{50} value of 61 $\pm$ 5 μM and a slope of 3.6 $\pm$ 0.6 (Table 1; Fig. 2A). Also, saturation binding assays for [^{3}H]5-HT at concentrations from 0.09 to 48 nM were carried out with or without PNU-69176E at 10 μM (Fig. 2B). Without the drug, [^{3}H]5-HT binding linearly increased and showed no sign of saturation, even at 48 nM [^{3}H]5-HT. In contrast, in the presence of PNU-69176E (10 μM), 5-HT binding data fitted to one site-binding model with a K_D of 17 $\pm$ 0.8 nM and maximal binding of 32 $\pm$ 0.8 pmol/mg of protein, which accounts for nearly 80% of the total binding site, as estimated from [^{3}H]mesulergine binding.

Binding of [^{3}H]mesulergine, an antagonist, to the 5-HT$_{2C}$ receptor, on the other hand, was not appreciably affected by PNU-69176E at concentrations from 3 to 25 μM (Fig. 3, A and B). The K_D for [^{3}H]mesulergine (2 $\pm$ 0.2 nM) was largely unchanged, ranging from 95 to 115% of the control value. Its B_{max} (43 pmol/mg of protein) also remained unchanged with PNU-69176E at concentrations up to 10 μM, but at 25 μM it decreased by approximately 45%, probably because of the sensitivity of the mesulergine binding site to the amphipathic property of PNU-69176E at high concentrations, as noted above (Fig. 3B).

Typically, G protein-coupled receptors interact with agonists via high- and low-affinity sites, and their relative affinities can be examined with competition experiments using a radioactive antagonist. Displacement of [^{3}H]mesulergine by 5-HT at 5-HT$_{2C}$ receptors, however, fitted well to a single site-binding model with a K_i of 159 $\pm$ 12 nM (Fig. 2). This monophasic profile indicates more than 90% of receptors existing in low-affinity states for 5-HT, leaving only a negligible receptor population in high-affinity states, probably caused by high-receptor density of the cloned cells (Alberts et al., 1999). PNU-69176E concentration-dependently shifted the displacement curve to the left (Fig. 3C). The K_i for 5-HT decreased from 159 $\pm$ 12 to 86 $\pm$ 10, 36 $\pm$ 3, 10 $\pm$ 1, and 6.4 $\pm$ 0.9 nM in the presence of PNU-69176E at 2.5, 5, 10, and 20 μM, respectively. Such parallel shifts of the displacement curve may indicate that the whole receptor population un-

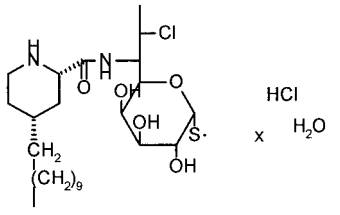

Fig. 1. Chemical structure of PNU-69176E.

dergoes gradual and uniform conformational changes in the presence of the drug. In the presence of PNU-69176E at 20 μM, the affinity of 5-HT (6.4 ± 0.9 nM) approached that of high-affinity sites as measured with [^{3}H]5-HT (K_D = 5 nM) (Julius et al., 1988; Alberts et al., 1999). Moreover, the ability of PNU-69176E to enhance 5-HT affinity was reversible. We treated 5-HT$_{2C}$ membranes with PNU-69176E at 10 μM for 30 min and then washed out upon dilution (4-fold) and ultracentrifugation. In such treated membranes, 5-HT replaced [^{3}H]mesulergine with a K_i of 128 ± 33 nM, which was not appreciably different from that of membranes which were not exposed to PNU-69176E at all.

Not only 5-HT but also other agonists improved their affinity to the 5-HT$_{2C}$ receptor in the presence of PNU-69176E. From competition experiments with [^{3}H]mesulergine in the presence of PNU-69176E at 10 μM, we found that the K_i values of Org 37684, 1-(3-chlorophenyl)piperazine hydrochloride, 2,5-dimethoxy-4-iodoamphetamine, and 5-carboxamidotryptamine decreased by 12-, 8-, 7, and 3.4-fold, respectively: from 342 ± 28 to 29 ± 4 nM for Org 37684, from 228 ± 12 to 32 ± 2 nM for 2,5-dimethoxy-4-iodoamphetamine, from 523 ± 81 to 64 ± 12 nM for 1-(3-chlorophenyl)piperazine hydrochloride, and from 4081 ± 612 to 1210 ± 185 nM for 5-carboxamidotryptamine. In the same experiments, the K_i for 5-HT decreased from 167 ± 15 to 14 ± 3 nM, approximately 12-fold. This indicates the universal action of PNU-69176E on 5-HT$_{2C}$ agonists, albeit to somewhat differential degrees.

Agonist binding to analogous 5-HT and dopamine receptors was not affected by PNU-69176E at 10 μM. The drug

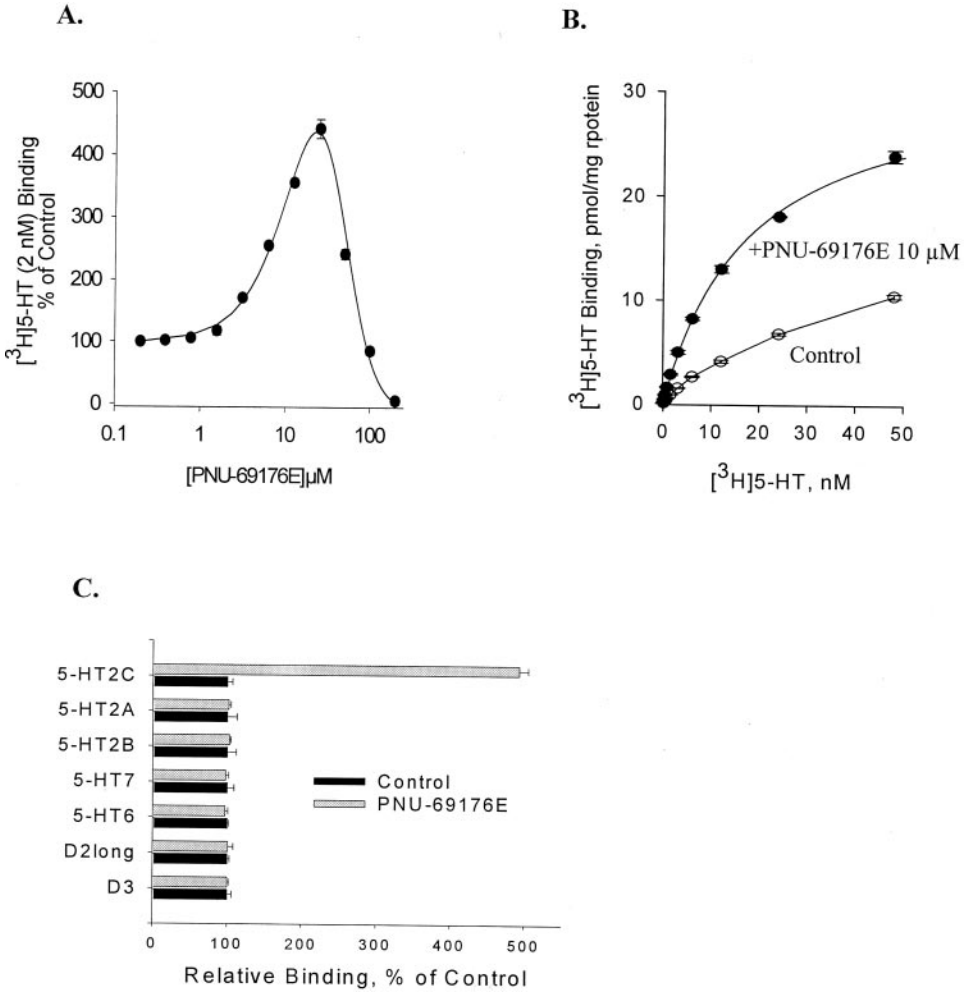

Fig. 2. Effects of PNU-69176E on [^{3}H]5-HT binding to 5-HT$_{2C}$ and to analogous 5-HT receptors and [^{3}H]quinpirole binding to dopamine receptors as expressed in HEK293 cells. A, profile for [^{3}H]5-HT (2 nM) binding with PNU-69176E at various concentrations. Binding experiments were carried out at 23°C for 1 h in HEK293 cell membranes expressing the human 5-HT$_{2C}$ receptor. Nonspecific binding was estimated in the presence of clozapine at 20 μM. The solid line in the plot represents the data fitting to a two-site logistic equation (Table 1). B, binding profiles for [^{3}H]5-HT at various concentrations with or without 10 μM PNU-69176E. The binding data in the absence of PNU-69176E showed no sign of saturation up to the concentration of [^{3}H]5-HT of 48 nM, but in the presence of PNU-69176E, it fitted to one-site binding model with a K_D of 17 ± 0.8 nM and maximal binding of 32 ± 0.8 pmol/mg of protein. C, effects of PNU-69176E on [^{3}H]5-HT (2 nM) binding to 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_{2C}$, 5-HT$_6$ and 5-HT$_7$ receptors and [^{3}H]quinpirole (5 nM) binding to human dopamine D2-long and D3 receptors expressed in HEK293 cells. Nonspecific binding was obtained in the presence of excess antagonist for each receptor. The data represent a composite of three duplicate concentration-response profiles (A and C) and two duplicate profiles (B).

TABLE 1

Analysis of biphasic concentration-response profiles for the actions of PNU-69176E on [^{3}H]5-HT binding (Fig. 2) and GTPγ [^{35}S] binding (Fig. 4) to the human 5-HT$_{2C}$ receptor in HEK293 cell membranes using a two-site logistic equation

Effects of PNU-69176E at various concentrations on [^{3}H]5-HT (2 nM) binding to the 5-HT$_{2C}$ receptor were measured in HEK293 cell membranes expressing the receptor using filtration techniques. Their effects on GTPγ [^{35}S] binding were measured in the same membrane preparation and as a function of drug concentrations with or without mesulergine in excess (100 μM), and they were normalized to the level observed with 5-HT at a saturating concentration (10 μM). Mesulergine by itself showed no appreciable effects on GTPγ [^{35}S] binding, but it blocked PNU-69176E–induced GTPγ [^{35}S] binding.

	[^{3}H]5-HT Binding	GTPγ[^{35}S] Binding
E_{max} (% increase)	355 ± 37	71 ± 5
EC_{50}	6.3 ± 1 μM (slope=2.3)	7.7 ± 0.6 μM (slope=2.5)
IC_{50}	61 ± 5 μM (slope=3.6)	49 ± 2 μM (slope=3.4)

showed no appreciable effect on [^{3}H]5-HT (2 nM) binding to 5-HT$_{2A}$, 5-HT$_{2B}$, 5-HT$_6$, and 5-HT$_{7A}$ receptors and also no effect on [^{3}H]quinpirole binding to dopamine D2-long and D3 receptors (Fig. 2C).

Functionally, cloned 5-HT$_{2C}$ receptors in HEK293 cells couple to both pertussis toxin-insensitive G$_{q/11}$ (Julius et al., 1988) and pertussis toxin-sensitive G$_i$ subtypes of G proteins (Alberts et al., 1999). GDP/GTP exchange at Gα subunits is an early step for G protein activation and could be monitored with [^{35}S]GTPγS (a slowly hydrolyzable analog) binding to Gα_i. The exchange at G$\alpha_{q/11}$ is not considerable because of their much slower turnover rates in isolated states (Pang and Sternweis, 1990; Smrcka et al., 1991). In HEK293-A cells, 5-HT concentration-dependently enhanced mesulergine-sensitive [^{35}S]GTPγS binding, whereas mesulergine by itself showed no appreciable effects on the basal [^{35}S]GTPγS binding (Alberts et al., 1999). The basal, mesulergine-sensitive [^{35}S]GTPγS binding, however, increased as a function of PNU-69176E concentrations (Fig. 4), and its concentration-response profile was biphasic and similar to that observed with [^{3}H]5-HT binding. At concentrations less than 25 μM, the drug progressively increased mesulergine-sensitive [^{35}S]GTPγS binding, but at concentrations greater than 25 μM, it gradually decreased the [^{35}S]GTPγS binding, and at 200 μM the drug largely abolished it. Peak stimulation by PNU-69176E was observed at 25 μM, with a net increase of 71 $\pm$ 5% (~500 fmol [^{35}S]GTPγS binding per mg of protein), as normalized to maximal stimulation by 5-HT (10 μM). The concentration profile fitted again to a two-site logistic equation (Fig. 4). The stimulatory phase for PNU-69176E displayed an EC$_{50}$ value of 7.7 $\pm$ 0.6 μM and a slope of 2.5 $\pm$ 0.3, and its inhibitory phase exhibited an IC$_{50}$ value of 49 $\pm$ 2 μM and a slope of 3.4 $\pm$ 0.9 (Table 1). These parameters are very similar to those obtained from similar analysis of PNU-69176E–stimulated [^{3}H]5-HT binding data (see above). This indicates a common, underlying mechanism(s) by which PNU-69176E affects [^{3}H]5-HT and [^{35}S]GTPγS binding to 5-HT$_{2C}$ receptors. It should be noted that in HEK293 cell membranes without heterologous expression of 5-HT$_{2C}$ receptors, no mesulergine-sensitive [^{35}S]GTPγS binding was induced by PNU-69176E at concentrations ranging from 2.5 to 20 μM.

Signaling responses for 5-HT$_{2C}$ receptors could also be examined with transient IP$_3$ release in intact cells during a short incubation period (e.g., 1 min) or [^{3}H]IP accumulation during a longer incubation period (e.g., 30 min) in the presence of lithium and pargeline. In this study, 5-HT (10 μM) transiently increased IP$_3$ releases in HEK293 cells, reaching a peak at the incubation time of 45 s. PNU-69176E (10 μM) by itself also increased IP$_3$ releases with the same time course and a peak that reached 71% of the maximal 5-HT (10 μM) response (2.4 $\pm$ 0.4 pmol/well) (Fig. 5). Similar results were obtained with [^{3}H]IP accumulation. Both 5-HT and PNU-69176E increased [^{3}H]IP accumulation as a function of time in cells labeled with [*myo*-^{3}H]inositol (Fig. 5). Maximal accumulation of [^{3}H]IP was observed at the incubation time of 30 min, and PNU-68176E (10 μM) alone induced [^{3}H]IP accumulation up to 83% of that observed with 5-HT (10 μM)

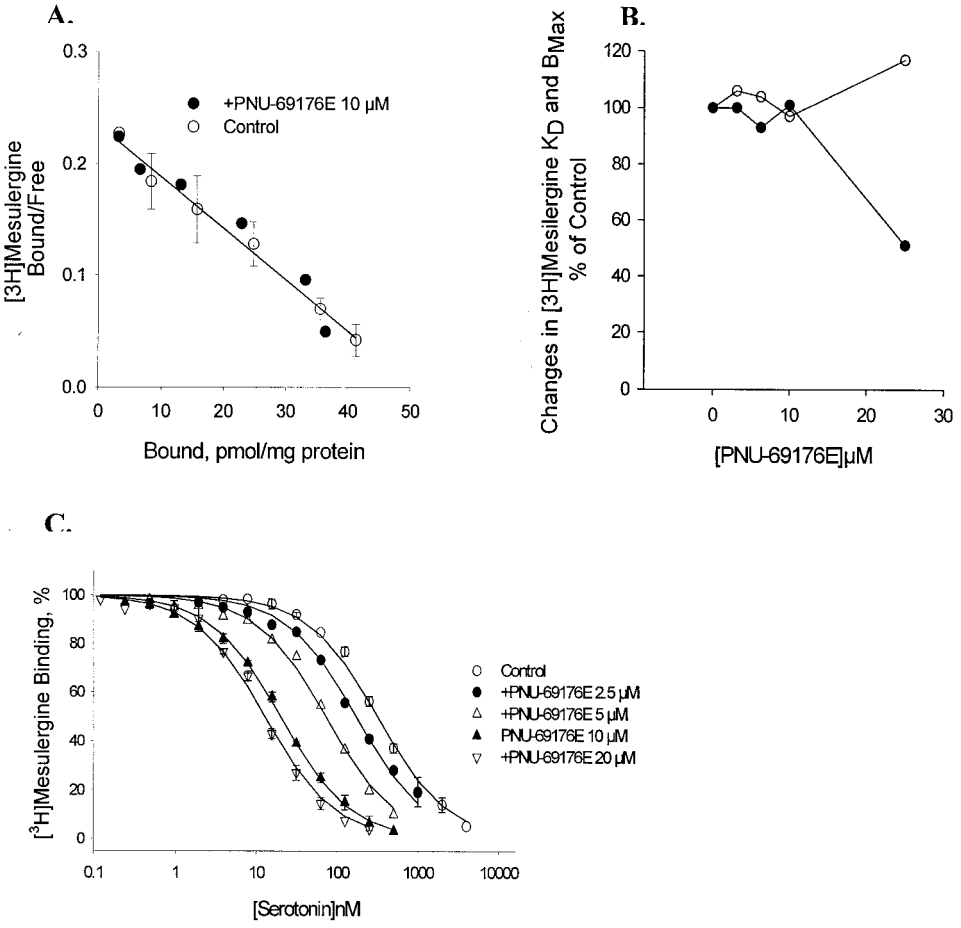

Fig. 3. Effects of PNU-69176E on [^{3}H]mesulergine (antagonist) binding to 5-HT$_{2C}$ receptors and on displacement of [^{3}H]mesulergine binding by 5-HT (low-affinity sites). A, binding data for [^{3}H]mesulergine at various concentrations with or without PNU-69176E at 10 μM were analyzed using Scatchard analysis. PNU-69176E (10 μM) produced no appreciable effects on K_D (2.0 $\pm$ 0.2 nM) and maximal binding (45 $\pm$ 3 pmol/mg of protein) for [^{3}H]mesulergine. B, similar analysis of [^{3}H]mesulergine binding as a function of PNU-69176E concentrations from 3 to 25 μM. No changes in K_D (○) and B_{max} (●) were noted, except for a 45% reduction of B_{max} with 25 μM PNU-69176E, probably because of its amphipathic property. C, 5-HT monophasically displaced [^{3}H]mesulergine (3 nM) binding, and the displacement data fitted to a model for a single binding site. The 5-HT K_i decreased from 159 $\pm$ 12 to 86 $\pm$ 10, 36 $\pm$ 3, 10 $\pm$ 1, and 6.4 $\pm$ 0.9 nM, when the concentration of PNU-69176E was progressively increased to 2.5, 5, 10, and 20 nM, respectively. The plots in A represent two triplicate ([^{3}H]mesulergine) or one triplicate ([^{3}H]mesulergine + PNU-69176E 10 μM) concentration-response profiles. The plots in B represent two duplicate concentration-response profiles.

(Fig. 5). The drug responses were blocked by mesulergine (10 μM), an antagonist to 5-HT$_{2C}$. These results are consistent with the view that the drug renders 5-HT$_{2C}$ receptors constitutively active.

To investigate potential functional interactions between PNU-690176E and 5-HT, we examined [^{35}S]GTPγS binding as a function of 5-HT concentrations with or without PNU-69176E (10 μM) (Fig. 4B). 5-HT concentration-dependently increased [^{35}S]GTPγS binding with an EC$_{50}$ value of 27 $\pm$ 4 nM. PNU-69176E at 10 μM visibly shifted the 5-HT profile upward. After subtraction of the portion induced by PNU-69176E alone, the 5-HT profile fitted to a single rectangular hyperbola with an EC$_{50}$ value of 10 $\pm$ 1.2 nM for 5-HT and a maximal level of 120 $\pm$ 5%, as normalized to that of 5-HT alone (Fig. 4B). This indicates that PNU-69176E (10 μM) potentiates the 5-HT action by decreasing the EC$_{50}$ value from 27 to 10 nM and increasing maximal stimulation by 20%.

We also examined the effect of 5-HT at submaximal (5 nM) and saturating concentrations on IP$_3$ production with or without PNU-69176E. Peak IP$_3$ level was measured with a 45-s exposure to 5-HT. At a submaximal concentration of 5

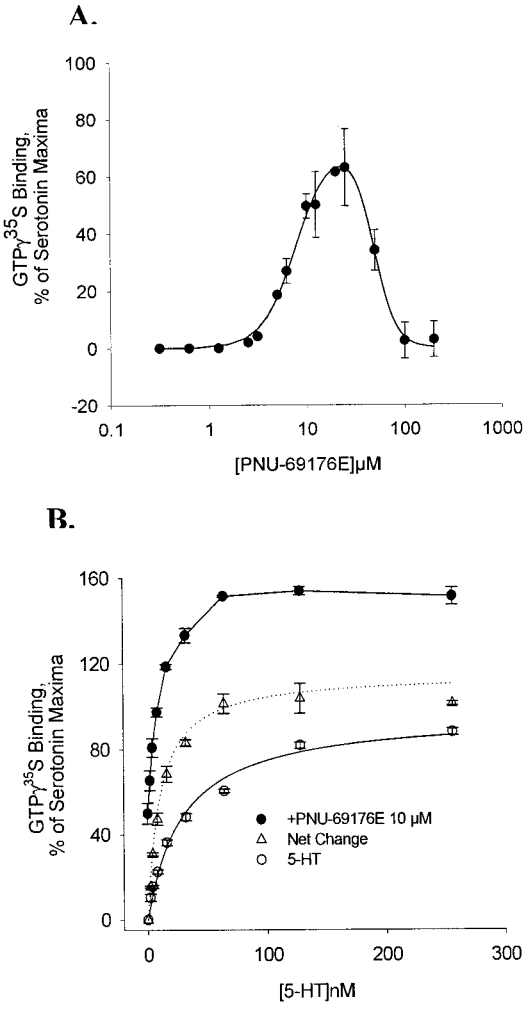

Fig. 4. Effects of PNU-69176E on [^{35}S]GTPγS binding in membranes from HEK293 cells expressing the human 5-HT$_{2C}$ receptor. A, [^{35}S]GTPγS binding was measured as a function of drug concentrations with or without mesulergine in excess (100 μM) and was normalized to the level observed with 5-HT at a saturating concentration (10 μM). Metergoline by itself showed no appreciable effects on [^{35}S]GTPγS binding but blocked PNU-69176E–induced [^{35}S]GTPγS binding. Amounts of mesulergine-sensitive [^{35}S]GTPγS binding were plotted as a function of drug concentrations, and fitted to a two-site logistic equation (Table 1 and text). B, 5-HT concentration-response profiles for [^{35}S]GTPγS binding in the presence or absence of PNU-69176E at 10 μM. To determine potential interactions of 5-HT and PNU-69176E, the amount of [^{35}S]GTPγS binding induced by PNU-69176E (10 μM) alone was subtracted from the 5-HT dose-response profiles with the drug. The data thus obtained fitted to a single rectangular hyperbolic equation (dashed line). PNU-69176E increased E$_{max}$ to 120 $\pm$ 5% compared with that obtained with 5-HT alone and decreased the EC$_{50}$ value from 27 $\pm$ 4 to 10 $\pm$ 2 nM.

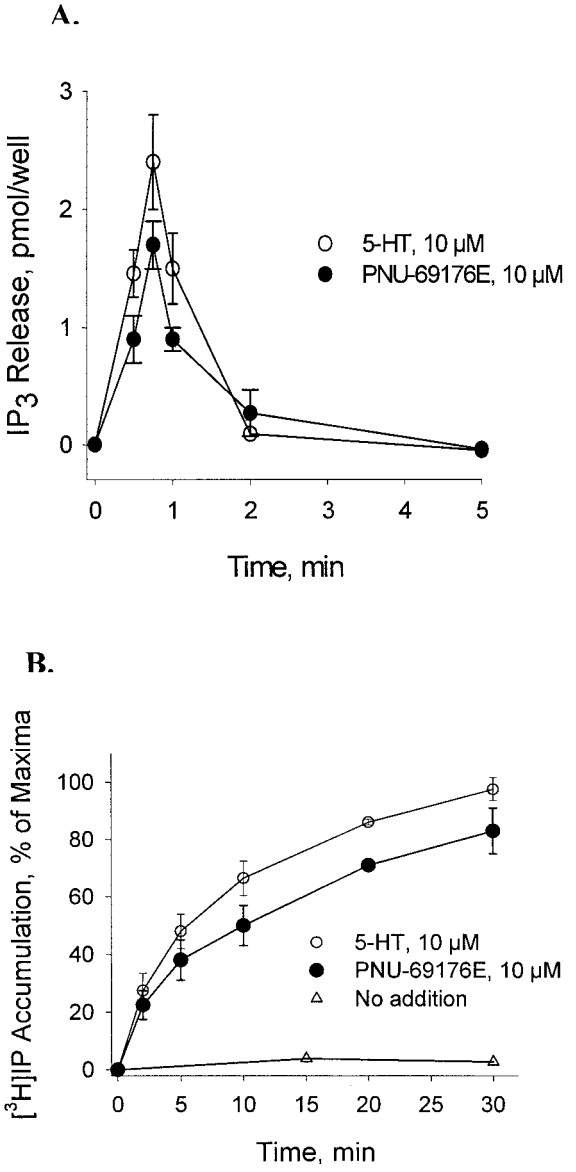

Fig. 5. IP$_3$ release or [^{3}H]IP accumulation induced by PNU-69176E alone in HEK293 cells heterologously expressing the human 5-HT$_{2C}$ receptors. A, IP$_3$ release was measured using a FlashPlate assay kit (PerkinElmer Life Sciences) upon treatment of cells with 10 μM 5-HT or 10 μM PNU-69176E for indicated durations (from 10 to 300 s) at room temperature. A transient increase of IP$_3$ release was observed, reaching a peak in 45 s and then returning to the basal level in 5 min. B, [^{3}H]IP accumulation was measured in cells prelabeled with [*myo*-^{3}H]inositol upon exposure to PNU-69176E at 10 μM or 5-HT 10 μM for 30 min, following a procedure described elsewhere (Berridge et al., 1982). The basal level of [^{3}H]IP accumulation was negligible during a 30-min incubation period and was not reduced in the presence of mesulergine (antagonist) at 100 μM. The data in A represent the mean $\pm$ S.E. from three triplicate concentration-response profiles, and those in B represent the mean $\pm$ S.E. from two quadruplicate concentration-response profiles.

nM, 5-HT induced an IP_3 release of 0.6 ± 0.1 pmol/well, and PNU-69176E (10 μM) alone induced an IP_3 release of 1.3 ± 0.2 pmol/well. In combination of the two, the peak IP_3 level reached a nearly maximal level of 1.8 ± 0.2 pmol/well. At a saturating concentration of 200 nM, 5-HT increased IP_3 release by 2.0 ± 0.2 pmol/mg of protein, and its action was not augmented by PNU-69175E at 10 μM (2 ± 0.1 pmol/mg of protein). This suggests that PNU-69176E and 5-HT share the same IP_3 signaling pathways, but their functional potentiation was not as evident as with [^{35}S]GTPγS binding, probably because of involvements of downstream amplification and threshold steps for IP_3 release.

In the course of studying the pharmacology of the 5-HT$_{2C}$ receptor, we obtained several mammalian cell lines, HEK293, SH-EP, and NIH3T3, stably expressing the receptor at various receptor densities, as estimated from maximal binding of [^{3}H]mesulergine (antagonist) (Table 2). The highest receptor density was 45 ± 3 pmol/mg of protein for the HEK293 cell line we studied (HEK293-A), followed by SH-EP-A, NIH3T3, and HEK293-B at receptor densities of 12.4 ± 2, 11.9 ± 0.6, and 6.6 ± 0.1 pmol/mg of protein, respectively. Despite widely variable receptor densities, all of these cell lines showed robust agonist-induced [^{35}S]GTPγS binding, which was blocked by *N*-ethylmaleimide (100 μM), an inhibitor of G_i and G_o subtypes of G proteins (data not shown). Also, PNU-69176E enhanced the affinity of 5-HT to low-affinity sites and increased the basal, mesulergine-sensitive [^{35}S]GTPγS binding. The K_i value for 5-HT low-affinity sites ranged from 159 to 223 nM in these cell lines and decreased in the presence of PNU-69176E (10 μM) to10 to 32 nM (Table 2). The drug (10 μM) increased mesulergine-sensitive [^{35}S]GTPγS binding by 23 to 50% as normalized to that of 5-HT at 10 μM (Table 1). We conclude that the positive allosteric modulation of 5-HT$_{2C}$ receptors by PNU-69176E was not dependent on receptor density or on specific cell lines.

Structurally, PNU-69176E consists of two moieties, a long alkyl chain (undecyl) and a polar moiety including the α-D-galactopyranoside (Fig. 1). Analogs of PHA-69176E with a shorter alkyl chain (methyl to hexyl) showed no effect on [^{3}H]5-HT binding to 5-HT$_{2C}$ receptors (Table 3). PNU-68607E (methyl), PNU-65287E (ethyl), PNU-63502E (propyl), PNU-61734E (*n*-butyl), PNU-62804E (*t*-butyl), PNU-67220E (pentanoyl), and PNU-62344E (*n*-hexyl) at 10 μM did not stimulate [^{3}H]5-HT binding to 5-HT$_{2C}$ receptors. Also long alkyl amides, PNU-8750 (*N*-(4-acetoamido-1-naphthylsulfonyl), PNU-33078 [(*N*-[2-(dimethylamino)ethyl]-*N*-methyl)], PNU-43240 (*N,N*-diethyldodecanamide), and PNU-170158 (*N*-phenyl dodecanamide) at 10 μM failed to stimulate [^{3}H]5-HT binding to 5-HT$_{2C}$ receptors (Table 3). These results indicate that the undecyl chain and the specific polar group seem to be essential for PNU-68176E to exert positive allosteric modulation on 5-HT$_{2C}$ receptors.

Discussion

Allosteric modulations have been reported for several types of G protein-coupled receptors. Muscarinic M_1 and M_2 receptors were allosterically modulated by gallamine, pancuronium, and alcuronium, affecting local ligand binding sites (Tuček and Prošta, 1995). Various biogenic amine receptors interact with Na^+ and Zn^{2+} via the aspartate residue at their second transmembrane segment, leading to changes in the binding properties of some ligands (Schetz and Sibley, 2000). Various serotonergic receptors, 5-HT$_{1A}$, 5-HT$_{2A}$, and 5-HT$_{2C}$, as expressed in *Xenopus laevis* oocytes, have been reported to show oleamide-sensitive receptor signals (Thomas et al., 1997), although this was not reproducible in our hands in mammalian expression systems (W. B. Im, C.L. Chio, G. L. Alberts, unpublished observations). At PGE_2 receptors, L-171837 reportedly enhanced [^{3}H]PGE_2 binding but blocked PGE_2-induced [^{35}S]GTPγS binding (Carriere et al., 2000), probably indicating complex conformational perturbations via multiple interaction sites. Overall, these earlier studies have indicated the presence of allosteric modulatory sites at G protein-coupled receptors, but modulatory actions through these sites were limited to local impacts on certain ligand-binding sites, species-dependent modulations, or nonspecific functional perturbations. In this study, we discovered a positive allosteric modulator that was highly selective for 5-HT$_{2C}$ receptors. PNU-69176E profoundly enhanced [^{3}H]5-HT binding to the human 5-HT$_{2C}$ receptor by selectively increasing the 5-HT affinity to its low-affinity sites (more than 20-fold), with no effect on antagonist binding, and it also rendered the receptor to be constitutively active, as measured with mesulergine-sensitive [^{35}S]GTPγS binding, transient IP_3 release, and [^{3}H]IP accumulation. These actions of PNU-69176E were not dependent on receptor density or specific cell lines, as shown with several mammalian cell lines (HEK293, NIH3T3, and SH-EP) at various receptor densities (6 to 45 pmol/mg of protein).

Interestingly, the modes of action for PNU-69176E are considerably different from those for conventional allosteric modulators of membrane receptors interacting with a single class of high-affinity sites (e.g., benzodiazepines). First, concentration-response profiles for PNU-69176E showed a Hill

TABLE 2

Effect of PNU-69176E (10 μM) on 5-HT binding (low-affinity sites) in competition with [^{3}H]mesulergine and 5-HT-induced GTPγ [^{35}S] binding in several mammalian cell lines expressing human or rat 5-HT$_{2C}$ receptors at various densities

data represent the mean ± S.E. from two duplicate concentration-response profiles. Receptor density was estimated from maximal binding of [^{3}H]mesulergine to 5-HT$_{2C}$.

Human 5-HT$_{2C}$ Receptors	Receptor Density[a]	K_i for 5-HT Binding[a]		GTPγ[^{35}S] Binding	
		5-HT	+PNU-69176E[b]	5-HT E_{max}	+PNU-69176E[b]
	pmol/mg protein		*nM*	*fmol/mg of protein*	*% of 5-HT*
HEK293-A	45 ± 3	159 ± 12	10 ± 1	724 ± 36	50 ± 3
SH-EP-A	12.4 ± 2	168 ± 7	19 ± 1	437 ± 39	23 ± 3
NIH3T3	11.9 ± 0.6	176 ± 10	11 ± 1	282 ± 19	50 ± 4
HEK293-B	6.6 ± 1	223 ± 16	32 ± 3	213 ± 8	35 ± 4

[a] Low-affinity sites for 5-HT as obtained from competition experiments using [^{3}H]mesulergine and 5-HT at various concentrations.
[b] PHA-69176E at 10 μM.

TABLE 3

Comparison of PNU-69176E analogs for their ability to enhance [^{3}H]5-HT binding to 5-HT$_{2C}$ receptors in membranes from HEK293 cells expressing the receptor

Test ligands share the core head group α -D-galacto-octopyranoside, methyl-7-chloro-6,7,8-trideoxy-6-[(2-piperidinyl)carbonyl]amino group, but each has a different alkyl substitutent at the 4 position of the piperidinyl group, or shares the dodecanamide group but with different head groups attached at the amine group.

Compounds		[^{3}H]5-HT Binding
	Type of Alkyl Chains	% *Change*
PNU-61734E	*n*-Butyl	−1
PNU-62344E	*n*-Hexyl	+1
PNU-62804E	*t*-Butyl	+1
PNU-63502E	*n*-Propyl	0
PNU-65287E	Ethyl	+2
PNU-67220E	*n*-Pentanoyl	−2
PNU-68607E	Methyl	−4
PNU-69176E	*n*-Undecyl	+393
	Type of Head Groups	
PNU-8750	*N*-(4-Acetoamido-1-naphthylsulfonyl)	+2
PNU-33078	*N*-[2-(Dimethylamino)ethyl]-*N*-methyl	0
PNU-43240	*N*,*N*-diethyl	−32
PNU-170158	*N*-Phenyl	−8

coefficient of nearly 3, indicating multiple cooperative binding sites. Second, the drug induced gradual and uniform conformational changes in the receptor population instead of converting a fractional population to high-affinity states, probably reflecting gradual occupancy of its multiple binding sites. Finally, structurally, PNU-69176E resembles amphipathic lipid metabolites with a long alkyl chain and a polar head group, both of which seem to be essential for its modulatory actions on 5-HT$_{2C}$. Thus, various amphipathic lipid metabolites could have modulatory action on 5-HT$_{2C}$. In this respect, it is noteworthy that cloned 5-HT$_{2C}$ receptors expressed in mammalian cells, e.g., NIH3T3 cells, reportedly display some constitutive activity, as monitored with clozapine- or mesulergine-sensitive basal inositol accumulation in intact cells (Barker et al., 1994). However, no constitutive activity of the 5-HT$_{2C}$ receptor was detected in isolated membranes from NIH3T3 or HEK293 cells, as measured by [^{35}S]GTPγS binding and [^{3}H]IP accumulation. It is conceivable that such a constitutive activity could be induced by specific lipid metabolites of relatively short half-lives, thus detectable only in intact cells.

Constitutive activation of G protein-coupled receptors has been frequently reported on mutations at various regions of receptors. This study shows another route of constitutive activation of G protein-coupled receptors, namely allosteric modulation by specific amphipathic compounds and perhaps certain lipid metabolites.

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Address correspondence to: Dr. Wha Bin Im, BiologyII/Neurobiology, 0216-209-512, Pharmacia, 301 Henrietta Street, Kalamazoo, MI 49007. E-mail: wbim@am.pnu.com