0026-895X/01/6006-1349–1355$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:1349–1355, 2001

Vol. 60, No. 6
1060/946982
Printed in U.S.A.

Allosteric Modulation of the Human 5-HT$_{7A}$ Receptor by Lipidic Amphipathic Compounds

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Received April 24, 2001; accepted September 7, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Human 5-HT$_{7A}$ receptors positively modulated adenylyl cyclases via G$_s$ subtypes of G proteins in human embryonic kidney 293 cells, and bound 5-hydroxytryptamine (HT) with high and low affinity (K_I values of 1.5 ± 0.3 and 93 ± 4 nM). More than 60% of 5-HT$_{7A}$ receptors, however, displayed the high-affinity 5-HT binding with no sensitivity to 5′-guanylylimidodiphosphate. In this study, we found that select amphipathic agents affected the high-affinity 5-HT binding to 5-HT$_{7A}$. Oleic acid at low concentrations (<15 μM), but not palmitic, stearic, and arachidonic acids, increased maximal [^{3}H]5-HT binding without affecting its K_D value and [^{3}H]mesulergine (antagonist) binding. Fatty acid-free bovine serum albumin (FF-BSA), a scavenger of fatty acids and lipid metabolites, substantially reduced maximal [^{3}H]5-HT binding (no change in K_D value and antagonist binding) but lost its action upon treatment with inactive stearic acid. FF-BSA and oleic acid produced no appreciable effects on [^{3}H]5-HT binding to analogous 5-HT receptors 5-HT$_{1D}$ and 5-HT$_{2C}$. Among various lysophospholipids, lysophosphatidyl choline (50 μM) decreased maximal [^{3}H]5-HT binding, and a similar zwitterion, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 0.1%), increased it (no change in K_D). Functionally, 5-HT–induced guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate (GTPγ^{35}S) binding was enhanced by oleic acid and CHAPS, but reduced by FF-BSA and lysophosphatidyl choline; the amphipathic agents and FF-BSA did not affect dopamine-induced GTPγ^{35}S binding at D1, a prototypic G$_s$-coupled receptor. At 5-HT$_{7A}$, oleic acid, FF-BSA, CHAPS, and lysophosphatidyl choline also brought about corresponding changes in the half-maximal 5-HT concentration for cAMP production, without affecting the maximal and basal levels. We propose that endogenous, amphipathic lipid metabolites may modulate 5-HT$_{7A}$ receptors allosterically to promote high-affinity 5-HT binding and to enable receptors to couple more efficiently to G$_s$ subtypes of G proteins.

5-HT$_7$ Receptors are G protein-coupled receptors with seven transmembrane segments, (Bard et al., 1993; Ruat et al., 1993; Shen et al., 1993), and exist in three alternatively spliced isoforms (Heidmann et al., 1997; Jasper et al., 1997; Stam et al., 1997; Heidmann et al., 1998). The predominant isoform, 5-HT$_{7A}$, is localized in discrete limbic regions (thalamus and hypothalamus) of the brain as well as peripheral tissues, including coronary artery and certain gastrointestinal tissues (Meyerhof et al., 1993; To et al., 1995; Leung et al., 1996). Multiple physiological roles have been proposed for the receptor, such as regulation of circadian rhythms [because of its presence in the suprachiasmatic nucleus of the hypothalamus (Lovenberg et al., 1993; Kawahara et al., 1994)], mood and emotions [because of its limbic location and its high-affinity interactions with antipsychotics and antidepressants (Adham et al., 1998; Meyerhof et al., 1993; To et al., 1995)], and also migraine genesis [because of its smooth muscle relaxing activity (Leung et al., 1996; Cushing et al., 1996)]. At cellular level, the 5-HT$_{7A}$ receptor, when expressed heterologously in mammalian cells, positively modulates adenylyl cyclases (Bard et al., 1993; Ruat et al., 1993; Shen et al., 1993; Adham et al., 1998) via activation of G$_s$ subtypes of G proteins. We confirmed here that the human 5-HT$_{7A}$ receptor as expressed heterologously in human embryonic kidney (HEK) 293 cells mediates the cholera toxin-sensitive cAMP production. At the same time, we observed that most receptors (> 60%) bound 5-HT with high affinity. Classically, high-affinity agonist binding represents the phenotype for G protein-bound receptors, but was unusual for 5-HT$_{7A}$ in its marked abundance and insensitivity to 5′-guanylylimidodiphosphate (GppNHp), as observed here. In this study, we explored whether phospholipid metabolites and similar amphipathic compounds could modulate the 5-HT$_{7A}$ receptor, a membrane-embedded receptor. Such possibilities were hinted at by earlier reports that oleamide and oleic acid influenced 5-HT$_{7A}$ receptors expressed in HeLa cells (Hedlund et al., 1999). In addition, our preliminary experiments showed that fatty acid-free bovine serum albumin (FF-BSA), a scavenger of lipid metabolites, markedly affected binding of [^{3}H]5-HT, but not [^{3}H]mesulergine (antagonist), to 5-HT$_{7A}$.

ABBREVIATIONS: HT, hydroxytryptamine; GppNHp, 5′-guanylylimidodiphosphate; HEK, human embryonic kidney; FF-BSA, fatty acid-free bovine serum albumin; GTPγ^{35}S, guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate; NEM, *N*-ethylmaleimide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 5-CT, 5-carboxamidotryptamine maleate.

Materials and Methods

The cDNA for the human 5-HT_{7A} receptor has been cloned into a PCI-Neo mammalian expression vector. The vector was used for transfection of HEK293 cells, using Ca^{2+} phosphate precipitation techniques. Cells were selected for a month in the presence of G-418 (400 μg/ml). Transcripts for 5-HT_{7A} were robustly detected with reverse transcription-polymerase chain reaction (using 3′ rapid amplification of cDNA ends reaction) from transfected but not naive cells. Cell membranes were prepared by standard procedures including homogenization and differential centrifugation as described elsewhere (Pregenzer et al., 1993). Binding of radioactive ligands was measured in membranes expressing recombinant receptors, using filtration techniques as described elsewhere (Pregenzer et al., 1993). Briefly, [^{3}H]mesulergine and [^{3}H]5-HT binding were measured in the medium containing 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 20 mM HEPES/Tris, pH 7.4, the radioactive ligand at varying concentrations (0.1 to 20 nM for typical binding profiles), and 20 μg of membrane protein, in a total volume of 500 μl at 23°C for 60 min. Reaction mixtures were filtered over Whatman GF/B filters under vacuum. Filters were washed three times with 4 ml of ice-cold 50 mM Tris/HCl buffer, pH 7.4. Nonspecific binding was estimated in the presence of excess unlabeled mesulergine (10 μM). Competition experiments with [^{3}H]mesulergine (2 nM) were carried out in the presence of test compounds at various concentrations under the same conditions.

GTPγ^{35}S binding was measured with the use of the procedures reported earlier (Chabert et al., 1994; Pregenzer et al., 1997), in the medium containing 25 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.003% digitonin, 2 nM GTPγ^{35}S (5 to 3 $\times$ 10^5 cpm/assay), and 10 μg of membrane protein in a volume of 200 μl. Digitonin was used at trace amounts here, solely to make the membranes permeable to GTPγ^{35}S. Test ligands were included at 10 μM, unless indicated otherwise. Membranes were preincubated with 100 μM 5′-adenylylimidodiphosphate for 30 min, 10 μM GDP for 10 min on ice, and then were added to the rest of reaction components. In some experiments, membranes were treated with *N*-ethylmaleimide (NEM) at 100 μM, and excess NEM was neutralized with β-mercaptoethanol at the end of 30-min incubation. Reaction mixtures were incubated at 30° for 30 min and were filtered over Whatman GF/B filters under vacuum. Filters were washed three times with 4 ml of ice-cold buffer containing 100 mM NaCl, 20 mM Tris/HCl, pH 8.0, and 25 mM $MgCl_2$. Agonist-induced GTPγ^{35}S binding was obtained by subtracting that observed without agonists. The binding data were analyzed using nonlinear regression method (Sigma Plot), and presented with mean values ± S.E.

Cellular changes in cAMP were measured using a FlashPlate assay kit from PerkinElmer Life Science Products (Boston, MA). Briefly, cells were grown in a 96-well plate to about 80%confluence, washed three times with phosphate-buffered saline, and treated with test ligands for 30 min. cAMP in cell lysates were measured using the competition between ^{125}I-cAMP and nonradioactive antigen for a fixed number of antibody binding sites in microplates coated with solid scintillant.

GTPγ^{35}S-bound Gα subunits were identified following the method described elsewhere (Okamoto et al., 1992) with a modification (Alberts, et al., 1999): the activation of receptors with serotonin in the presence of GTPγ^{35}S before membrane solubilization with detergents. Briefly, membranes were incubated in the presence of GTPγ^{35}S (4 nM) and serotonin (10 μM) under conditions identical to those for GTPγ^{35}S binding as described above. Treated membranes were solubilized with an equal volume of a buffer containing 100 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl and 0.6% CHAPS for 30 min on ice, and were diluted to a final CHAPS concentration of 0.125%. An aliquot of the mixtures (typically 300 μl) was transferred to a well in a 96-well plate that had been coated successively with goat anti-rabbit antibodies (1:100 dilution), bovine serum albumin (5 mg/ml), and one of the affinity-purified rabbit antibodies for various Gα subunits (1:200 dilution). After incubation for 1 h at room temperature and washing, each well was counted for ^{35}S using a standard scintillation cocktail and a β-counter. The antibodies we used here include those specific for Gα_i (the C-terminal sequence, 345–354), Gα_s (the C-terminal sequence, 385–394), G$\alpha_{q/11}$ (the common C-terminal sequence, QLNLKEYNLV), or Gα_{13} (the sequence 367–377) from Calbiochem (San Diego, CA). The mouse monoclonal anti-

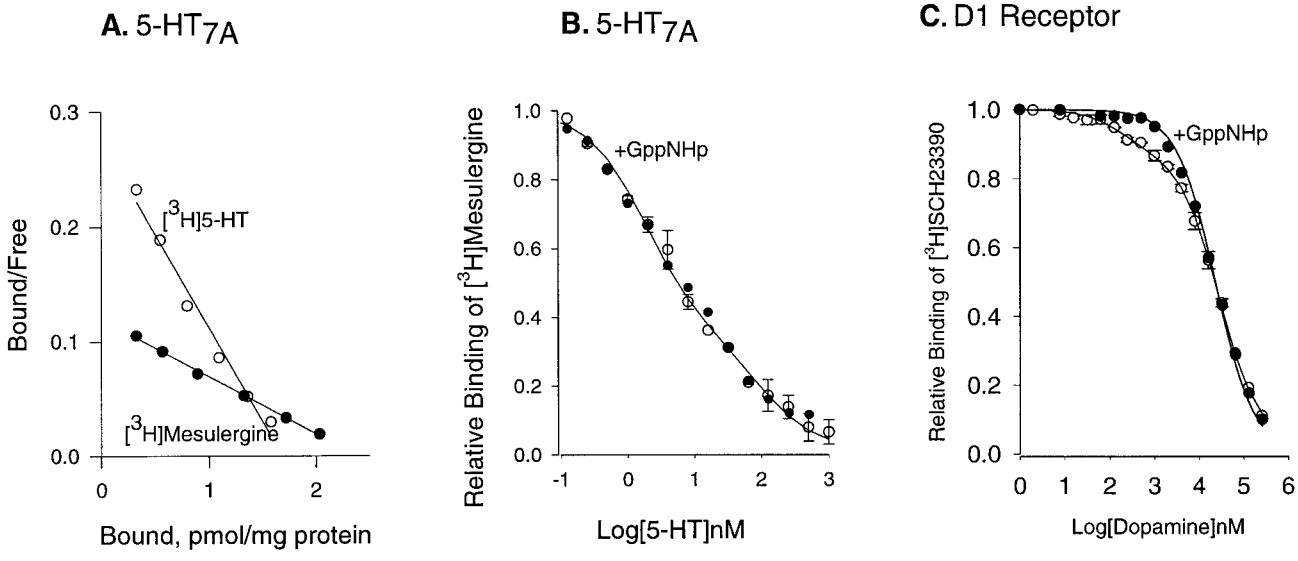

Fig. 1. Comparison of high- and low-affinity binding of 5-HT to 5-HT_{7A} and of dopamine to D1 receptors expressed heterologously in HEK293 cells. A, we analyzed [^{3}H]5-HT or [^{3}H]mesulergine binding to the human 5-HT_{7A} receptor at various concentrations, using Scatchard plot. B and C, competition experiments were carried out with [^{3}H]mesulergine at 4 nM and 5-HT at 0.1 to 1000 nM in cell membranes expressing 5-HT_{7A} receptors and with [^{3}H]SCH23390 at 2 nM and dopamine at 10 to 50,000 nM in cell membranes expressing D1 receptors. [^{3}H]Mesulergine binding was blocked by 5-HT (○) in a biphasic manner (solid line) with a K_I value of 1.8 ± 0.3 (high affinity) accounting for 64% of the total [^{3}H]mesulergine binding and with a K_I value of 93 ± 4 nM (low affinity). Treatment with 10 μM GppNHp did not alter the biphasic profile (●). [^{3}H]SCH23390 binding was inhibited by dopamine in a biphasic manner (solid line) with a K_I value of 53 ± 16 (high affinity) accounting for 12% of the [^{3}H]SCH23390 binding and with a K_I value of 7576 ± 574 nM (low affinity). Treatment with 10 μM GppNHp abolished high-affinity binding. Now, the data (●) fitted to a one-site binding model with a K_I value of 6237 ± 448 nM. The data represent mean ± S.E.M (n = 3).

body raised against bovine G_o protein was obtained from Chemicon (Temecula, CA). Agonist-induced GTPγ^{35}S binding was computed by subtracting the level observed without test agonists. Stock solutions were prepared in 0.1% ascorbic acid for monoamines, in dimethyl sulfoxide for lysophospholipids, and in alcohol for fatty acids and oleamide. Dilutions were made in borosilicate glass tubes, and final concentrations of dimethyl sulfoxide or alcohol were less than 0.5%. CHAPS and FF-BSA were prepared in the assay buffer. Reactions were carried out in polypropylene microtubes.

Results

Binding of [^{3}H]5-HT and [^{3}H]mesulergine (antagonist) to the human 5-HT$_{7A}$ receptor, when expressed heterologously in HEK293 cells, fitted well to a one-site binding model (linearity) with dissociation constants (K_D) of 1.5 ± 0.1 and 4.3 ± 0.1 nM, respectively, and maximal binding values of 1.7 ± 0.2 (Fig. 1) and 2.6 ± 0.1 pmol/mg of protein, respectively (Fig. 1). With naive HEK293 cells, we observed no detectable levels of [^{3}H]mesulergine and [^{3}H]5-HT binding under the same conditions. We also carried out competition experiments with [^{3}H]mesulergine at 4 nM and 5-HT at various concentrations (Fig. 1). The displacement data fitted to a model of two binding sites, high-affinity sites with a K_I value of 1.8 ± 0.3 nM (similar to the K_D for 5-HT), accounting for 64% of [^{3}H]mesulergine binding sites, and low-affinity sites with a K_I value of 93 ± 4 nM. 5-Carboxamidotryptamine maleate (5-CT), another agonist, displayed a similar biphasic profile, with high-affinity sites with a K_I value of 1.6 ± 0.2 nM accounting for 65% of [^{3}H]mesulergine binding sites and low-affinity sites with a K_I value of 76 ± 3 nM (Table 1). This indicates that more than 60% of the total receptors, when estimated from maximal [^{3}H]mesulergine (an antagonist) binding, displayed the high-affinity 5-HT binding. Classically, this phenotype represents G protein-associated receptors but was unusual for 5-HT$_{7A}$ in its marked abundance. Several antagonists, on the other hand, displaced [^{3}H]mesulergine binding in monophasic patterns (e.g., methiothepin, lisuride, and metergoline, with K_I values of 0.25 ± 0.04, 0.33 ± 0.05, and 0.97 ± 0.05 nM, respectively) (Table 1). GppNHp (10 μM), a nonhydrolyzable GTP analog, often uncouples receptor-G protein interactions, but it produced no appreciable changes in the relative populations of low- and high-affinity sites for 5-HT, as shown in the biphasic displacement pattern of [^{3}H]mesulergine by 5-HT (Fig. 1). On the other hand, GppNHp abolished high-affinity dopamine binding to the D1 dopamine receptor (a prototypic G_s coupled receptor) when expressed in the same cell line (HEK293 cells) at a similar receptor density, 2.5 ± 0.2 pmol/mg protein. Dopamine reduced [^{3}H]SCH23390 (antagonist with a K_D value of 0.66 ± 0.05 nM) binding to D1 receptors by 12 ± 2% with a K_I value of 53 ± 16 nM, but this high-affinity site disappeared in the presence of GppNHp, leaving only low-affinity sites with a K_I value of 7576 ± 574 nM (Fig. 1).

We also examined agonist-induced GTPγ^{35}S binding to Gα subunits. At 5-HT$_{7A}$, 10 μM 5-HT–induced GTPγ^{35}S binding increased as a function of GTPγ^{35}S concentrations (0.5 to 20 nM), with an EC_{50} value of 2.2 ± 0.8 nM and maximal binding of 270 ± 32 fmol/mg of protein (Fig. 2). Similar values were observed with dopamine-activated D1 dopamine receptors (2.6 ± 0.3 nM and 330 ± 53 fmol/mg of protein) (Fig. 2). Methiothepin (100 μM) by itself produced no effect on the basal level, but blocked 1 μM 5-HT–induced GTPγ^{35}S binding at 5-HT$_{7A}$ (24 ± 4% over the basal) whereas 100 μM SCH23390 blocked dopamine-induced GTPγ^{35}S binding at D1 (Fig. 2). Moreover, at 5-HT$_{7A}$, the methiothepin-sensitive GTPγ^{35}S binding was primarily associated with $G\alpha_s$, as detected with the immobilization method with various Gα-specific antibodies (Okamoto et al., 1992). The relative levels of GTPγ^{35}S association with the antibodies specific for $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_{13}$ were 24 ± 3, 5 ± 2, 1 ± 1, 4 ± 4, and 0 ± 1%, respectively, above the basal binding (no 5-HT in anti-$G\alpha_s$-coated wells) (Fig. 2).

Generally, G_i/G_o subtypes are the most abundant and active cellular G proteins contributing to GTPγ^{35}S binding. To evaluate independently the potential involvements of G_i/G_o subtypes at 5-HT$_{7A}$, membranes were treated with 100 μM NEM, an established, selective inhibitor of G_i/G_o subtypes (Winslow et al., 1987; Nakajima et al., 1990; Alberts et al., 1999). NEM treatment decreased the basal GTPγ^{35}S (2 nM) binding by 21 ± 3, but with no appreciable effect on the net 5-HT-induced GTPγ^{35}S binding (Fig. 2). This further supports the primary association of 5-HT$_{7A}$ with G_s but not G_i/G_o subtypes.

We examined the effects of lipid metabolites and similar amphipathic compounds on the 5-HT$_{7A}$ receptor, because such agents have been reported to allosterically modulate various membrane receptors (Koenig and Martin, 1992; Hedlund et al., 1999). FF-BSA, a scavenger of lipid metabolites, produced a noticeable, concentration-dependent reduction in [^{3}H]5-HT binding, with little effect on [^{3}H]mesulergine (antagonist) binding to 5-HT$_{7A}$ (Fig. 3). At 3%, FF-BSA decreased [^{3}H]5-HT (2 nM) binding by 46 ± 4%. Scatchard analysis showed a decrease in maximal 5-HT binding from 1.7 ± 0.2 to 1.1 ± 0.1 pmol/mg of protein, with no appreciable

TABLE 1

Comparison of binding and functional properties of serotonergic ligands at the human 5-HT$_{7A}$ receptor heterologously expressed in HEK293 cells

Competition binding experiments using 2 nM [^{3}H]mesulergine were carried out with several serotonergic ligands at various concentrations in HEK293 cell membranes expressing 5-HT$_{7A}$ receptors. Only displacement data for 5-HT and 5-CT were fitted to a two-site binding model; the rest were fitted to a one-site binding model. The values in parentheses represent the relative fraction of high- and low-affinity sites. The IC_{50} values from dose-response profiles were converted to K_I (Cheng and Prusoff, 1973). Intrinsic efficacy for ligands was estimated from an increase in cellular cAMP in intact cells or in 2 nM GTPγ^{35}S binding in isolated membranes and normalized for that obtained with 10 μM serotonin. The data represent mean ± S.E.M. (n = 3).

Compounds	Ligand Binding, K_I		Serotonin Efficacy	
	1st site	2nd site	cAMP	GTPγ^{35}S
	nM		%	
5-HT	1.8 ± 0.3 (0.64)	93 ± 4 (0.35)	100	100
5-CT	1.6 ± 0.2 (0.67)	76 ± 30 (0.32)	89 ± 12	116 ± 2
Methiothepin	0.25 ± 0.04		0	7 ± 4
Metergoline	0.97 ± 0.05		2	7
Lisuride	0.33 ± 0.05		0	6

change in its K_D value (1.2 ± 0.2 nM; Table 2 and Fig. 4). Among fatty acids tested, only oleic acid at concentrations less than 15 μM increased 2 nM [^{3}H]5-HT binding by 24 ± 5% without affecting [^{3}H]mesulergine binding. Oleic acid at 15 μM increased maximal [^{3}H]5-HT binding from 1.7 ± 0.2 to 2.2 ± 0.2 pmol/mg of protein but showed no effect on its K_D value (1.3 ± 0.2 nM) (Fig. 4). At higher concentrations, however, oleic acid gradually inhibited both [^{3}H]5-HT and [^{3}H]mesulergine (4 nM) binding. This could arise from its ability to disturb ligand binding sites as an amphipathic compound. Stearic acid at 30 μM showed no effects, but at higher concentrations, it decreased both [^{3}H]5-HT and [^{3}H]mesulergine binding (Fig. 3). Similar profiles were observed with palmitic, arachidonic, and myristic acids (data not shown). It seems that FF-BSA could decrease [^{3}H]5-HT binding by two ways, scavenging lipid metabolites such as oleic acid or possible protein-protein interactions with receptors. When fatty acid binding sites at FF-BSA were saturated with innocuous stearic acid (10 μM), FF-BSA lost its action on [^{3}H]5-HT binding (Fig. 3). This indicates that the FF-BSA action may arise from its scavenging of stimulatory lipid metabolites. Furthermore, oleic acid and FF-BSA produced no appreciable effects on [^{3}H]5-HT binding to analogous 5-HT receptors (i.e., gorilla 5-HT$_{1D}$ and human 5-HT$_{2C}$ receptors) when expressed heterologously in HEK293 cells. In the presence of oleic acid at 15 μM, for example, [^{3}H]5-HT binding to 5-HT$_{1D}$ and 5-HT$_{2C}$ was 104 ± 4 and 100 ± 4% of control, respectively; with FF-BSA at 3%, binding was 97 ± 2 and 104 ± 5%, respectively.

It has been reported that oleamide and oleic acid at 0.1 μM decrease allosterically the affinity of [^{3}H]5-HT for rat 5-HT$_{7A}$ receptors by 2- to 3-fold in HeLa cells, without affecting maximal binding (Hedlund et al., 1999). Here oleamide and oleic acid at the concentration range 0.01 to 1 μM showed no appreciable effects on [^{3}H]5-HT and [^{3}H]mesulergine binding to human 5-HT$_{7A}$ receptors in HEK293 cells. At micromolar concentrations, oleic acid (<15 μM) stimulated [^{3}H]5-HT binding in HEK293 cells, and oleamide (>50 μM) blocked gradually both [^{3}H]5-HT and [^{3}H]mesulergine binding (Fig. 3). These differences in receptor sensitivity could be ascribed to various factors, including species variations in rat and human receptors and possibly different surrounding lipid environments in the HeLa and HEK293 cells. In fact, the rat 5-HT$_{7A}$ in HeLA cells displayed a K_D value for 5-HT (12.68 nM) nearly 10-fold greater than the human counterpart in HEK293 cells (1.5 nM) (Hedlund et al., 1999).

We also studied lysophospholipids, which are also generated from hydrolysis of phospholipids by phospholipases. Various lysophospholipids (oleoyl and stearyl) at 15 μM or less showed no selective effects on [^{3}H]5-HT or mesulergine binding. Even at higher concentrations (50 μM), only lysophosphatidyl choline decreased selectively [^{3}H]5-HT binding (no effect on [^{3}H]mesulergine binding) by reducing maximal [^{3}H]5-HT binding from 1.7 ± 0.2 to 1.2 ± 0.2 pmol/mg of protein, but not its K_D value (1.4 ± 0.1 nM) (Table 2). Lysophosphatidyl glycerol decreased both [^{3}H]5-HT and [^{3}H]mesulergine binding (Fig. 3), and similar profiles were observed with lysophosphatidyl inositol and lysophosphatidyl serine (data not shown). Lysophosphatidyl ethanolamine and lysophosphatidic acid at concentrations up to 100 μM marginally inhibited [^{3}H]5-HT and mesulergine binding, with decreases of less than 20%.

Another nondenaturing zwitterionic detergent, CHAPS at 0.2% or less, markedly increased [^{3}H]5-HT binding, but marginally decreased [^{3}H]mesulergine binding (Fig. 3). In the presence of 0.1% CHAPS, maximal [^{3}H]5-HT binding increased from 1.7 ± 0.2 to 2.6 ± 0.1 pmol/mg of protein, with no appreciable change in its K_D value (1.4 ± 0.1 nM compared with 1.5 ± 0.1 nM). For [^{3}H]mesulergine binding, 0.1% CHAPS showed no effect on maximal binding (2.5 ± 0.2 compared with 2.6 ± 0.1 pmol/mg of protein) but increased its K_D value from 4.3 ± 0.1 to 6.9 ± 0.4 nM. The opposite effects by lysophaphatidyl choline and CHAPS, two similar zwitterions, suggest that their actions arise from specific interac-

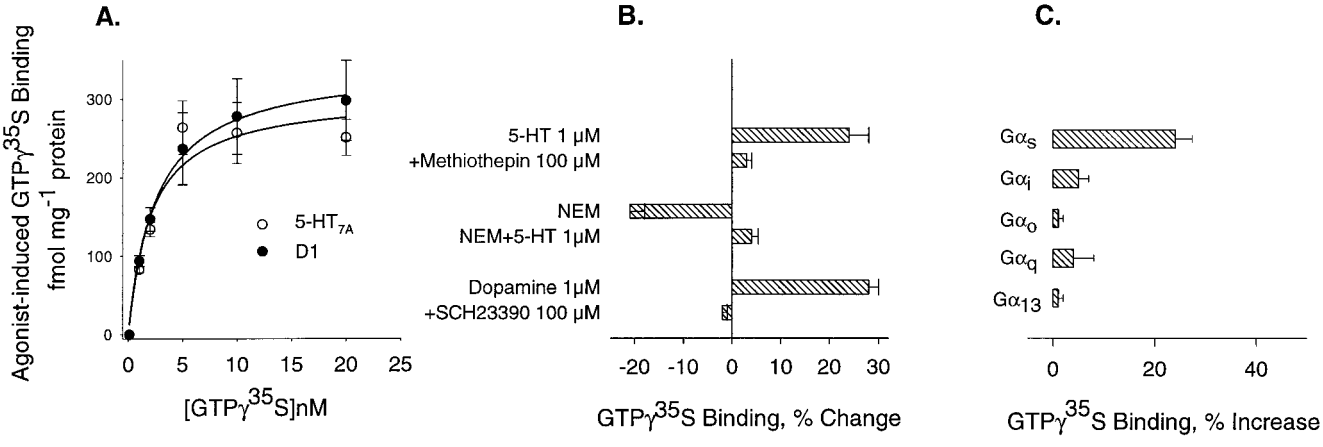

Fig. 2. Agonist-induced GTPγ^{35}S binding in HEK293 cell membranes expressing the human 5-HT$_{7A}$ or dopamine D1 receptors, and immobilization of GTPγ^{35}S-bound Gα subunits with various Gα-selective antibodies. A. Enhancement of GTPγ^{35}S binding by 5-HT or dopamine at 10 μM (a saturating concentration) was measured as a function of the concentration of GTPγ^{35}S ranging from 1 to 20 nM. Solid lines represent data fitted to a single hyperbolic rectangular, using Sigma Plot (See Text). B. The 5-HT (1 μM)-induced GTPγ^{35}S (2 nM) binding at 5-HT$_{7A}$ was blocked by its antagonist, methiothepin (100 μM), and was not affected by NEM, a selective inhibitor of G$_i$/G$_o$ subtypes of G proteins. NEM alone decreased the basal binding by 21%. The dopamine-induced GTPγ^{35}S (2 nM) binding at D1 was blocked by its antagonist, SCH23390 (100 μM). C. Agonist-induced association of GTPγ^{35}S with Gα subunits was monitored upon immobilization with various Gα-specific antibodies. Membranes were treated with 5-HT (10 μM) in the presence of GTPγ^{35}S at 2 nM for 30 min, and then solubilized with CHAPS at 0.3%. Solubilized Gα subunits were immobilzed with various Gα-specific antibodies. The data presented as percent increase from the basal value observed without agonist treatment, and are the mean ± S.E.M (n = 3).

tions with receptors, not from general membrane perturbations.

Functionally, these agents also affected 5-HT-induced GTPγ^{35}S binding. FF-BSA (3%) and 50 μM lysophosphatidyl choline reduced 10 μM 5-HT–induced GTPγ^{35}S binding to 56 ± 4 and 55 ± 10%, respectively, as normalized to that observed with 10 μM 5-HT, whereas oleic acid and CHAPS increased the GTPγ^{35}S binding to 137 ± 5 and 133 ± 7%, respectively. In the analogous Gs-coupled D1 receptor, however, 15 μM oleic acid, 3% FF-BSA, 50 μM lysophosphatidyl choline, and 0.1% CHAPS produced no appreciable effects on 10 μM dopamine-induced, SCH-23390-sensitive GTPγ^{35}S binding (96 ± 28, 95 ± 3, 83 ± 16, and 109 ± 22%, respectively).

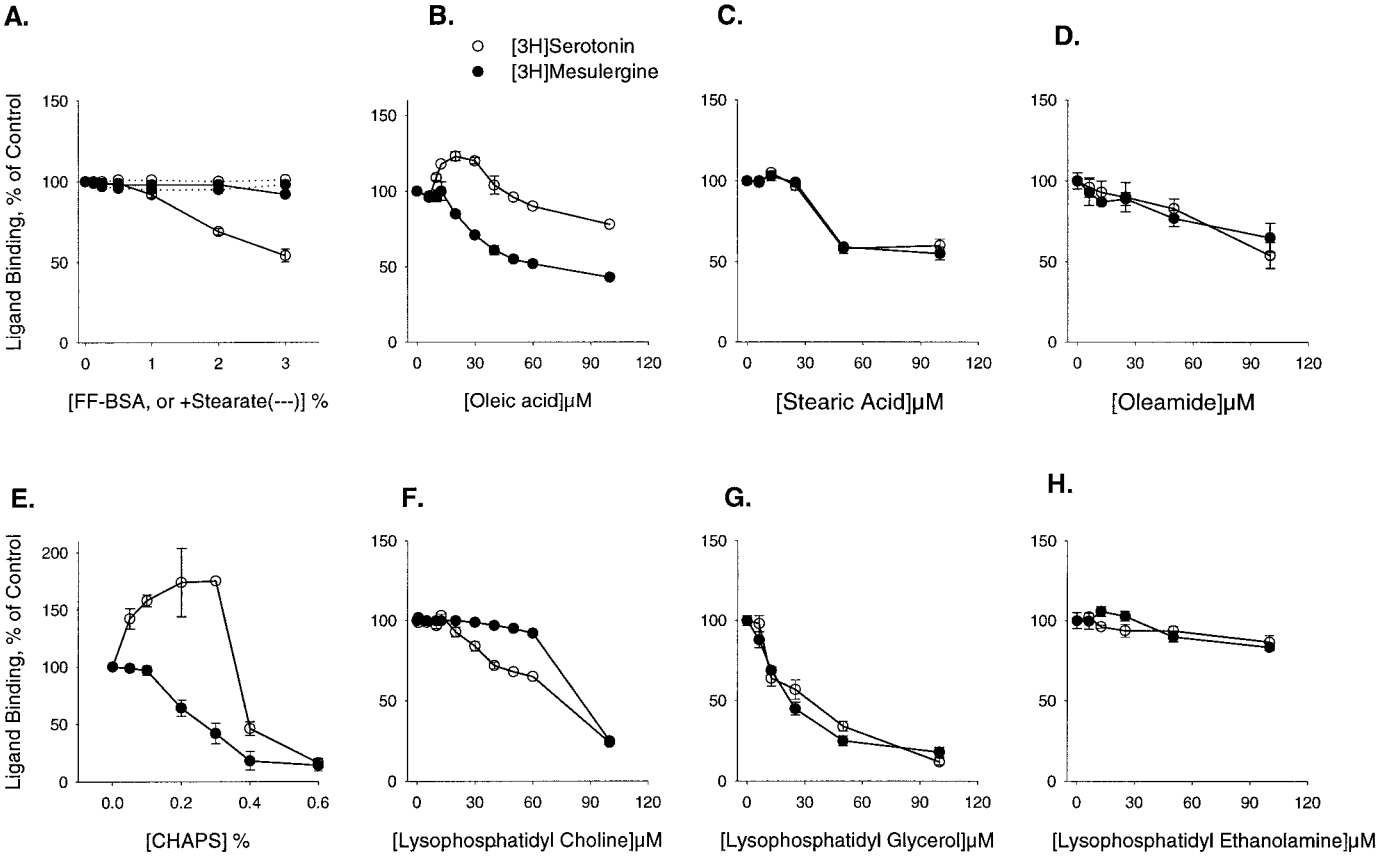

Fig. 3. Effects of FF-BSA and various lipid amphipathic compounds on [^{3}H]5-HT and [^{3}H]mesulergine binding to the 5-HT$_{7A}$ receptor expressed in HEK293 cells. Binding of 2 nM [^{3}H]5-HT and 4 nM [^{3}H]mesulergine to the 5-HT$_{7A}$ receptor in isolated membranes was measured in the presence or absence of the indicated amphipathic compounds at the concentration range from 1 to 100 μM, using filtration techniques as described under *Materials and Methods*. Nonspecific binding was measured in the presence of excess mesulergine (10 μM), and used to compute specific binding, which constitutes more than 90% of the total binding. Relative changes in the specific binding of [^{3}H]5-HT and [^{3}H]mesulergine were obtained upon normalization to control values. A, effects of FF-BSA (solid lines) and 10 μM stearate-saturated FF-BSA (dotted lines); B, oleic acid; C, stearic acid; D, oleamide; E, CHAPS; F, lysophosphatidyl choline; G, lysophosphatidyl glycerol; H, lysophosphatidyl ethanolamine. The data represent the mean ± S.E.M. (n = 3).

TABLE 2

Effect of various amphipathic compounds on [^{3}H]5-HT binding, cAMP production, and GTPγ^{35}S Binding at human 5-HT$_{7A}$ receptors expressed in HEK293 cells

Cellular cAMP changes were monitored using a FlashPlate assay kit from PerkinElmer in HEK 293 cells in a 96-well plate. A standard curve with various concentrations of cAMP was constructed, following the vendor's protocol. The basal levels of cAMP were less than 0.2 pmol/well and were not appreciably affected by addition of the listed compounds. The 5-HT–induced GTPγ^{35}S binding was measured in isolated HEK 293 cell membranes in the presence or absence of 10 μM 5-HT and 2 nM GTPγ^{35}S. Methiothepin at 100 μM blocked 5-HT–induced GTPγ^{35}S binding and by itself showed no effect. The data represent the mean ± S.E.M. (n = 3).

	[^{3}H]5-HT Binding		cAMP Production		GTPγ^{35}S Binding of 5-HT
	K_D	B_{max}	EC_{50}	E_{max}	
	nM	*pmol/mg protein*	*nM*	*pmol/well*	%
Control	1.5 ± 0.1	1.7 ± 0.2	8.1 ± 0.7	9.1 ± 0.1	100
CHAPS[a]	1.4 ± 0.1	2.6 ± 0.1*	3.7 ± 0.4**	8.4 ± 0.3	133 ± 7
Oleic acid, 15 μM	1.3 ± 0.2	2.2 ± 0.2*	4.0 ± 0.4**	9.0 ± 0.5	137 ± 5
FF-BSA, 3%	1.2 ± 0.2	1.1 ± 0.1*	13.9 ± 1.2**	9.1 ± 0.8	56 ± 4
LPC (50 μM)	1.4 ± 0.1	1.2 ± 0.2*	15.8 ± 1.6**	8.3 ± 0.3	55 ± 10

[a] 0.2% for [^{3}H]5-HT Binding, but 0.1% for cAMP and GTPγ^{35}S binding.
*, $p < 0.01$ from t test
**, $p < 0.05$ from t test

We also examined how these agents affect cAMP accumulation at 5-HT_{7A} in the HEK293 cell. Typically, 5-HT increased cAMP production in a concentration-dependent manner with an EC_{50} value of 8.1 ± 0.7 nM and maximal stimulation of 9 ± 1 pmol/well in a 96-well plate (Fig. 4). With cholera toxin treatment (5 μg/ml culture for overnight), the basal level of cAMP increased from 0.1 to 4 pmol/well, but 5-HT produced no responses; the covalent modification of G_s subtypes by cholera toxin (ADP-ribosylation) rendered them into permanently activated states (Milligan, 1988). FF-BSA at 3% and lysophosphatidyl choline at 50 μM showed no effects on the basal cAMP production (<0.2 pmol/well) but increased the 5-HT concentration (EC_{50}) from 8.1 to 13.9 ± 1.2 and 15.8 ± 1.6 nM, respectively, without affecting maximal cAMP production (9.1 ± 0.8 and 8.3 ± 0.3 pmol/well, respectively; Fig. 4 and Table 2). On the other hand, 15 μM oleic acid and 0.1% CHAPS decreased the EC_{50} value from 8.1 ± 0.7 to 3.7 ± 0.4 and 4 ± 1 nM, respectively, but with little effect on maximal production (8.4 ± 0.3 and 9 ±1 pmol/well, respectively) and on basal cAMP production.

Discussion

The human 5-HT_{7A} receptor is a G_s-coupled receptor; when expressed heterologously in HEK293 cells, more than 60% of its population displayed high-affinity [^{3}H]5-HT binding. We discovered here that the high-affinity agonist binding decreased in the presence of FF-BSA and increased in the presence of oleic acid without appreciable effects on antagonist binding. The actions of FF-BSA and oleic acid seem to be highly selective for 5-HT_{7A}, judging from the following observations. 1) FF-BSA and oleic acid showed no appreciable effects on [^{3}H]5-HT binding to analogous 5-HT receptors (5-HT_{1D} and 5-HT_{2C}). 2) Structural analogs of oleic acid, such as palmitic, stearic, and arachidonic acids, were not effective. 3) FF-BSA lost its action when preincubated with inactive 10 μM stearic acid, supporting its role in scavenging endogenous lipid metabolites rather than protein-protein interaction. 4) FF-BSA and oleic acid reduced maximal binding for 5-HT, not the K_D value, indicating their selective action at receptors. 5) Oleic acid was effective at concentrations (<15 μM) too low to form micellar structures, and its action was not mimicked by lysophospholipids, another class of amphipathic lipid metabolites. 6) In general, the human 5-HT_{7A} receptor seems to be susceptible to allosteric modulations by amphipathic agents. For example, lysophosphatidyl choline decreased maximal 5-HT binding for 5-HT (not antagonist binding), and CHAPS, a similar zwitterion, increased it. From these results, we propose that endogenous amphipathic compounds, probably including oleic acid and others that remain unidentified, may allosterically modulate 5-HT_{7A} to increase high-affinity 5-HT binding.

In addition, the amphipathic compounds at concentrations that selectively affected [^{3}H]5-HT binding also influenced 5-HT-induced GTPγ^{35}S binding at 5-HT_{7A}. Oleic acid and CHAPS increased and FF-BSA and lysophosphatidyl choline decreased 5-HT-induced GTPγ^{35}S binding. No similar actions by these agents at D1 (a prototypic G_s-coupled receptor) also support the view of their specific interactions with 5-HT_{7A}, but not with G proteins in the signaling pathways.

In this study, we also observed that GppNHp produced no appreciable effect on the high-affinity agonist binding to 5-HT_{7A} but abolished high-affinity dopamine binding to D1. Two possibilities can be cited: 1) At 5-HT_{7A}, a certain portion could be sensitive to GppNHp but not detectable because the overwhelming majority of high-affinity binding was insensitive to the guanine nucleotide. 2) 5-HT_{7A} receptors could form a very tight complex with Gs that could not be destabilized by GppNHp. A similar situation has been proposed for

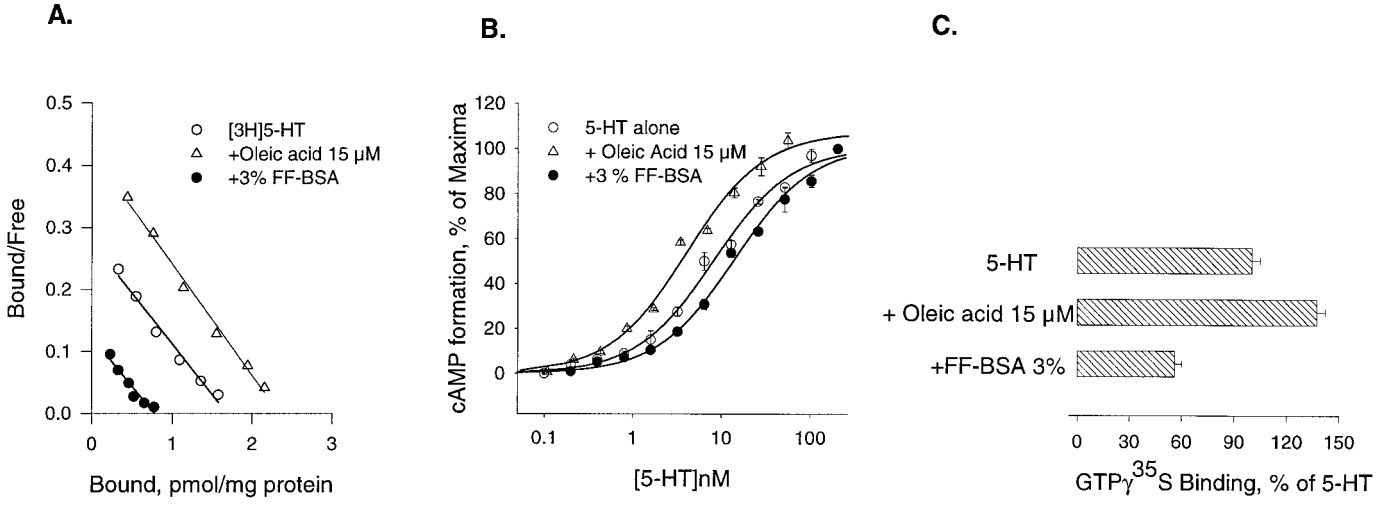

Fig. 4. Effects of FF-BSA and CHAPS on 5-HT binding parameters, 5-HT-induced cAMP production and GTPγ^{35}S binding. A, Scatchard analysis of binding data for [^{3}H]5-HT at various concentrations in cell membranes expressing 5-HT_{7A} receptors without (□) or with FF-BSA (3%) (●) or oleic acid (15 μM) (△). Binding experiments were carried out with filtration techniques at room temperature. [^{3}H]5-HT concentration varied from 0.25 to 10 nM. Nonspecific binding was measured in the presence of excess mesulergine (10 μM) and was used to compute specific binding. The solid lines represent linear regression analysis using Sigma Plot (see Table 2 for parameters). B, 5-HT–induced cAMP production was measured in intact cells using FlashPlate assay kit from PerkinElmer without (○) or with FF-BSA (3%) or oleic acid (15 μM). Cells were treated with 5-HT from 0.5 to 500 nM for 30 min in the presence of 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase. The data fitted to the equation of single rectangular hyperbola from Sigma plot (solid lines). Maximal cAMP accumulation reached about 8 to 9 pmol/well in a 96-well plate. C. 5-HT (10 μM)-induced 2 nM GTPγ^{35}S binding was measured without or with FF-BSA (3%) or oleic acid (15 μM) and normalized to control. The data represent the mean ± S.E.M. (n = 3).

Mel$_{1a}$-metatonin receptor and G$_i$ (Roka et al., 1999). Further study will be needed.

In summary, endogenous amphipathic lipid metabolites that could be scavenged by FF-BSA, such as oleic acid, seem to allosterically modulate 5-HT$_{7A}$ to enhance its high-affinity agonist binding, at least in part, and to confer more efficient coupling with G$_s$ subtypes of G proteins. Their interactions with 5-HT$_{7A}$ seem to be specific, judging from the narrow structural requirement for modulators (oleic acid and CHAPS) and their inactivity at analogous 5-HT and dopamine receptors.

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