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Detailed Pharmacological Characterization of 5-HT_{1A}-Receptor-Mediated [^{35}S]GTPγS Binding in Rat Hippocampal Membranes

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Abstract. 5-HT-stimulated [^{35}S]GTPγS binding to rat hippocampal membranes was pharmacologically characterized. Signal/noise ratio or percent increase over basal was optimized with 100 μM GDP, 2 – 10 mM $MgCl_2$, and 150 – 200 mM NaCl. However, we preferred the standard condition (20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl: Condition I) to the alternative one (100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl: Condition II) because 1) absolute values of basal and 5-HT-sensitive bindings decreased with higher concentrations of GDP and NaCl; 2) EC_{50} values determined under Condition II were 2 – 6 fold higher than those under Condition I; 3) some partial agonists had less intrinsic activities in the presence of higher concentrations of GDP; and 4) Inhibitory effects of WAY100635 were complete under Condition I, while incomplete under Condition II. Pharmacological profile of concentration-dependent stimulation by a series of 5-HT ligands and concentration-dependent inhibition of 5-HT-stimulated binding by several 5-HT-receptor antagonists clearly indicated that this response under Condition I was mediated solely through 5-HT_{1A} receptors. Although caution should be paid especially to the apparent intrinsic activities susceptible to the assay conditions, this method appears useful to investigate functional coupling between 5-HT_{1A} receptors and their coupled G proteins in native hippocampal membranes.

Keywords: 5-HT_{1A} receptor, G protein, [^{35}S]GTPγS binding, hippocampus, intrinsic activity

Introduction

5-Hydroxytryptamine (serotonin, 5-HT) elicits diverse physiological responses as a neurotransmitter or neuromodulator in the mammalian central nervous system through multiple distinct receptor subtypes. To date, it has been revealed that the 5-HT-receptor superfamily is composed of at least fourteen members, which have been classified based on gene structure, amino acid sequence homology, and intracellular signaling cascades (1, 2). All subtypes except one (5-HT_3) are metabotropic receptors with the characteristic seven hydrophobic transmembrane domains, which couple to diverse intracellular signaling cascades via guanine nucleotide-binding proteins (G proteins). Of these, 5-HT_{1A} receptors have been extensively investigated and characterized in many pharmacological, biochemical, electrophysiological, and behavioral experiments, especially since the introduction of the 5-HT_{1A}-receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). 5-HT_{1A} receptors are densely distributed in limbic brain areas, notably the hippocampus, lateral septum, cortical areas (particularly cingulate and entorhinal cortex), and also the mesencephalic raphe nuclei (both dorsal and median raphe nuclei).

The interaction between 5-HT_{1A} receptors and G proteins in native brain membranes has been investigated in an indirect manner for long time, that is, by the modulating effects of GTP or its nonhydrolyzable analogs on the binding sites labeled by an agonistic radioligand such as [^{3}H]8-OH-DPAT. Subsequently, 5-HT_{1A}-receptor-mediated G protein activation was successfully detected and characterized in rat hippocampal membranes by utilizing one of the functional characteristics of the heterotrimeric G proteins, that is, high-affinity GTPase activity (3, 4). Although the

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pharmacological profile of this response (4) clearly indicated the involvement of 5-HT_{1A} receptors associated with the inhibition of adenylyl cyclase activity (5), the maximal increase above the unstimulated basal high-affinity GTPase activity induced by 5-HT was only 10 – 20%. Such a low signal/noise ratio seemed not to permit us to determine the accurate intrinsic activity of a probable partial agonist nor to proceed to probe the G protein subtype(s) involved in this response.

The heterotrimeric G proteins work as a molecular switch. Agonist-bound receptor facilitates GDP release from the α subunit of G protein (G_α) and replacement with GTP (GTP-GDP exchange: on-switch step), and the activated G proteins return to the inactive state following the hydrolysis of GTP by high-affinity GTPase intrinsic to G_α and the reassociation of GDP-bound G_α and $\beta\gamma$ subunits of G protein ($G_{\beta\gamma}$) (off-switch step) (6). Recently, the on-switch step has been widely utilized to measure the agonist-induced binding of the nonhydrolyzable analog of GTP, [^{35}S]guanosine-5'-*O*-(γ-thio)-triphosphate ([^{35}S]GTPγS), to G_α subunits, which assesses the functional interaction between several receptors and their coupled G proteins, especially when derived from the $G_{i/o}$ type, in cultured cells as well as native brain membranes (7). 5-HT_{1A}-receptor-mediated [^{35}S]GTPγS binding was reported in the membranes prepared from Chinese hamster ovary (CHO) cells expressing human 5-HT_{1A} receptors in 1996 (8, 9); and thereafter, there have been many reports utilizing this technique in this cell line and others such as HeLa cells (10 – 16), C6-glioma cells (10, 11, 13, 14), and Cos-7 cells (17). This technique has also been adapted to native membranes prepared from the brain of rats (18 – 23) and humans (24 – 28). However, these reports usually used only a limited range of ligands, and there have been several discrepancies among the studies. The aim of the present investigation was to establish the standard method of measuring 5-HT_{1A}-receptor-mediated [^{35}S]GTPγS binding in rat hippocampal membranes and to characterize it pharmacologically in detail with the help of an extensive series of 5-HT-receptor agonists and antagonists.

Materials and Methods

Membrane preparation

Male Sprague-Dawley rats weighing 200 – 250 g were killed by decapitation and their brains were quickly removed. The hippocampus dissected on ice from the brain of a rat was homogenized in 5 ml of ice-cold TED buffer (5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) containing 10% (w/v) sucrose by 20 strokes with a motor-driven Teflon/glass tissue grinder. All the following centrifuge procedures were carried out at 4°C. Subsequent to centrifugation of the homogenate at 1,000 × *g* for 10 min, the supernatant was decanted to another centrifuge tube, whereas the pellet was vortexed in 5 ml of TED/sucrose buffer followed by another centrifugation at 1,000 × *g* for 10 min. The combined supernatant was washed twice by being centrifuged at 9,000 × *g* for 20 min and resuspended in 10 ml of TED buffer. The suspension was kept on ice for 30 min followed by the final centrifugation at 35,000 × *g* for 10 min, and the resulting pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) to produce the homogenate with a protein concentration ranging from 1.0 to 2.0 mg/ml. The homogenate was divided into aliquots in plastic tubes, frozen quickly on fine-grained dry ice, and stored at −80°C until use.

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding experiments were performed essentially as described previously (29). On the day of the experiment, hippocampal membranes were thawed slowly on ice and diluted with 50 mM Tris-HCl buffer (pH 7.4). Aliquots (100 μl) of the diluted membranes equivalent to 10 – 20 μg protein were incubated, unless indicated otherwise, at 30°C for 60 min in 500 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 nM [^{35}S]GTPγS, 20 μM GDP, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 0.2 mM dithiothreitol, 100 mM NaCl, and various concentrations of a 5-HT-receptor agonist and/or antagonist. In some experiments, the concentrations of GDP, $MgCl_2$, and NaCl in the assay buffer were varied according to the experimental objectives. The reaction was terminated by rapid filtration through glass fiber filters (GF/B; Whatman Int., Maidstone, UK) using a Brandel cell harvester with twice washing with 5 ml ice-cold washing buffer (50 mM Tris-HCl, pH 7.4). The radioactivity content of the filters was counted in 8 ml scintillation cocktail Emulsifier-Scintillator Plus (Packard Bioscience, Groningen, The Netherlands) by a liquid scintillation counter. The non-specific binding was measured in the presence of 100 μM unlabeled GTPγS, which was subtracted from the total binding to define the specific [^{35}S]GTPγS binding.

Data analysis

All results except for Fig. 5 were presented as the mean ± S.E.M. values of separate experiments, each performed in duplicate. The concentration-dependent increase in specific [^{35}S]GTPγS bindings by a 5-HT-receptor ligand was expressed as a percent increase above the basal unstimulated binding and analyzed by means of a non-linear regression method (sigmoid

model with a variable slope and with a bottom value fixed to zero) using the commercially available program GraphPad PRISM™ (GraphPad, San Diego, CA, USA) to produce the concentration eliciting the half-maximal effect (EC_{50}) and the percent maximal increase above the basal binding ($\%E_{max}$). The inhibitory curve of an antagonist against the fixed concentration of an agonist was expressed as percent of the increase in the binding by the agonist alone and also analyzed with a non-linear regression method with a top value fixed to 100% and a bottom value either fixed to zero or unfixed to produce the concentration eliciting the half-maximal inhibition (IC_{50}). Antagonist potency (K_b) for an antagonist was calculated by the following equation (30):

$$K_b = IC_{50} \div \{[2 + (A / EC_{50})^b]^{1/b} - 1\}$$

where A and b are the agonist concentration and Hill coefficient of the agonist stimulation isotherm, respectively. The EC_{50} and K_b values were normalized to negative logarithmic values as pEC_{50} and pK_b, respectively.

Materials

[^{35}S]GTPγS (1,250 Ci/mmol) was purchased from Du Pont NEN Research Products (Boston, MA, USA). The following compounds were generous gifts from pharmaceutical companies: flesinoxan HCl, Solvay Pharmaceutical Co. (Weesp, The Netherlands); ipsapirone HCl, Bayer AG (Wuppertal, Germany); and tandospirone citrate, Sumitomo Pharmaceutical Co. (Osaka). 6-Chloro-2[piperidinyl-4-thio]pyridine HCl (anpirtoline), 3-[4-(4-chlorophenyl)piperazin-1-yl]-1,1-diphenyl-2-propanol HCl (BRL15572), 1,4-dihydro-3-(1,2,3,6-tetrahydro-4-pyridinyl)-5*H*-pyrrolo[3,2-*b*]pyridin-5-one 2HCl (CP93129), 5-propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-pyrrolo[3,2-*b*]pyridine HCl (CP94253), 3-[3-(2-dimethylaminoethyl)-1*H*-indol-5-yl]-*N*-(4-methoxybenzyl)acrylamide (GR46611), 3-[3-(dimethylamino) propyl]-4-hydroxy-*N*-[4-(pyridinyl) phenyl] benzamide 2HCl (GR55562), *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide HCl (GR127935), 2-[5-[3-(4-methylsulfonylamino)benzyl-1,2,4-oxadiazol-5-yl]-1*H*-indol-3-yl]ethanamine (L694247), methylergometrine maleate, 5-methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole hemisuccinate (RU24969), and *N*-(3-trifluoromethylphenyl)piperazine HCl (TFMPP) were purchased from Tocris Cookson, Ltd. (Bristol, UK). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Effects of GDP, $MgCl_2$, and NaCl on 5-HT-stimulated [^{35}S]GTPγS binding

In order to probe the optimum experimental conditions for 5-HT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes, the effects of various concentrations of GDP, $MgCl_2$, and NaCl were determined. As shown in Fig. 1, increasing concentrations of GDP in the assay medium dramatically reduced the specific [^{35}S]GTPγS bindings both in the absence and presence of 10 μM 5-HT, but with a slightly different sensitivity. As a result, the increase in specific [^{35}S]GTPγS binding induced by 10 μM 5-HT, which was undetectable in the absence of GDP, made an appearance in the presence of GDP at submicromolar and higher concentrations (Fig. 1, inset). The percent increase over the basal binding induced by 10 μM 5-HT appeared to reach the maximum at 100 μM GDP, while the maximal absolute increases expressed as fmol per mg protein per 60 min were obtained in the presence of 1 – 30 μM GDP.

The specific [^{35}S]GTPγS binding to hippocampal

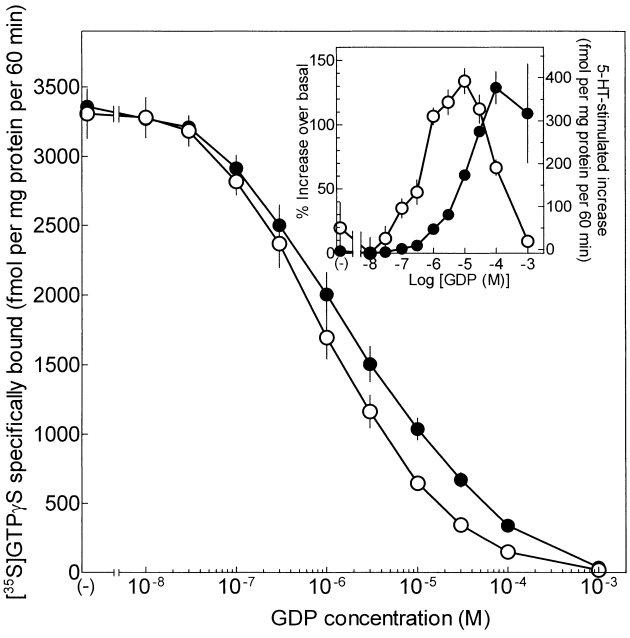

Fig. 1. Effects of GDP on 5-HT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes. Specific [^{35}S]GTPγS binding in the absence (open circles) and presence (closed circles) of 10 μM 5-HT was determined in the presence of various concentrations of GDP. The assay condition except for GDP was standardized with 5 mM $MgCl_2$ and 100 mM NaCl. Results are expressed as mean ± S.E.M. values of three independent experiments, each performed in duplicate. Inset: Increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed as percent (closed circles, left ordinate) or as absolute value (open circles, right ordinate) over the respective basal binding at various concentrations of GDP.

membranes was strictly dependent on the existence of Mg^{2+} in the assay medium (Fig. 2). Additionally, the increase in specific [^{35}S]GTPγS binding induced by 10 μM 5-HT was also dependent on the presence of Mg^{2+}, with the optimum $MgCl_2$ concentrations of 2 – 10 mM (Fig. 2, inset).

The addition of increasing concentrations of NaCl induced concentration-dependent reduction of specific [^{35}S]GTPγS bindings both in the absence and presence of 10 μM 5-HT (Fig. 3). The increment of specific [^{35}S]GTPγS binding stimulated by 10 μM 5-HT per se was gradually reduced by the addition of increasing concentrations of NaCl up to 200 mM, while the 5-HT-elicited increase expressed as percent over the basal binding was inversely augmented with increasing concentrations of NaCl (Fig. 3, inset).

Effects of several 5-HT-receptor agonists on [^{35}S]GTPγS binding under different assay conditions

Simply based on the results shown in Figs. 1 – 3, the optimum experimental condition to maximize the signal/noise ratio of 5-HT-stimulated [^{35}S]GTPγS binding to rat hippocampal membranes appeared to contain 100 μM (or higher concentrations of) GDP, 2 – 10 mM $MgCl_2$, and 150 – 200 mM NaCl in the assay medium. As this condition is somewhat different from that applied to the $GABA_B$-receptor-mediated [^{35}S]GTPγS binding in rat brain membranes (i.e., 20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl) (29), we determined concentration-response curves for several representative 5-HT-receptor agonists under the two different assay conditions. In the presence of 20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl (Condition I), 5-HT stimulated specific [^{35}S]GTPγS binding in a concentration-dependent manner with a mean EC_{50} value of 74 nM ($pEC_{50} = 7.13 \pm 0.03$, n = 7) and a $\%E_{max}$ of 104.9 ± 6.6 (Fig. 4). Under the condition of 100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl (Condition II), the concentration-response curve for 5-HT-stimulated [^{35}S]GTPγS binding was shifted rightward and the mean EC_{50} value was calculated to be 370 nM ($pEC_{50} = 6.43 \pm 0.06$, n = 4). The $\%E_{max}$ value under this condition was 154.7 ± 8.8, significantly higher than that determined under Condition I ($P<0.01$; non-paired two-tailed Student's *t*-test).

Similarly, the mean EC_{50} values for 5-carboxamidot-

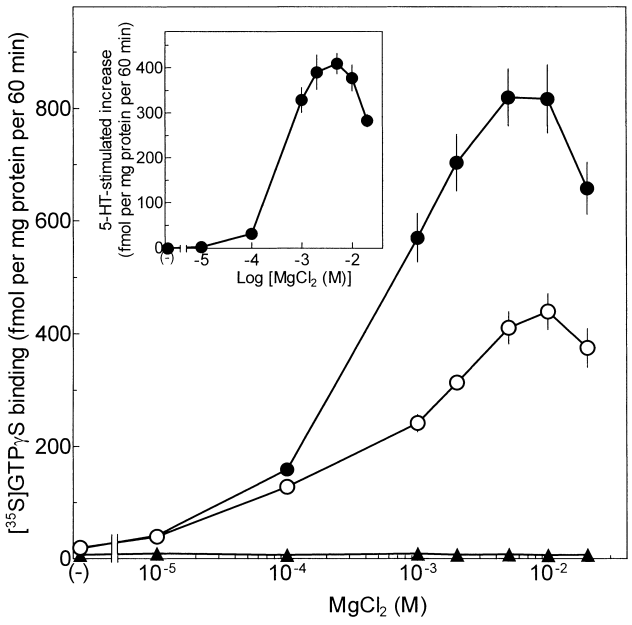

Fig. 2. Effects of $MgCl_2$ on 5-HT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes. [^{35}S]GTPγS binding in the absence (open circles) or presence (closed circles) of 10 μM 5-HT, and in the presence of 100 μM GTPγS (nonspecific binding) (closed triangles), was determined at various concentrations of $MgCl_2$. The assay condition except for $MgCl_2$ was standardized with 20 μM GDP and 100 mM NaCl. Results are expressed as mean ± S.E.M. values of three independent experiments, each performed in duplicate. Inset: Increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed as fmol per mg protein per 60 min at various concentrations of $MgCl_2$.

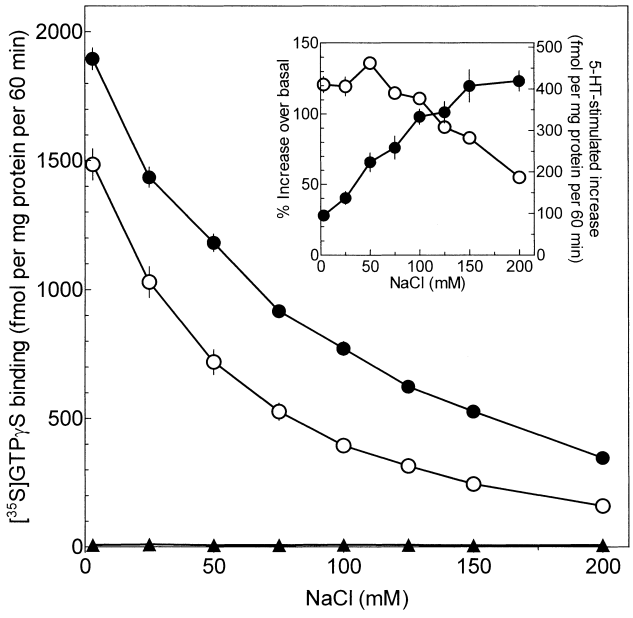

Fig. 3. Effects of NaCl on 5-HT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes. [^{35}S]GTPγS binding in the absence (open circles) and presence (closed circles) of 10 μM 5-HT, and in the presence of 100 μM GTPγS (nonspecific binding) (closed triangles), was determined at various concentrations of NaCl. The assay condition except for NaCl was standardized with 20 μM GDP and 5 mM $MgCl_2$. Results are expressed as mean ± S.E.M. values of three independent experiments, each performed in duplicate. Inset: Increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed as percent (closed circles, left ordinate) or as absolute value (open circles, right ordinate) over the respective basal binding at various concentrations of NaCl.

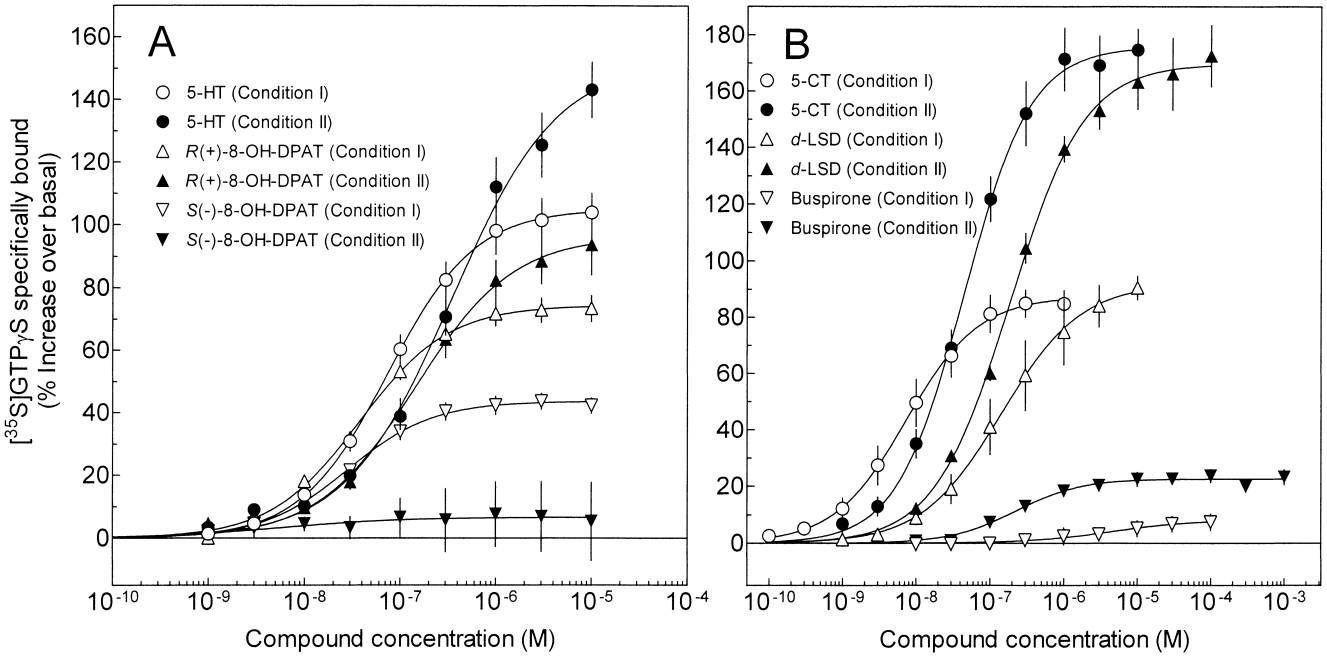

Fig. 4. Effects of several 5-HT-receptor agonists on [^{35}S]GTPγS binding in rat hippocampal membranes. Specific [^{35}S]GTPγS binding was measured in the presence of various concentrations of a test compound either under Condition I (20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl) (open symbols) or under Condition II (100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl) (closed symbols). Results are expressed as mean ± S.E.M. values of percent increase over the respective basal binding obtained from 3 – 7 independent experiments, each performed in duplicate. A: 5-HT (circles), *R*(+)-8-OH-DPAT (triangles), and *S*(−)-8-OH-DPAT (inverse triangles). B: 5-CT (circles), *d*-LSD (triangles), and buspirone (inverse triangles).

ryptamine maleate (5-CT), *R*(+)-8-OH-DPAT, and lysergic acid diethylamide (*d*-LSD) determined under Condition II were 2 – 6 fold higher than those obtained under Condition I (Fig. 4). It seemed difficult to compare exactly the EC_{50} values between the two different experimental conditions for *S*(−)-8-OH-DPAT and buspirone because of scant increases in [^{35}S]GTPγS binding by these compounds under either condition.

Regarding the $\%E_{max}$ values, it was hard to generalize the trend observed under the two different assay conditions. Thus, the $\%E_{max}$ values for 5-CT, *d*-LSD, and *R*(+)-8-OH-DPAT determined under Condition II were greater than those under Condition I, as in the case of 5-HT, whereas the inverse phenomenon was observed for *S*(−)-8-OH-DPAT (Fig. 4). To obtain further insight concerning the effects of GDP on the $\%E_{max}$ values, the increases in specific [^{35}S]GTPγS binding induced by these compounds at a maximally effective concentration (10 μM for 5-CT and 100 μM for 5-HT, *d*-LSD, *R*(+)-8-OH-DPAT, *S*(−)-8-OH-DPAT, and buspirone) were determined in the presence of various concentrations of GDP (Fig. 5). When expressed as percent of the respective maximal increase induced by 100 μM 5-HT, the intrinsic activity of a test compound depended on the concentration of GDP. 5-CT behaved as a full agonist throughout all concentrations of GDP examined. The partial agonist properties of *R*(+)-8-OH-DPAT were also constant in spite of GDP concentrations, with an intrinsic activity of 0.7 – 0.8. Interestingly, *d*-LSD was almost a full agonist in the presence of GDP ≥ 20 μM, but a partial agonist with an intrinsic activity of ca. 0.6 at the lowest concentration (4 μM) of GDP. The two partial agonists with lower intrinsic activities, *S*(−)-8-OH-DPAT and buspirone became less efficacious as the concentrations of GDP were increased.

In addition to the fact that most agonists showed lower potencies and that some partial agonists had less intrinsic activities in the presence of higher concentrations of GDP, Condition I was the same as in our previous reports (23, 29). Because of this, Condition I was preferred and chosen as a standard assay condition in the following experiments, although the $\%E_{max}$ values for 5-HT and some 5-HT-receptor agonists were higher under Condition II.

Effects of a series of 5-HT-receptor agonists on [^{35}S]GTPγS binding under Condition I

Under Condition I, concentration-response curves were drawn for a series of 5-HT-receptor ligands. As shown in Table 1, the most potent agonists in this assay system were 5-CT and *R*(+)-lisuride hydrogen maleate, with a mean EC_{50} of 7.7 and 8.1 nM, respectively. The

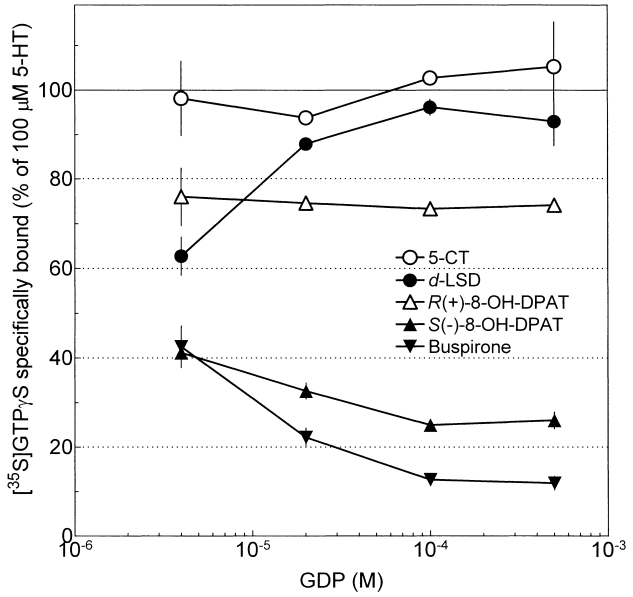

Fig. 5. Effects of GDP on maximal stimulation of [^{35}S]GTPγS binding by several 5-HT-receptor agonists in rat hippocampal membranes. Specific [^{35}S]GTPγS binding in the absence and presence of the test compounds at maximally effective concentration was determined in the presence of various concentrations of GDP. The assay condition except for GDP was standardized with 5 mM $MgCl_2$ and 100 mM NaCl. Increase in specific [^{35}S]GTPγS binding by 10 μM 5-CT (open circles), 100 μM *d*-LSD (closed circles), 100 μM *R*(+)-8-OH-DPAT (open triangles), 100 μM *S*(−)-8-OH-DPAT (closed triangles), and 100 μM buspirone (inverse closed triangles) is expressed as percent of the maximal increase induced by 100 μM 5-HT. Results are expressed as the mean ± S.E.M. of triplicate determinations of one experiment.

selective 5-HT$_{1A}$-receptor agonists, *N*,*N*-dipropyl-5-carboxamidotryptamine maleate (*N,N*-DP-5CT), flesinoxan, and *p*-aminophenylethyl-*m*-trifluomethylphenyl piperazine (PAPP) also stimulated [^{35}S]GTPγS binding with relatively high potencies. Some reportedly selective 5-HT$_{1B/1D}$-receptor agonists such as L694247, GR46611, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo-[1,2-*a*]quinoxaline maleate salt (CGS12066A), anpirtoline, and CP94253 were shown to be agonists, but with lower potencies. The %E$_{max}$ values were various and

Table 1. Agonist properties of [^{35}S]GTPγS binding in rat hippocampal membranes

Compound	n	pEC$_{50}$	EC$_{50}$ (nM)	%E$_{max}$
5-CT	3	8.12 ± 0.13	7.7	87.4 ± 4.6
(5-CT)	3	(7.35 ± 0.03)	(45)	(175.9 ± 10.2)
R(+)-Lisuride	4	8.09 ± 0.14	8.1	50.5 ± 9.4
Dihydroergotamine	4	7.91 ± 0.05	12	110.2 ± 4.4
N,*N*-DP-5CT	4	7.82 ± 0.08	15	47.6 ± 8.7
S(−)-8-OH-DPAT	4	7.54 ± 0.03	29	43.7 ± 2.6
(*S*(−)-8-OH-DPAT)	4	(—[a])	(—[a])	(—[a])
(±)-8-OH-DPAT	4	7.49 ± 0.02	32	76.6 ± 5.5
R(+)-8-OH-DPAT	6	7.43 ± 0.02	37	74.3 ± 4.1
(*R*(+)-8-OH-DPAT)	4	(6.83 ± 0.02)	(147)	(96.8 ± 9.3)
Flesinoxan	4	7.31 ± 0.18	49	38.6 ± 3.3
5-HT	7	7.13 ± 0.03	74	104.9 ± 6.6
(5-HT)	4	(6.43 ± 0.06)	(370)	(154.7 ± 8.8)
d-LSD	4	7.04 ± 0.05	90	94.5 ± 2.8
(*d*-LSD)	4	(6.73 ± 0.04)	(180)	(170.3 ± 11.4)
PAPP	4	6.91 ± 0.15	120	55.3 ± 2.3
L694247	4	6.82 ± 0.07	150	124.3 ± 5.1
R(+)-UH301	4	6.80[b]	160	5.3[b]
Methylergometrine	4	6.63 ± 0.06	230	66.5 ± 6.2
RU24969	4	6.58 ± 0.05	260	61.2 ± 2.2
GR46611	7	6.54 ± 0.07	290	110.1 ± 5.5
5-MeOT	4	6.50 ± 0.09	320	59.1 ± 5.3
Metergoline	4	6.48 ± 0.04	330	36.2 ± 3.4
5-MeO-*N*,*N*-DMT	4	6.32 ± 0.04	480	52.0 ± 6.2
Tandospirone[c]	4	6.28 ± 0.03	530	37.3 ± 2.5
CGS12066A	5	6.10 ± 0.13	800	65.1 ± 5.9
Spiroxatrine	4	6.09 ± 0.09	810	39.5 ± 3.3
Methysergide	4	5.95 ± 0.07	1,100	38.4 ± 5.4
mCPP[c]	4	5.86 ± 0.06	1,400	29.6 ± 3.0
BRL54443	4	5.42 ± 0.03	3,800	86.3 ± 8.9
Tryptamine	4	5.37 ± 0.06	4,300	61.6 ± 2.9
Anpirtoline	4	5.34 ± 0.12	4,600	48.0 ± 10.8
Buspirone[c]	4	5.34[b]	4,600	8.2[b]
(Buspirone)	4	(6.58 ± 0.15)	(260)	(23.5 ± 1.6)
CP94253	3	5.24 ± 0.22	5,800	57.2 ± 17.9
TFMPP	4	4.79 ± 0.14	16,000	23.2 ± 4.5
CP93129	5	<5	>10,000	—[d]
(±)-DOI	4	<5	>10,000	—[d]
BW723C86	4	<5	>10,000	—[d]
N-Methylquipazine	4	<5	>10,000	—[d]
BMY7378	4	—[a]	—[a]	—[a]
Ipsapirone	4	—[a]	—[a]	—[a]
S(−)-UH301	4	—[a]	—[a]	—[a]
WB4101	4	—[a]	—[a]	—[a]
Methiothepin	4	—[a]	—[a]	—[a]
(±)-DOB	4	—[a]	—[a]	—[a]
NAN190	4	—[a]	—[a]	—[a]
S(−)-Pindolol	4	—[a]	—[a]	—[a]
Spiperone	4	—[a]	—[a]	—[a]
WAY100635	4	—[a]	—[a]	—[a]
SB224289	4	—[a]	—[a]	—[a]

Values in parentheses were determined under Condition II (100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl). All other values were determined under Condition I (20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl). [a] Inactive as an agonist. [b] Analyzed using the averaged values of replicated experiments collectively. [c] Reported in Odagaki et al. (23). [d] Apparently effective as an agonist, but its %E$_{max}$ unable to be determined due to the too low potency.

most agonists had intrinsic activities indicative of their partial agonist properties with the exception of some full agonists such as 5-CT, dihydroergotamine, *d*-LSD, L694247, GR46611, and 3-(1-methylpiperidin-4-yl)-1*H*-indol-5-ol maleate salt (BRL54443). Of the anxiolytics with 5-HT_{1A}-receptor agonist properties, tandospirone was the most efficacious agonist with a mean EC_{50} value of 530 nM and $\%E_{max}$ of 37.3 ± 6.6 (23). The stimulatory effects of buspirone were scarce to indicate this anxiolytic drug was the least efficacious partial agonist (23). Ipsapirone, another arylpiperazine derivative with properties of a partial 5-HT_{1A}-receptor agonist, was not effective in this assay system at all. Both enantiomers of 5-fluoro-8-hydroxy-2-dipropylamino-1,2,3,4-tetrahydronaphthalene HCl (UH-301) barely revealed agonist properties in the present study. The pEC_{50} values of 13 agonists determined in the present investigation were significantly correlated ($r = 0.76$, $P<0.01$) with those reported in the previous study, in which 5-HT_{1A}-receptor-mediated inhibition of cyclic AMP production was determined in mouse hippocampal neurons in primary culture (31).

Effects of a series of 5-HT-receptor antagonists on 5-HT_{1A}-receptor-mediated [^{35}S]GTPγS binding

Under Condition I, the increase in specific [^{35}S]GTPγS binding to rat hippocampal membranes induced by 1 μM 5-HT was determined in the presence of various concentrations of a test compound to produce the inhibition curve. As shown in Fig. 6, the selective 5-HT_{1A}-receptor antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl] ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate salt (WAY100635) inhibited completely the 5-HT-stimulated binding in a concentration dependent manner with a mean IC_{50} of 7.1 nM. The second most potent antagonist was methiothepin, followed by *S*(−)-cyanopindolol, *S*(−)-pindolol, and *S*(−)-propranolol. The selective 5-$HT_{1B/1D}$-receptor antagonist GR127935 and the selective 5-HT_{2A}-receptor antagonist ritanserin were very weak antagonists, and other compounds investigated were inactive as an antagonist (Table 2).

The inhibitory effects of WAY100635 were also examined under Condition II, that is, in the presence of 100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl (Fig. 6A, closed symbols). Under this condition, WAY100635 also potently inhibited the increase in

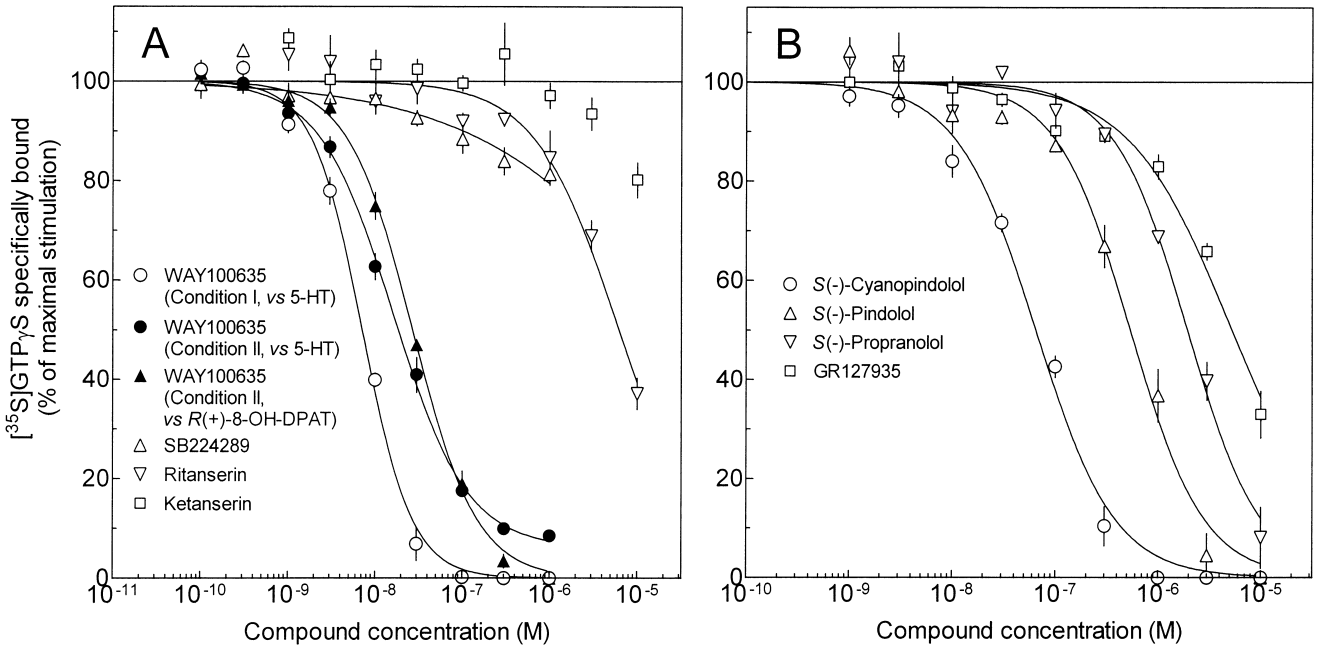

Fig. 6. Effects of several 5-HT-receptor antagonists on 5-HT- or *R*(+)-8-OH-DPAT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes. Specific [^{35}S]GTPγS binding was measured in the presence of various concentrations of a test compound and 1 μM 5-HT under Condition I (20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl) (open symbols). In some experiments, effects of WAY100635 were determined under Condition II (100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl) against 10 μM 5-HT (closed circles) or 10 μM *R*(+)-8-OH-DPAT (closed triangles). Results are expressed as the mean ± S.E.M. of percent values of the increase induced by 5-HT (1 or 10 μM) or *R*(+)-8-OH-DPAT (10 μM) obtained from 3 – 4 independent experiments, each performed in duplicate. A: WAY100635 (open and closed circles, closed triangles), SB224289 (open triangles), ritanserin (open inverse triangles), and ketanserin (open squares). B: *S*(−)-cyanopindolol (open circles), *S*(−)-pindolol (open triangles), *S*(−)-propranolol (open inverse triangles), and GR127935 (open squares).

Table 2. Antagonist properties of 5-HT-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes

Compound	n	pK_b	K_b (nM)
WAY100635	4	9.32 ± 0.03	4.8
(WAY100635)	4	(9.29 ± 0.04)	(5.1)
Methiothepin	3	8.48 ± 0.07	33
S(−)-Cyanopindolol	3	8.36 ± 0.05	44
S(−)-Pindolol	3	7.44 ± 0.08	370
S(−)-Propranolol	3	6.87 ± 0.05	1,300
GR127935	3	6.44 ± 0.07	3,600
Ritanserin	3	6.38 ± 0.07	4,200
SB224289	3	—[a]	—[a]
GR55562	3	—[a]	—[a]
BRL15572	3	—[a]	—[a]
Ketanserin	3	—[a]	—[a]
LY278584	3	—[a]	—[a]

Values in parentheses were determined under Condition II (100 μM GDP, 5 mM $MgCl_2$, and 150 mM NaCl). All other values were determined under Condition I (20 μM GDP, 5 mM $MgCl_2$, and 100 mM NaCl). [a] Inactive as an antagonist.

[^{35}S]GTPγS binding elicited by 10 μM 5-HT in a concentration-dependent manner. However, it should be noted that this inhibition appeared incomplete and some residual portion (6.2 ± 0.9%) was always observed. On the other hand, the inhibitory curve of WAY100635 against 10 μM *R*(+)-8-OH-DPAT was complete even under this assay condition, as demonstrated in Fig. 6A.

Discussion

As reported previously (7), exogenously added GDP was also essential to detect 5-HT-stimulated [^{35}S]GTPγS binding in the present study. The optimum GDP concentrations to obtain the highest signal/noise ratio were apparently 100 μM and higher. However, the absolute values of 5-HT-induced increment formed an inverse U-shape curve, with the maximum increases obtained at 1 – 30 μM GDP. We did not want to use high GDP concentrations because the relative experimental errors tended to increase in the presence of the highest concentrations of GDP, and thus we chose 20 μM GDP as the standard assay condition. This GDP concentration is similar to those reported in native mammalian brain membranes (10 – 50 μM) (18, 20, 22 – 24, 25 – 27), but much lower than that (300 μM) adopted in rat hippocampal and cerebral cortical membranes (19, 21).

The requirement of Mg^{2+} to detect specific binding, with the optimum concentrations of 2 – 10 mM for maximizing 5-HT-elicited increase, was quite similar to that of $GABA_B$-receptor-mediated [^{35}S]GTPγS binding in rat cortical membranes (29). Mg^{2+} ions have multiple actions on G protein events (6), and at least two separate actions of this ion have been demonstrated in agonist-induced [^{35}S]GTPγS binding (32). In contrast to the absolute requirement of Mg^{2+}, Na^+ ions were not essential to observe the specific [^{35}S]GTPγS binding nor the 5-HT-sensitive increase. However, the addition of increasing concentrations of NaCl led to reduction of the basal unstimulated binding, thus improving the signal/noise ratio as observed in previous studies (7). Na^+ ions are thought to bind to a conserved aspartate residue in the second transmembrane domain of G protein-coupled receptors (GPCRs) (33) and this favors uncoupling of the receptor-G protein complex, resulting in reduction of basal [^{35}S]GTPγS binding. Although the percent increase elicited by 10 μM 5-HT appeared to reach maximum in the presence of 150 – 200 mM NaCl, we preferred 100 mM NaCl since the absolute value of 5-HT-stimulated response was reduced obviously by higher concentrations of NaCl.

For several of the selected 5-HT ligands, concentration-response curves were drawn under both conditions. As expected, maximal response elicited by 5-HT was, when expressed as percent increase, much higher under Condition II than under Condition I. On the other hand, mean the EC_{50} value for 5-HT became 5-fold higher under Condition II. Similar phenomena were observed as to 5-CT, *d*-LSD, and *R*(+)-8-OH-DPAT, and the lowered poteincies under Condition II likely derived largely from the higher GDP concentration (10, 19, 24, 34). The higher concentration of NaCl (150 mM) of Condition II should barely contribute to the decreased potencies of 5-HT agonists, because it was reported that the effect of lowering Na^+ concentration on EC_{50} values for 5-HT agonists was, if any, very limited and not significant (25, 34). In C6-glial cells expressing human 5-HT$_{1A}$ receptors, the relative maximal effects of almost all 5-HT$_{1A}$-receptor agonists to potentiate [^{35}S]GTPγS binding were attenuated by increasing concentrations of GDP, with few exceptions like 5-CT (10). Similarly, differences in efficacy of μ-opioid agonists were enlarged by increasing GDP concentrations in rat thalamus and cultured cells (35). The GDP effects on the maximal increase by 5-CT (a full agonist), and partial agonists like buspirone and *S*(−)-8-OH-DPAT in this study are in agreement with these reports. On the other hand, in spite of its partial agonist properties, the relative efficacy of *R*(+)-8-OH-DPAT appeared constant (0.7 – 0.8) throughout 4 – 500 μM GDP. Most surprisingly, *d*-LSD appeared almost a full agonist in the presence of 20 – 500 μM GDP, whereas its maximum effect in the presence of 4 μM GDP was less than that of *R*(+)-8-OH-DAPT, a partial 5-HT$_{1A}$-receptor agonist. These results

indicate that the intrinsic activity of an agonist varies according to the G protein activation state modulated by various concentrations of GDP, and the pattern of GDP effects appears to be individual depending on the compound as well. This individuality should be taken into consideration in addition to the ratio of receptors to G proteins (36) when intrinsic activity of a compound is discussed. Indeed, Pauwels et al. (11) reported that the maximal [^{35}S]GTPγS binding in response to 5-HT$_{1B/1D}$ ligands in C6-glial cells expressing human 5-HT$_{1A}$ receptors were either unaffected or significantly enhanced by increasing concentrations of GDP, which was quite a different pattern compared to 5-HT$_{1A}$-receptor agonists (10). They speculated that the differential pattern is derived from involvement of different G protein subtypes in the increase in [^{35}S]GTPγS binding induced by 5-HT$_{1A}$ agonists and 5-HT$_{1B/1D}$ agonists. Whatever the molecular mechanisms involved, it is worthy to note that the intrinsic activity of an agonist should be cautiously determined under different experimental conditions in functional assays.

Under Condition I, WAY100635 potently and completely inhibited the binding stimulated by 1 μM 5-HT, while the inhibition was incomplete under Condition II with a residual portion hardly displaceable even at 1 μM. The incomplete inhibition of 5-HT- or 5-CT-stimulated [^{35}S]GTPγS binding by WAY100635 was also reported by other investigators (19, 20, 22), indicating involvement of some receptor type(s) other than 5-HT$_{1A}$ receptors. Although we are unaware of the exact cause of the appearance of WAY100635-insensitive residual binding only under Condition II, this phenomenon is another reason why we preferred Condition I.

Numerous studies by means of receptor autoradiography, in situ hybridization, and immunohistochemistry have shown that almost all 5-HT-receptor subtypes except for 5-HT$_{2B}$ receptors are expressed in rat hippocampus, at least at the mRNA levels (1). The profile of agonists and antagonists clearly indicates 5-HT-stimulated [^{35}S]GTPγS binding in the present study is mediated by 5-HT$_{1A}$ receptors. Thus, almost all selective and nonselective 5-HT$_{1A}$-receptor agonists stimulated the binding with various potencies, which were significantly correlated with those determined in the assay for 5-HT$_{1A}$-receptor-mediated inhibition of cyclic AMP production in mouse hippocampal neurons in primary culture (31). Ipsapirone and *R*(+)-enantiomer of UH301 were devoid of apparent agonistic effects against expectation. Meller et al. (20) reported ipsapirone activated [^{35}S]GTPγS binding in rat hippocampal membranes with an EC$_{50}$ of 79 nM. *R*(+)-UH301 was reported to behave as a full (37) or at least partial (38) agonist at 5-HT$_{1A}$ receptors. The standard condition (Condition I) adopted in the present study may be unsuitable for detecting agonist properties of these compounds. As pointed out by Pauwels et al. (11), a number of reportedly 5-HT$_{1B/1D}$ agonists also bind to 5-HT$_{1A}$ receptors to act as an agonist. Indeed, some 5-HT$_{1B/1D}$-receptor agonists, such as L694247, GR46611, CGS12066A, and CP94253, behaved as agonists with moderate to low potencies in this study. However, lack of involvement of 5-HT$_{1B/1D}$ receptors was ascertained by the fact that CP93129 was the least potent agonist and 5-HT-stimulated [^{35}S]GTPγS binding was barely inhibited by any of the selective 5-HT$_{1B/1D}$ antagonists examined. Similarly, the possibility of involvement of 5-HT-receptor subtypes other than 5-HT$_{1A}$ and 5-HT$_{1B/1D}$ was excluded by the profile of some compounds such as BRL54443, (±)-2,5-dimethoxy-4-bromoamphetamine HBr [(±)-DOB], (±)-2,5-dimethoxy-4-iodoamphetamine HCl [(±)-DOI], *N*-methylquipazine, ketanserin, and 1-methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1*H*-indazole-3-carboxamide maleate (LY278584) as an agonist or antagonist.

Recently, the concept of constitutive activity of GPCRs has been well appreciated to account for the phenomenon that several compounds originally classified as neutral antagonists behave as inverse agonists (39). Several reagents such as spiperone, methiothepin, and even WAY100635 were reported to act as inverse agonists at 5-HT$_{1A}$ receptors under certain experimental conditions (12, 15, 16, 34, 36, 40). However, it should be important to note that inverse agonist efficacy depends largely on experimental conditions and almost all examples of inverse agonism have been reported using cultured cells expressing high levels of receptors (39). In particular, receptor/G protein ratio (36) and NaCl concentrations (15, but see also 34) were critical to facilitate the efficacy of an inverse agonist to be captured. In previous reports using native membranes, we are unaware of any example of inverse agonism at 5-HT$_{1A}$ receptors (19, 20, 22). In the present study, no certain evidence was obtained indicative of inverse agonism, either. Although some antipsychotics were reported to behave as inverse agonists at human 5-HT$_{1A}$ receptors (16), the relevance of constitutive activity of 5-HT$_{1A}$ receptors in the physiological milieu is still uncertain and further studies are needed to clarify possible therapeutic implications of these antipsychotics as inverse agonists.

In conclusion, 5-HT-stimulated [^{35}S]GTPγS binding to rat hippocampal membranes was investigated carefully as to optimum experimental conditions, and receptor subtype involved in this response was characterized pharmacologically in detail with a series of 5-HT-receptor agonists and antagonists. Although

caution should be paid especially to the apparent intrinsic activities susceptible to the assay conditions, this method appears useful for investigating the functional interaction between 5-HT$_{1A}$ receptors and their coupled G proteins in native hippocampal membranes.

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