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5-HT_{1A} receptor-mediated G protein activation assessed by [^{35}S]GTPγS binding in rat cerebral cortex

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Abstract

To date, 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor-mediated functional assays (adenylyl cyclase inhibition, high-affinity GTPase activity and [^{35}S]guanosine-5′-*O*-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding) have been performed mainly in hippocampal membranes. In the current study, 5-HT-stimulated G protein activation assays were carried out in rat cerebral cortical membranes. High-affinity GTPase activity was stimulated by 5-HT, but not by 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT). By contrast, 5-HT- and 8-OH-DPAT-stimulated [^{35}S]GTPγS binding displayed sufficient dynamic range enough to warrant further pharmacological analysis. Under standard conditions, which were determined precisely in terms of the concentrations of GDP, $MgCl_2$ and NaCl, the profile of 5-HT-stimulated [^{35}S]GTPγS binding investigated using a series of 5-HT receptor agonists and antagonists clearly indicated the involvement of the 5-HT_{1A} receptor subtype. There appeared to be no evidence supporting the presence of regional heterogeneity in coupling efficiency between 5-HT_{1A} and G proteins in the hippocampus or cortex. This method is a useful tool for investigating functional coupling between postsynaptic 5-HT_{1A} receptors and G proteins in cerebral cortical membranes.

Keywords: Serotonin; 5-HT_{1A} receptor; G protein; [^{35}S]GTPγS binding; Cerebral cortex

1. Introduction

Activation of central 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptors elicits diverse molecular and cellular responses, such as stimulation and inhibition of adenylyl cyclase activity, activation of an inwardly rectifying K^+ current, modulation of the neuronal firing rate, modification of phosphatidylinositide turnover and increased intracellular Ca^{2+} mobilization (Albert et al., 1996; Barnes and Sharp, 1999; Olivier et al., 1999; Zifa and Fillion, 1992). The 5-HT_{1A} receptor is a member of the superfamily of G protein-coupled receptors, which have seven hydrophobic transmembrane domains. Functional interactions between these receptors and their coupled G proteins have been investigated in an indirect manner for a long time; for example, by examining the modulating effects of GTP or its nonhydrolyzable analogs on binding sites labeled by an agonistic radioligand, such as [^{3}H]8-hydroxy-2-(di-*n*-propylamino) tetralin ([^{3}H]8-OH-DPAT) (Hall et al., 1985; Harrington and Peroutka, 1990; Radja et al., 1992; Schlegel and Peroutka, 1986).

Heterotrimeric G proteins are activated by agonist-bound receptors through GTP binding to α subunits of G proteins (G_α), and are inactivated by GTP hydrolyzing activity intrinsic to G_α (Gilman, 1987). These molecular processes have allowed researchers to measure agonist-induced high-affinity GTPase activity (Odagaki and Fuxe, 1997) and [^{35}S]guanosine-5′-*O*-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding (Harrison and Traynor, 2003), in order to assess the functional interactions between several types of receptor and their coupled G proteins. In rat hippocampal membranes, stimulation of high-affinity GTPase activity mediated by 5-HT_{1A} receptors was reported previously (Odagaki and Fuxe, 1995a,b). More recently, 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding

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has been demonstrated in brain membranes prepared from the rat hippocampus (Alper and Nelson, 1998, 2000; Meller et al., 2000; Newman-Tancredi et al., 2003; Odagaki and Toyoshima, 2005; Odagaki et al., 2005), rat cerebral cortex (Alper and Nelson, 1998, 2000; Meller et al., 2000; Odagaki et al., 2005), human hippocampus (Elliott and Reynolds, 1999) and human cerebral cortex (González-Maeso et al., 2000, 2002a,b). However, probably because of the high density of 5-HT_{1A} receptors in limbic areas (Barnes and Sharp, 1999; Zifa and Fillion, 1992), the pharmacological characterization of such responses has been performed mainly in hippocampal membranes (Alper and Nelson, 1998; Meller et al., 2000; Newman-Tancredi et al., 2003; Odagaki and Fuxe, 1995b; Odagaki and Toyoshima, 2005). In the present investigation, we aimed to characterize the details of 5-HT_{1A} receptor-mediated G protein activation pharmacologically in rat cerebral cortical membranes. Furthermore, we compared the pharmacological profile of 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding in rat cerebral cortical membranes with that in hippocampal membranes, in order to evaluate the existence of regional heterogeneity in the 5-HT_{1A} receptors themselves or in 5-HT_{1A} receptor-dependent signaling cascades (Borsini et al., 1995; Dumuis et al., 1988; Hall et al., 1985; Marazziti et al., 2002; Nénonéné et al., 1996; Radja et al., 1992).

2. Materials and methods

2.1. Membrane preparation

The experimental protocols were reviewed and approved by the Animal Research Committee of Saitama Medical School, Japan. The animal care and use procedures conformed to the European Community Guidelines for the use of Experimental Animals (86/609/EEC). Male Sprague–Dawley rats weighing 200–250 g were killed by decapitation and their brains were removed immediately. The cerebral cortex from each brain was dissected on ice, 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 subsequent centrifuge procedures were carried out at 4 °C. Following centrifugation of the homogenate at 1000 ×*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 further centrifugation at 1000 ×*g* for 10 min. The combined supernatant was washed twice by centrifugation at 9000 ×*g* for 20 min and resuspension in 10 ml of TED buffer. The suspension was kept on ice for 30 min, followed by a 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.

2.2. High-affinity GTPase activity assay

The high-affinity GTPase activity was determined as described previously (Odagaki and Fuxe, 1997). In brief, thawed cerebral cortical membranes equivalent to 2.5–5.0 μg protein were incubated at 30 °C for 15 min in 100 μl of 50 mM Tris–HCl buffer (pH 7.4) containing 0.3 μM [γ-^{32}P]GTP, 2 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM adenylylimidodiphodphate, 5 mM phosphocreatine, 50 IU/ml creatine phosphokinase, 50 μg BSA, 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 0.5 mM cyclic AMP, 0.5 mM 3-isobutyl-1-methylxanthine and 100 mM NaCl. The enzyme reaction was terminated by transferring the tubes into an ice bath, immediately followed by the addition of 500 μl of 20 mM phosphoric acid containing 5% (w/v) activated charcoal. The tubes were kept chilled for about 10 min and then centrifuged at 20,600 ×*g* for 10 min. An aliquot (200 μl) from the supernatant fraction was pipetted onto the dry scintillant Deepwell LumaPlate (Packard Bioscience, Meriden, CT), and the radioactivity (cpm) of each sample was estimated after drying by scintillation counting for 5 min. The low-affinity GTPase activity was determined based on the GTP hydrolysis in the presence of 100 μM unlabeled GTP, which was subtracted from the total activity in order to define the high-affinity GTPase activity.

2.3. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding experiments were performed as described previously (Odagaki and Toyoshima, 2005; Odagaki et al., 2005). On the day of the experiment, the cerebral cortical 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 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, U.K.) using a Brandel cell harvester, followed by washing twice with 5 ml of ice-cold buffer (50 mM Tris–HCl, pH 7.4). The radioactivity content of the filters was counted in an 8-ml scintillation cocktail of Emulsifier–Scintillator Plus (Packard Bioscience, Groningen, Netherlands) using 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.

2.4. Data analysis

All results are presented as the mean±S.E.M. values of separate experiments performed in duplicate. The concentration-dependent increases in the high-affinity GTPase activity or the specific [^{35}S]GTPγS binding by a 5-HT receptor ligand were expressed as the percent increase above the basal unstimulated value. These data were analyzed using a non-linear-regression method (a sigmoid model with a variable slope and with a bottom value fixed to zero) with the commercially available program GraphPad PRISM™ (GraphPad, San Diego, CA), which calculated the concentration eliciting the half-maximal effect (EC_{50}) and the percent maximal increase above the basal value (%E_{max}). The inhibitory curve of an antagonist in the [^{35}S]GTPγS binding assay was expressed as the percent increase in the binding by 1 μM 5-HT. These data were also analyzed using a non-linear-regression method, with a top value fixed to 100% and a bottom value fixed to zero, in order to determine the concentration eliciting the half-maximal inhibition (IC_{50}). The antagonist potency (K_B) was calculated using the following equation (Lazareno and Birdsall, 1993):

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

where A and b are the agonist concentration and the 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.

2.5. Materials

[γ-^{32}P]GTP (30 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Du Pont NEN Research Products (Boston, MA). The following compounds were generous gifts from the named pharmaceutical companies: flesinoxan HCl (Solvay Pharmaceutical Co., Weesp, The Netherlands), ipsapirone HCl (Bayer AG, Wuppertal, Germany) and tandospirone citrate (Sumitomo Pharmaceutical Co., Osaka, Japan). All of the compounds in the following list were purchased from Tocris Cookson Ltd. (Bristol, UK): 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). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

3. Results

3.1. Effects of 5-HT and (±)-8-OH-DPAT on high-affinity GTPase activity

Under the experimental conditions used for previous studies on 5-HT-stimulated high-affinity GTPase activity in rat hippocampal membranes (Odagaki and Fuxe, 1995a,b), 5-HT was found to stimulate high-affinity GTPase activity in rat cerebral cortical membranes in a concentration-dependent manner, with a mean EC_{50} of 45 nM (pEC_{50}=7.35±0.11, n=4) and a %E_{max} of 14.1±2.2 (Fig. 1). By contrast, (±)-8-OH-DPAT only marginally increased high-affinity GTPase activity. These disappointing results compelled us to abandon further pharmacological characterization of the receptor subtype(s) mediating 5-HT-stimulated high-affinity GTPase activity in this brain area.

3.2. Optimization of 5-HT-stimulated [^{35}S]GTPγS binding

In the preliminary experiments, the percent maximum stimulation values of specific [^{35}S]GTPγS binding in rat cerebral cortical membranes in response to 5-HT and (±)-8-OH-DPAT were 40–50% and 20–30%, respectively, under the standard experimental conditions used in our previous studies of 5-HT_{1A} receptor-stimulated [^{35}S]GTPγS binding in rat hippocampal membranes (Odagaki and Toyoshima,

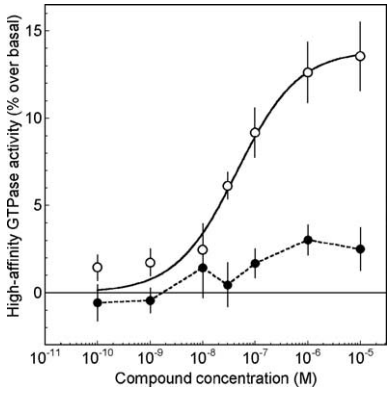

Fig. 1. Effects 5-HT and (±)-8-OH-DPAT on high-affinity GTPase activity in rat cerebral cortical membranes. High-affinity GTPase activity was measured in the presence of various concentrations of 5-HT (○) and (±)-8-OH-DPAT (●), and expressed as the mean±S.E.M. values of percent increase over the respective basal activity obtained from four independent experiments each performed in duplicate.

2005). Although these $\%E_{max}$ values were less than one-half of those in the hippocampal membranes [5-HT, 104.9±6.6% (n=7); (±)-8-OH-DPAT, 76.6±5.5% (n=4)], the results encouraged us to adopt this assay as a method of measuring functional G protein activation mediated through 5-HT receptor subtype(s) in rat cerebral cortical membranes.

Initially, the effects of various concentrations of GDP, $MgCl_2$ and NaCl on 5-HT-stimulated [^{35}S]GTPγS binding were examined, in order to determine the optimum condition in rat cerebral cortical membranes. As shown in Fig. 2, a 5-HT-sensitive increase in [^{35}S]GTPγS binding was detectable only in the presence of GDP, with optimum concentrations of 1–30 μM. Although the percent increase over the basal unstimulated level of specific [^{35}S]GTPγS binding by 10 μM 5-HT was maximized at a higher concentration (100 μM) of GDP, 20 μM GDP was chosen as a standard assay condition, because the basal binding in the presence of higher concentrations of GDP appeared to be excessively reduced and potentially had greater measurement fluctuations. Specific [^{35}S]GTPγS binding to cerebral cortical membranes was increased by the addition of $MgCl_2$ in a concentration-dependent manner with maximal effects observed at 5–10 mM $MgCl_2$ (Fig. 3). Moreover the increment caused by 10 μM 5-HT was strictly dependent on the existence of Mg^{2+} at optimum concentrations of 2–10 mM (Fig. 3, inset). Based on these results, 5 mM $MgCl_2$ was included as the standard assay condition. As shown in Fig. 4, specific [^{35}S]GTPγS binding, both in the absence and presence of 10 μM 5-HT, was reduced by increasing the concentrations of NaCl. The 5-HT-stimulated increment in specific [^{35}S]GTPγS binding tended to decrease and increase, when expressed as the difference in binding and the percent increase over basal binding, respectively, depending on the NaCl concentration (Fig. 4, inset). We chose 100 mM NaCl as the standard assay condition in the following experiments. These constituents (that is, 20 μM GDP, 5 mM $MgCl_2$ and 100 mM NaCl) were

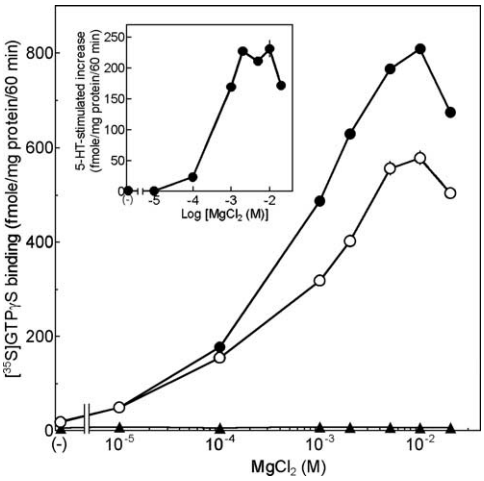

Fig. 3. Effects of $MgCl_2$ on 5-HT-stimulated [^{35}S]GTPγS binding in rat cerebral cortical membranes. [^{35}S]GTPγS binding in the absence (○) and presence (●) of 10 μM 5-HT, and in the presence of 100 μM GTPγS (nonspecific binding) (▲), was determined with various concentrations of $MgCl_2$. The assay conditions, except for $MgCl_2$, were standardized with 20 μM GDP and 100 mM NaCl. The results are expressed as the mean±S.E.M. values of three independent experiments each performed in duplicate. (Inset) The increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed in fmol/mg protein/60 min at various concentrations of $MgCl_2$.

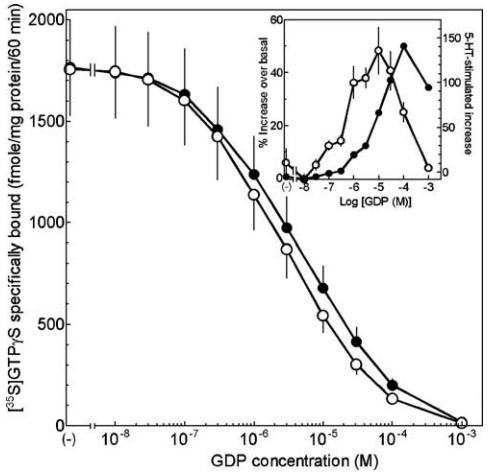

Fig. 2. Effects of GDP on 5-HT-stimulated [^{35}S]GTPγS binding in rat cerebral cortical membranes. Specific [^{35}S]GTPγS binding in the absence (○) and presence (●) of 10 μM 5-HT was determined with various concentrations of GDP. The assay conditions, except for GDP, were standardized with 5 mM $MgCl_2$ and 100 mM NaCl. The results are expressed as the mean±S.E.M. values of three independent experiments each performed in duplicate. (Inset) The increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed as a percent (●, left ordinate) or as an absolute value (○, right ordinate) over the respective basal binding at various concentrations of GDP.

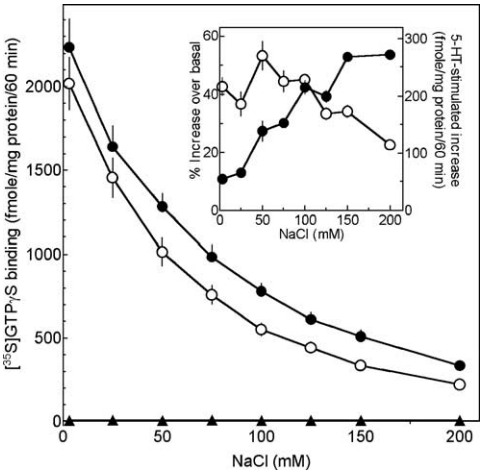

Fig. 4. Effects of NaCl on 5-HT-stimulated [^{35}S]GTPγS binding in rat cerebral cortical membranes. [^{35}S]GTPγS binding in the absence (○) and presence (●) of 10 μM 5-HT, and in the presence of 100 μM GTPγS (nonspecific binding) (▲), was determined with various concentrations of NaCl. The assay conditions, except for NaCl, were standardized with 20 μM GDP and 5 mM $MgCl_2$. The results are expressed as the mean±S.E.M. values of three independent experiments each performed in duplicate. (Inset) The increase in specific [^{35}S]GTPγS binding by 10 μM 5-HT is expressed as a percent (●, left ordinate) or as an absolute value (○, right ordinate) over the respective basal binding at various concentrations of NaCl.

consistent with the standard assay conditions used in our previous study on 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding in rat hippocampal membranes.

3.3. Agonistic effects of 5-HT receptor ligands on [^{35}S]GTPγS binding

Representative concentration-dependent increases in specific [^{35}S]GTPγS binding to rat cerebral cortical membranes in response to several 5-HT receptor agonists are depicted in Fig. 5. Endogenous ligand 5-HT stimulated the binding with a mean EC_{50} value of 63 nM (pEC_{50}=7.20±0.01, n=4) and a $\%E_{max}$ of 48.7±2.5. Both enantiomers of 8-OH-DPAT were also potent agonists with mean EC_{50} values of 40 and 26 nM for *R*(+)- and *S*(−)-isomer, respectively. However, the efficacy of *R*(+)-8-OH-DPAT ($\%E_{max}$=37.1±1.6, n=4) was three times greater than that of *S*(−)-isomer ($\%E_{max}$=12.2±2.2, n=5). By contrast, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo-[1,2-*a*]quinoxaline maleate salt (CGS12066A), which is a relatively selective 5-HT_{1B} receptor agonist (Neale et al., 1987; Schoeffter and Hoyer, 1989), was a less potent agonist in the present assay with a mean EC_{50} value of 550 nM (pEC_{50}=6.26±0.08, n=8). Furthermore, although anpirtoline, which is a highly potent agonist of the rat 5-HT_{1B} receptor (Schlicker et al., 1992), apparently stimulated the specific [^{35}S]GTPγS binding at higher concentrations, its EC_{50} value could not be determined due to the lack of saturability even at 100 μM. The selective 5-HT_2 receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine HCl [(±)-DOI] showed no agonist activity.

The profile of [^{35}S]GTPγS binding to rat cerebral cortical membranes with a series of 5-HT receptor ligands, including the above-mentioned compounds, is summarized in Table 1.

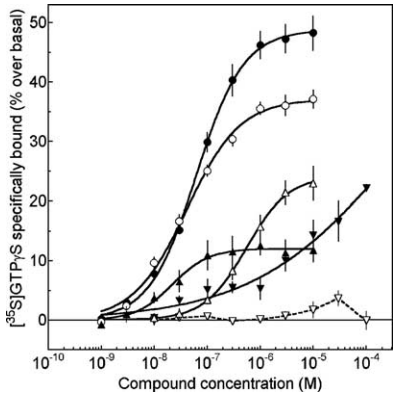

Fig. 5. Representative concentration-response curves for [^{35}S]GTPγS binding stimulated by several 5-HT receptor agonists in rat cerebral cortical membranes. Specific [^{35}S]GTPγS binding was measured in the presence of various concentrations of 5-HT (●), *R*(+)-8-OH-DPAT (○), *S*(−)-8-OH-DPAT (▲), CGS12066A (△), anpirtoline (▼), and (±)−DOI (▽). The results are expressed as the mean±S.E.M. values of the percent increase over the respective basal binding obtained from 4 to 8 independent experiments each performed in duplicate.

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

Compound	n	pEC_{50}	EC_{50} (nM)	$\%E_{max}$
R(+)-lisuride	4	8.20±0.19	6.3	26.1±2.4
5-CT	4	7.90±0.06	13	27.3±2.2
Dihydroergotamine	4	7.82±0.19	15	46.1±4.1
Flesinoxan	4	7.64±0.18	23	12.4±2.6
S(−)-8-OH-DPAT	5	7.59±0.09	26	12.2±2.2
R(+)-8-OH-DPAT	4	7.40±0.08	40	37.1±1.6
(±)-8-OH-DPAT	4	7.37±0.12	42	25.2±2.7
L694247	4	7.27±0.14	54	36.8±2.2
5-HT	4	7.20±0.01	63	48.7±2.5
d-LSD	4	7.08±0.06	84	25.5±1.0
N,*N*-DP-5CT	4	7.05±0.37	90	26.4±4.9
GR46611	4	6.89±0.02	130	40.7±3.5
PAPP	4	6.78±0.07	170	17.4±2.3
Methylergometrine	4	6.76±0.02	180	20.7±1.6
RU24969	4	6.61±0.04	250	24.3±1.8
5-MeO-*N*,*N*-DMT	4	6.61±0.13	250	20.3±2.2
5-MeOT	4	6.56±0.11	280	23.2±3.1
Metergoline	4	6.53±0.11	300	14.9±0.8
mCPP [a]	4	6.46±0.17	340	8.5±0.9
CP93129	4	6.38 [b]	420	8.7 [b]
Tandospirone [a]	5	6.28±0.13	520	10.2±0.9
CGS12066A	8	6.26±0.08	550	24.1±2.9
Methysergide	4	6.25±0.03	560	36.9±1.5
Tryptamine	4	5.61±0.09	2,500	21.2±2.1
Spiroxatrine	4	<6	>1000	– [c]
BRL54443	4	<6	>1000	– [c]
Anpirtoline	4	<6	>1000	– [c]
CP94253	4	<6	>1000	– [c]
BW723C86	4	<6	>1000	– [c]
R(+)-UH301	4	– [d]	– [d]	– [d]
S(-)-UH301	4	– [d]	– [d]	– [d]
Buspirone [a]	4	– [d]	– [d]	– [d]
Ipsapirone	4	– [d]	– [d]	– [d]
BMY7378	4	– [d]	– [d]	– [d]
WB4101	4	– [d]	– [d]	– [d]
TFMPP	4	– [d]	– [d]	– [d]
(±)-DOI	4	– [d]	– [d]	– [d]
(±)-DOB	4	– [d]	– [d]	– [d]
N-methylquipazine	4	– [d]	– [d]	– [d]
Methiothepin	4	– [d]	– [d]	– [d]
NAN190	4	– [d]	– [d]	– [d]
S(−)-pindolol	4	– [d]	– [d]	– [d]
Spiperone	4	– [d]	– [d]	– [d]
WAY100635	4	– [d]	– [d]	– [d]
SB224289	4	– [d]	– [d]	– [d]

[a] Reported in Odagaki et al. (2005).
[b] Analyzed using the averaged values of replicated experiments collectively.
[c] Apparently effective as an agonist, but its $\%E_{max}$ was not determined due to the too low potency.
[d] Inactive as an agonist.

3.4. Antagonistic effects of 5-HT receptor ligands on 5-HT-stimulated [^{35}S]GTPγS binding

Concentration-dependent inhibition curves of several 5-HT receptor antagonists against the increase in specific [^{35}S]GTPγS binding to rat cerebral cortical membranes elicited by 1 μM 5-HT are shown in Fig. 6. The most potent antagonist in this assay system was *N*-[2-[4-(2-

methoxyphenyl)-1-piperazinyl]ethyl]-*N*-2-pyridinylcyclohexanecarboxamide maleate salt (WAY100635), which is a potent and selective neutral antagonist of 5-HT_{1A} receptors (Fletcher et al., 1996; Forster et al., 1995), with a pIC_{50} value of 8.15±0.07 (n=3). Several β-adrenoceptor antagonists showed weaker activity than WAY100635 in the following rank order: *S*(−)-cyanopindolol>*S*(−)-pindolol>*S*(−)-propranolol. The potent and selective 5-HT_2 receptor antagonist, ketanserin tartrate, showed minimal activity as an antagonist in the present experimental system.

The antagonistic effects of a series of compounds against 5-HT-induced [^{35}S]GTPγS binding to cerebral cortical membranes are listed according to their mean K_B values in Table 2.

3.5. Correlations of affinities and efficacies between rat hippocampal membranes and cerebral cortical membranes

The pEC_{50} values of the agonists and the pK_B values of the antagonists determined in the present study were correlated with those obtained through the pharmacological characterization of 5-HT_{1A} receptor-mediated [^{35}S]GTPγS binding to rat hippocampal membranes (Odagaki and Toyoshima, 2005). There was a highly significant correlation (r=0.95, P<0.001, n=29) between the two brain regions (Fig. 7A). Comparing the $\%E_{max}$ values of agonists also revealed a significant correlation (r=0.77, P<0.001, n=23) between the cerebral cortical and hippocampal membranes (Fig. 7B).

4. Discussion

In the present study, the functional activation of heterotrimeric G proteins elicited by activated 5-HT receptor(s) in rat cerebral cortical membranes was investigated by means of agonist-induced [^{35}S]GTPγS binding (Harrison and Traynor, 2003). We also initially attempted to utilize agonist-induced high-affinity GTPase activity (Odagaki and Fuxe, 1997). However, although 5-HT stimulated high-affinity GTPase activity to a considerable extent, even in cerebral cortical membranes, the lack of a significant effect of 8-OH-DPAT compelled us to abandon any further characterization of this response.

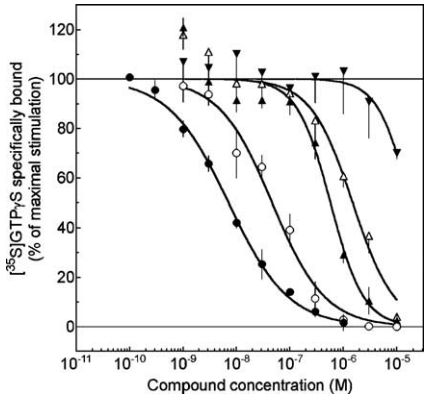

Fig. 6. Representative inhibitory curves for 5-HT-stimulated [^{35}S]GTPγS binding inhibited by several 5-HT receptor antagonists in rat cerebral cortical membranes. Specific [^{35}S]GTPγS binding was measured in the presence of 1 μM 5-HT and various concentrations of WAY100635 (●), *S*(−)-cyanopindolol (○), *S*(−)-pindolol (▲), *S*(−)-propranolol (△), and ketanserin (▼). The results are expressed as the mean±S.E.M. of percent values of the increase induced by 1 μM 5-HT obtained from three independent experiments each performed in duplicate.

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

Compound	n	pK_B	K_B (nM)
WAY100635	3	9.38±0.7	0.42
S(−)-cyanopindolol	3	8.56±0.16	2.8
Methiothepin	3	8.48±0.07	3.3
S(−)-pindolol	3	7.48±0.07	33
GR127935	4	7.36±0.14	44
S(−)-propranolol	3	7.06±0.10	87
Ritanserin	3	6.86±0.15	140
SB224289	4	–[a]	–[a]
GR55562	3	–[b]	–[b]
BRL15572	3	–[b]	–[b]
Ketanserin	3	–[b]	–[b]
LY278584	3	–[b]	–[b]

[a] Mean pIC_{50}<7.
[b] Mean pIC_{50}<6.

The 5-HT-induced inhibition of adenylyl cyclase activity is a well-established method for exploring the 5-HT_{1A} receptor-mediated physiological consequences in hippocampal membranes (Cornfield et al., 1988; De Vivo and Maayani, 1986; Mørk and Geisler, 1990; Schoeffter and Hoyer, 1988). By contrast, few previous reports have attempted the detailed pharmacological characterization of 5-HT-induced adenylyl cyclase inhibition in cerebral cortical membrane. The forskolin-stimulated adenylyl cyclase activity in rat cerebral cortical membranes was inhibited by 5-HT or 5-carboxamidotryptamine (5-CT) by 10–23% (Borsini et al., 1995; De Vivo and Maayani, 1990; Marazziti et al., 2002; Palego et al., 1999). This response appeared to be mediated through 5-HT_{1A} receptors, based on the findings of Palego et al. (1999) that *R*(+)-8-OH-DPAT also inhibited the enzyme activity and that this activity was completely antagonized by 1 μM WAY100635. However, another group failed to reveal significant inhibitory effects of 8-OH-DPAT (Borsini et al., 1995). Furthermore, the inhibitory effects of buspirone, which is an anxiolytic agent with 5-HT_{1A} receptor partial agonist properties (Tunnicliff, 1991), were also reported to be lacking or minimal (De Vivo and Maayani, 1990; Marazziti et al., 2002). Mørk and Geisler (1990) investigated the effects of several 5-HT receptor ligands, including 8-OH-DPAT and 5-CT, on adenylyl cyclase activity, either in the absence or presence of Ca^{2+}, in rat cerebral cortical membranes; however the results were too complicated to interpret them clearly as the responses mediated via single receptor subtype.

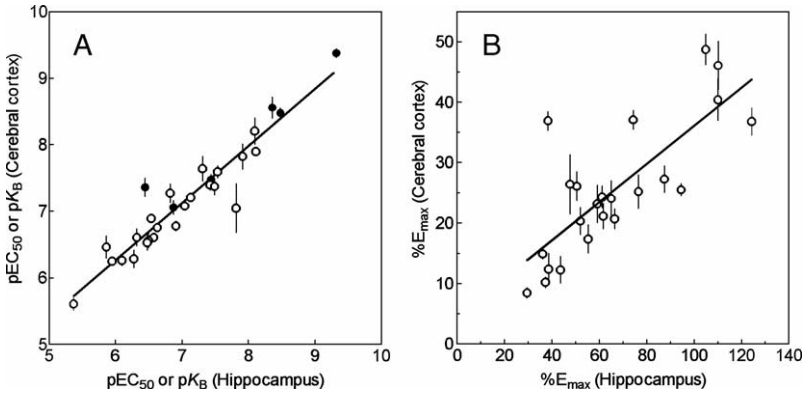

Fig. 7. Correlations of affinities and efficacies between rat hippocampal membranes and cerebral cortical membranes. (A) Correlation between the pEC_{50} values of agonists (○) and the pK_B values of antagonists (●) determined in the present study, and those determined in rat hippocampal membranes (Odagaki and Toyoshima, 2005). (B) Correlation between the $\%E_{max}$ values of agonists determined in the present study and those determined in rat hippocampal membranes (Odagaki and Toyoshima, 2005).

The agonist-induced [^{35}S]GTPγS binding assay (Harrison and Traynor, 2003) is now widely utilized as a simple method to assess the functional activation of G proteins coupled with several receptor subtypes. Although the $\%E_{max}$ values for 5-HT and (±)-8-OH-DPAT in rat cerebral cortical membranes were less than one-half of those determined in hippocampal membranes, the response was sufficient to encourage further pharmacological characterization. The experimental conditions necessary to optimize the 5-HT-stimulated increase in specific [^{35}S]GTPγS binding in cerebral cortical membranes were similar to those reported in hippocampal membranes (Odagaki and Toyoshima, 2005). Although the percent increase over the basal binding elicited by 10 μM 5-HT was not necessarily maximized, we chose 20 μM GDP, 5 mM $MgCl_2$ and 100 mM NaCl as our standard assay conditions, in order to avoid the potential enhancement of experimental errors due to the reduced basal binding in the presence of higher concentrations of GDP and NaCl.

Under these conditions, specific [^{35}S]GTPγS binding to rat cerebral cortical membranes was increased in a concentration-dependent manner by 5-HT itself and many 5-HT receptor agonists. The profile of these agonists clearly indicates the involvement of the 5-HT_{1A} receptor subtype in this response. The compounds that were reported to have extremely high affinities (K_i < 10 nM) for 5-HT_{1A} receptors, such as 5-CT, *N*,*N*-dipropyl-5-carboxamidotryptamine maleate (*N*,*N*-DP-5CT), 8-OH-DPAT, dihydroergotamine, flesinoxan, lysergic acid diethylamide (*d*-LSD), lisuride, 5-HT, RU24969, 5-methoxy-*N*,*N*-dimethyltryptamine (5-MeO-*N*,*N*-DMT), *p*-aminophenylethyl-*m*-trifluomethylphenyl piperazine (PAPP), and metergoline (Zifa and Fillion, 1992), stimulated the binding with mean EC_{50} values ≦300 nM. By contrast, selective agonists of 5-HT receptors other than the 5-HT_{1A} subtype—for example, CP93129 (5-HT_{1B}), CGS12066A (5-HT_{1B}), 3-(1-methylpiperidin-4-yl)-1*H*-indol-5-ol maleate salt (BRL54443) (5-$HT_{1E/1F}$), anpirtoline (5-HT_{1B}), CP94253 (5-HT_{1B}), 1-[5(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl (BW723C86) (5-HT_{2B}), (±)-DOI (5-$HT_{2A/2C}$), (±)-2,5-dimethoxy-4-bromoamphetamine HBr [(±)-DOB] (5-$HT_{2A/2C}$) and *N*-methylquipazine (5-HT_3)—were inactive or less potent agonists in this assay system.

The pharmacological profile of antagonists against the increase in specific [^{35}S]GTPγS binding to rat cerebral cortical membranes elicited by 1 μM 5-HT also strongly indicates the involvement of 5-HT_{1A} receptors. The most potent inhibitor among the compounds examined was WAY100635, which is a selective 5-HT_{1A} receptor antagonist (Fletcher et al., 1996; Forster et al., 1995). Several β-adrenoceptor antagonists, which are also known to be potent antagonists of 5-HT_{1A} and 5-HT_{1B} receptors (Olivier et al., 1999; Zifa and Fillion, 1992), inhibited the 5-HT-stimulated [^{35}S]GTPγS binding with a rank order of potency of *S*(−)-cyanopindolol > *S*(−)-pindolol > *S*(−)-propranolol. By contrast, compounds that are known to act as selective antagonists of 5-HT receptor subtypes other than 5-HT_{1A}—for example, GR127935 (5-$HT_{1B/1D}$), ritanserin (5-HT_{2A}), 1′-methyl-5-([2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl)-2,3,6,7-tetrahydro-spiro(furo[2,3-f]indole-3,4′-piperidine) (SB224289) (5-HT_{1B}), GR55562 (5-HT_{1B}), BRL15572 (5-HT_{1D}), ketanserin (5-HT_2), and 1-methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-1*H*-indazole-3-carboxamide maleate (LY278584) (5-HT_3)—had little or no inhibitory effects on the 5-HT-elicited binding.

Several previous reports have indicated that 5-HT_{1A} receptors in the cerebral cortex are distinct from those in the hippocampus. Dumuis et al. (1988) have shown that some compounds, such as buspirone, ipsapirone, methysergide and metergoline, are potent agonists of 5-HT_{1A} receptors, which inhibit cyclic AMP production in mouse hippocampal neurons in primary culture, while they act as competitive antagonists of the same category of receptors in cerebral cortical neurons. These authors have also shown that 8-OH-DPAT is a weak partial agonist in cortical neurons but a potent full agonist in hippocampal neurons (Dumuis et al., 1988). Hall et al. (1985) have demonstrated pharmacologically

diverse characteristics in [^{3}H]8-OH-DPAT binding to membranes from the rat hippocampus, striatum and cerebral cortex with respect to the sensitivity to cations or guanine nucleotides. Buspirone, 8-OH-DPAT and 1-[2-(2-thenoylamino)ethyl]-4[1-(7-methoxynaphtyl)]-piperazine (S14671) were active agonists in the assay for the inhibition of forskolin-stimulated adenylyl cyclase activity only in rat hippocampal membranes and not in frontal cortical membranes. By contrast, 1-[2-[4-(3-trifluoromethylphenyl)piperazin-1-yl]ethyl]benzimidazol (flibanserin) was a full agonist in both brain regions (Borsini et al., 1995). In human brain membranes, WAY100635 inhibited forskolin-stimulated adenylyl cyclase activity only in hippocampal membranes but not in prefrontal cortical membranes (Marazziti et al., 2002). Furthermore, it has been reported that [^{3}H]8-OH-DPAT binding is differentially alkylated in hippocampal and cerebral cortical membranes (Nénonéné et al., 1996). In the present study, the pEC_{50} and pK_B values determined in rat cerebral cortical membranes were significantly correlated with those determined in hippocampal membranes. The correlation coefficient (r) of 0.95 is close to unity, thereby strongly suggesting that pharmacologically identical 5-HT_{1A} receptors are involved in both brain regions. The effects of varied concentrations of GDP, $MgCl_2$ and NaCl on 5-HT-stimulated [^{35}S]GTPγS binding in cerebral cortical membranes were also similar to those in hippocampal membranes (Odagaki and Toyoshima, 2005). Thus, when 5-HT-stimulated [^{35}S]GTPγS binding was taken as an index of 5-HT_{1A} receptor activation, there appeared to be no evidence supporting the existence of heterogeneity of 5-HT_{1A} receptors between the hippocampus and the cerebral cortex.

The $\%E_{max}$ values of agonists in the two brain regions were also significantly correlated with each other ($r=0.77$). Although this result is consistent with the above-mentioned notion that identical 5-HT_{1A} receptors are involved in the response in both brain regions, the correlation coefficient was only modest compared with that for the affinities of compounds between the two brain regions. This phenomenon probably derives from the fact that many heterogeneous receptors are expressed in native brain regions. The compounds that were examined in the present study were not necessarily selective 5-HT_{1A} receptor agonists, and the stimulatory effects of such nonselective agonists might involve G protein activation through receptor subtypes other than 5-HT_{1A} receptors. Furthermore, the intrinsic activity of an agonist has been shown to vary depending on several factors such as the presence of receptor reserve and the degree of efficiency of signal transduction mechanisms (Boddeke et al., 1992; Meller et al., 1990; Raymond et al., 1992; Varrault and Bockaert, 1992; Varrault et al., 1992; Yocca et al., 1992). Moreover, experimental conditions, such as the GDP concentrations in the assay buffer (Odagaki and Toyoshima, 2005; Pauwels et al., 1997, 1998), might also contribute to the lowered correlation coefficient.

In the present assay system, the stimulatory effects of anxiolytic arylpiperazine derivatives that were known to be selective agonists of 5-HT_{1A} receptors were ambiguous, which was contrary to our expectations. Of the compounds examined, only tandospirone showed distinct agonistic properties with a mean EC_{50} of 520 nM and a $\%E_{max}$ of 10.2, which corresponded to an intrinsic activity of 0.21 relative to 5-HT. No obvious stimulatory effect was observed for buspirone or ipsapirone. In previous studies, tandospirone was reported to act as an efficacious 5-HT_{1A} receptor agonist with an intrinsic activity of 0.84–0.87 (Tanaka et al., 1995; Yabuuchi et al., 2004). However, in our experiments, tandospirone behaved as a partial agonist in the [^{35}S]GTPγS binding assay with a lower intrinsic activity (values of 0.57 and 0.36 relative to 5-HT, in Chinese hamster ovary cells expressing human 5-HT_{1A} receptors and rat hippocampal membranes, respectively) (Odagaki and Toyoshima, 2005; Odagaki et al., 2005). There have been several previous reports of the agonistic effects of buspirone and ipsapirone on 5-HT_{1A} receptors in various functional assays with different degrees of efficacy. Although it is generally accepted that buspirone and ipsapirone are 5-HT_{1A} receptor agonists, limited or absent effects of these compounds as 5-HT_{1A} receptor agonists have sometimes been reported (Boddeke et al., 1992; Borsini et al., 1995; De Vivo and Maayani, 1990; Dumuis et al., 1988; Marazziti et al., 2002; Odagaki and Toyoshima, 2005; Odagaki et al., 2005; Raymond et al., 1992). In addition to the regional preference of the hippocampus over the cerebral cortex for the detection of the 5-HT_{1A} receptor agonist properties of buspirone and ipsapirone (Dumuis et al., 1988), the assay medium in the present study contained GDP, which was necessary for detecting the agonist-induced activation of [^{35}S]GTPγS binding (Harrison and Traynor, 2003; Fig. 2 in the present study). In general, the intrinsic activity of 5-HT_{1A} receptor partial agonists decreases as the concentration of GDP in the assay buffer increases (Odagaki and Toyoshima, 2005; Pauwels et al., 1997). Thus, the standard assay conditions adopted in the present study might have been inappropriate for detecting the partial 5-HT_{1A} receptor agonist properties of these anxiolytic arylpiperazine derivatives.

In conclusion, the profile of 5-HT-stimulated [^{35}S]GTPγS binding to rat cerebral cortical membranes clearly indicates that this response is derived from G protein activation through the 5-HT_{1A} receptor subtype. There appears to be no evidence supporting the presence of regional heterogeneity in the coupling efficiency of 5-HT_{1A} receptors to G proteins in hippocampal and cerebral cortical membranes. Considering the insufficient effects of 8-OH-DPAT on forskolin-stimulated adenylyl cyclase activity (Borsini et al., 1995) as well as on high-affinity GTPase activity (this study) and the complicated biphasic effects of 5-HT ligands on Ca^{2+}-stimulated adenylyl cyclase activity (Mørk and Geisler, 1990) in cerebral cortical membranes, the 5-HT-stimulated [^{35}S]GTPγS binding described in the present report provides a useful tool to investigate the functional coupling between postsynaptic 5-HT_{1A} receptors and their coupled heterotrimeric G proteins in this brain region.

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