Differential discrimination of G-protein coupling of serotonin$_{1A}$ receptors from bovine hippocampus by an agonist and an antagonist

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Abstract We have studied the effect of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolyzable analogue of GTP, on agonist and antagonist binding to bovine hippocampal 5-hydroxytryptamine (5-HT)$_{1A}$ receptor in native membranes. Our results show that the specific binding of the agonist is inhibited with increasing concentrations of GTP-γ-S along with a reduction in binding affinity. In sharp contrast to this, antagonist binding to 5-HT$_{1A}$ receptor shows no significant reduction and remains invariant over a large range of GTP-γ-S concentrations. The binding affinity of the antagonist also remains unaltered. This shows that the agonist and the antagonist differentially discriminate G-protein coupling of 5-HT$_{1A}$ receptors from bovine hippocampus.

Key words: 5-Hydroxytryptamine$_{1A}$ receptor; 8-Hydroxy-2-(di-*N*-propylamino)tetralin; 4-(2′-Methoxy)-phenyl-1-(2′-(*N*-2′′-pyridinyl)-*p*-fluorobenzamido)ethyl-piperazine; G-protein coupling; Guanosine-5′-*O*-(3-thiotriphosphate); Bovine hippocampus

1. Introduction

Serotonin (5-hydroxytryptamine (5-HT)) is an intrinsically fluorescent [1], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems [2]. Serotonergic signalling appears to play a key role in the generation and modulation of various cognitive and behavioral functions including sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression and learning [3–5]. Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders and obsessive compulsive disorder [4,6,7].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [8]. Serotonin receptors are members of a superfamily of seven transmembrane domain receptors [9] that couple to GTP binding regulatory proteins (G-proteins). Among the various types of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor subtype has been the most extensively studied for a number of reasons [10]. We have recently partially purified and solubilized the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form [11] and have shown modulation of receptor binding by metal ions [10] and alcohols [12].

Since most seven transmembrane domain receptors are coupled to G-proteins [13], guanine nucleotides are known to regulate agonist binding. The 5-HT$_{1A}$ receptor is negatively coupled to the adenylate cyclase system through G-proteins [14]. We report here that agonist binding to the 5-HT$_{1A}$ receptor is sensitive to guanine nucleotides. However, antagonist binding to the 5-HT$_{1A}$ receptor is found to be insensitive to guanine nucleotides. This could be due to the binding of the agonist only to those receptors which are coupled to G-proteins, while the antagonist binds to all receptors irrespective of their state of G-protein coupling.

2. Materials and methods

Fresh bovine brains were obtained from a local slaughterhouse within 10 min of death and the hippocampal region was carefully dissected out. The hippocampi were immediately flash frozen in liquid nitrogen and stored at −70°C until further use. Native membranes were prepared as described earlier [12]. Bovine hippocampal tissue (∼120 g) was homogenized as 10% (w/v) in a polytron homogenizer in buffer A (2.5 mM *tris*-(hydroxymethyl)aminomethane (Tris), 0.32 M sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol *bis*(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 0.02% sodium azide, 0.24 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900×*g* for 10 min at 4°C. The supernatant was filtered through four layers of cheese cloth and the pellet was discarded. The supernatant was further centrifuged at 50 000×*g* for 20 min at 4°C. The resulting pellet was suspended in 10 volumes of buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4) using a hand-held Dounce homogenizer and centrifuged at 50 000×*g* for 20 min at 4°C. This procedure was repeated until the supernatant was clear. The final pellet was resuspended in a minimum volume of 50 mM Tris buffer (pH 7.4), homogenized using a Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70°C for radioligand binding assays.

Agonist binding assays were performed with varying concentrations of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) (Boehringer Mannheim, Germany) as follows. Tubes in triplicate containing 1 mg of total protein were incubated for 1 h at room temperature with 0.29 nM [^{3}H]8-hydroxy-2-(di-*N*-propylamino)tetralin (OH-DPAT) (DuPont New England Nuclear, Boston, MA, USA: specific activity 127.0 Ci/mmol) in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4). Non-specific binding was determined by performing the assay in the presence of 10 μM unlabelled serotonin. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0 μm pore size) 2.5 cm diameter glass microfiber filters (Whatman International, Kent, UK) which were pre-soaked in 0.3% polyethylenimine for 3 h [15]. The filters were then washed three times with 3 ml of ice-cold water, dried and the retained radioactivity was measured in a Packard Tri-Carb 1500 scin-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; *p*-MPPF, 4-(2′-methoxy)-phenyl-1-(2′-(*N*-2′′-pyridinyl)-*p*-fluorobenzamido)ethyl-piperazine; *p*-MPPI, 4-(2′-methoxy)-phenyl-1-(2′-(*N*-2′′-pyridinyl)-*p*-iodobenzamido)ethyl-piperazine; OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; PMSF, phenylmethylsulfonyl fluoride; Tris, *tris*-(hydroxymethyl)aminomethane

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tillation counter using 5 ml of scintillation fluid. Antagonist binding assays in the presence of GTP-γ-S were performed as above using [^{3}H]4-(2′-methoxy)-phenyl-1-(2′-(*N*-2′′-pyridinyl)-*p*-fluorobenzamido)-ethyl-piperazine (*p*-MPPF) (DuPont New England Nuclear, Boston, MA, USA; specific activity 64.6 Ci/mmol) as the radioligand. The assay tubes contained 0.5 nM [^{3}H]*p*-MPPF in a total volume of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, pH 7.4). Non-specific binding was determined by performing the assay in the presence of 10 μM unlabelled 4-(2′-methoxy)-phenyl-1-(2′-(*N*-2′′-pyridinyl)-*p*-iodobenzamido)ethyl-piperazine (*p*-MPPI) (a kind gift from Dr V. Bakthavachalam, National Institute of Mental Health Chemical Synthesis Program, Research Biochemicals International). Protein concentration was determined using bicinchoninic acid reagent (Pierce, Rockford, IL, USA) [16].

Saturation binding assays were carried out using varying concentrations (0.1–7.5 nM) of radiolabelled agonist ([^{3}H]OH-DPAT) or antagonist ([^{3}H]*p*-MPPF) using native membranes containing 1 mg of total protein. Non-specific binding was measured in the presence of 10 μM unlabelled 5-HT (for agonist) or *p*-MPPI (for antagonist). Binding assays were carried out at room temperature as mentioned above in the presence of high (100 μM) and low (1 nM) concentrations of GTP-γ-S. Control experiments were carried out without GTP-γ-S. Binding data were analyzed as described earlier [10]. The concentration of bound ligand was calculated from the equation:

$$RL^* = 10^{-9} \times B/(V \times SA \times 2220)\ M$$

where B = bound radioactivity in desintegrations per minute (dpm) (i.e. total dpm − non-specific dpm), V is the assay volume in ml and SA is the specific activity of the radioligand. Scatchard plots (i.e. plots of RL*/L* versus RL*) were analyzed using Sigma-Plot (version 3.1) in an IBM PC. The dissociation constants (K_d) were obtained from the negative inverse of the slopes, determined by linear regression analysis of the plots ($r = 0.92$–0.99). The binding parameters shown in Table 2 were obtained by averaging the results of three independent experiments while saturation binding data shown in Figs. 3 and 4 are from representative experiments.

3. Results and discussion

Among the various types of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor subtype has been the most extensively studied. One of the major reasons for this is the early availability of a highly selective agonist, OH-DPAT, that allows extensive biochemical, physiological and pharmacological characterization of the receptor [17]. Fig. 1 shows the inhibition of specific OH-DPAT binding to bovine hippocampal 5-HT$_{1A}$ receptor in native membranes by GTP-γ-S, a non-hydrolyzable analogue of GTP, in a characteristic concentration-dependent manner [10]. This shows that the bovine hippocampal 5-HT$_{1A}$ receptor is coupled to G-proteins and GTP-γ-S induces a transition of the receptor from a high affinity to a low affinity state. It has previously been reported that OH-DPAT binds to the high affinity binding sites of only that population of 5-HT$_{1A}$ receptors which is coupled to G-proteins [18].

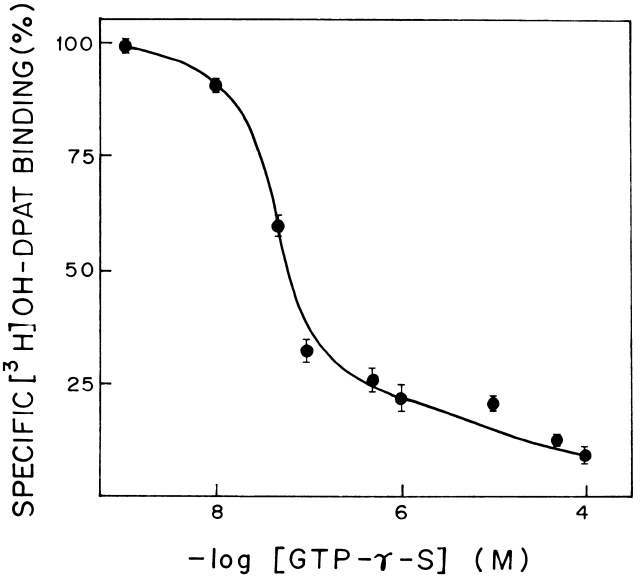

Fig. 1. Effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist [^{3}H]OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The data points are the means ± S.E.M. of triplicate points from three independent experiments. See Section 2 for other details.

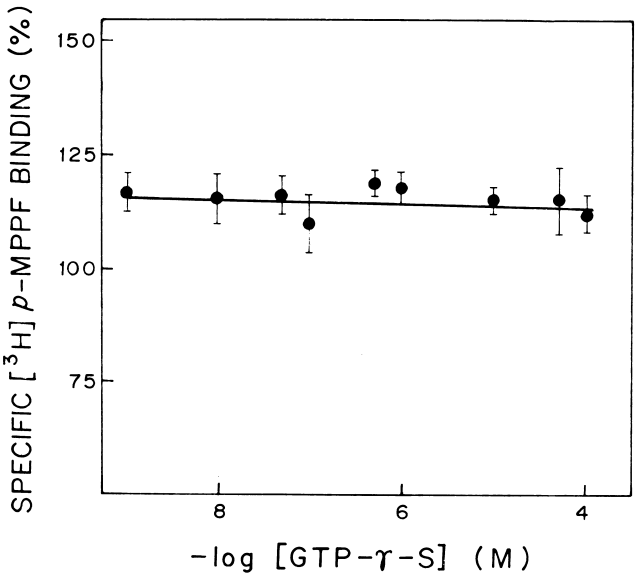

Fig. 2. Effect of increasing concentrations of GTP-γ-S on the specific binding of the antagonist [^{3}H]*p*-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The data points are the means ± S.E.M. of triplicate points from four independent experiments. See Section 2 for other details.

Although selective 5-HT$_{1A}$ agonists (e.g. OH-DPAT) have been discovered more than a decade ago [17], the development of selective 5-HT$_{1A}$ antagonists has been relatively slow and less successful. Recently, *p*-MPPI and *p*-MPPF have been introduced as selective antagonists for the 5-HT$_{1A}$ receptor [19–22]. These compounds bind specifically to 5-HT$_{1A}$ receptor with a high affinity. Fig. 2 shows the effect of varying concentrations of GTP-γ-S on specific *p*-MPPF binding to the 5-HT$_{1A}$ receptors in native membranes. In sharp contrast to what is observed with agonist binding, the antagonist binding shows no dependence on GTP-γ-S over a large range of concentrations (1 nM–100 μM) used, i.e. the antagonist binding is independent of GTP-γ-S. Furthermore, there is a slight (10–

Table 1
Specific activities for [^{3}H]OH-DPAT and [^{3}H]*p*-MPPF binding to 5-HT$_{1A}$ receptors from bovine hippocampal membranes[a]

Ligand	Specific binding activity[b] (fmol/mg of protein)
[^{3}H]OH-DPAT (agonist)	76.2 ± 6.4
[^{3}H]*p*-MPPF (antagonist)	120.9 ± 11.3

[a]For details of binding assays, see Section 2.
[b]Data reported are mean ± S.E.M. of five independent experiments.

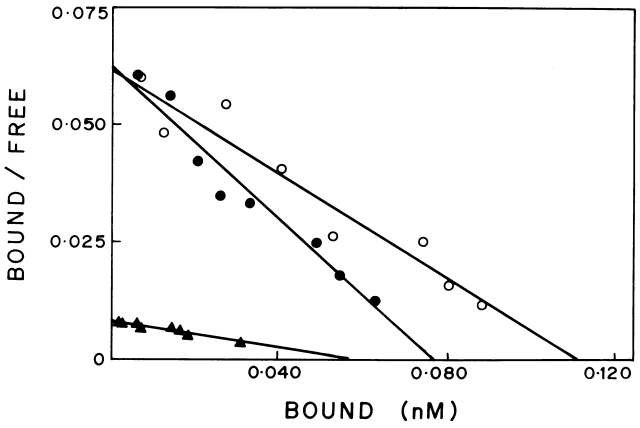

Fig. 3. Scatchard analysis of specific binding of [^{3}H]OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence of 100 μM (▲), 1 nM (●) GTP-γ-S and without (○) GTP-γ-S. The concentration of [^{3}H]OH-DPAT ranged from 0.1 to 7.5 nM. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Section 2 for other details.

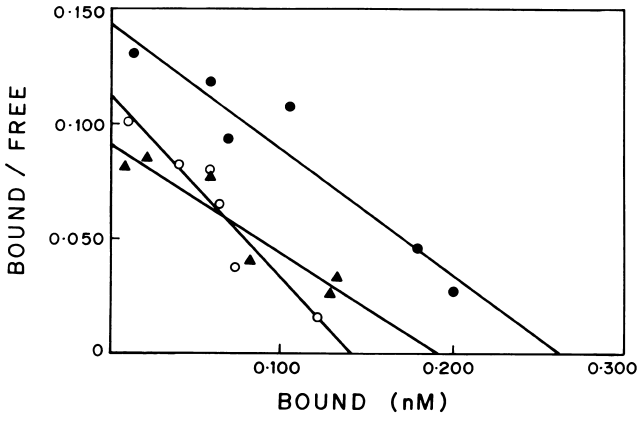

Fig. 4. Scatchard analysis of specific binding of [^{3}H]*p*-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence of 100 μM (▲), 1 nM (●) GTP-γ-S and without (○) GTP-γ-S. The concentration of [^{3}H]*p*-MPPF ranged from 0.1 to 7.5 nM. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Section 2 for other details.

20%) increase in binding in the presence of GTP-γ-S. Table 1 shows that the specific activity obtained using the agonist [^{3}H]OH-DPAT is 76.2 fmol/mg protein while that obtained using the antagonist [^{3}H]*p*-MPPF is 120.9 fmol/mg. There is thus a ~60% increase in specific activity when [^{3}H]*p*-MPPF is used. This further suggests that while the agonist [^{3}H]OH-DPAT binds to only that population of 5-HT$_{1A}$ receptors that is coupled to G-proteins [18], the antagonist [^{3}H]*p*-MPPF binds to both G-protein-coupled and free receptor giving rise to a higher specific activity. Comparing the specific activity values obtained with the agonist and the antagonist, therefore, can provide an idea of the extent of G-protein coupling of 5-HT$_{1A}$ receptors in the system.

Figs. 3 and 4 show the Scatchard analysis of the specific binding of [^{3}H]OH-DPAT and [^{3}H]*p*-MPPF to the 5-HT$_{1A}$ receptor in bovine hippocampal membranes in the presence of high and low concentrations of GTP-γ-S. The binding parameters under these conditions are summarized in Table 2. The binding affinity of [^{3}H]OH-DPAT shows a considerable reduction at high concentrations (100 μM) of GTP-γ-S, confirming that the receptor is in a low affinity state at high GTP-γ-S concentrations. This is in agreement with Fig. 1 which shows that at high GTP-γ-S concentrations, the low affinity form of the receptor predominates. Table 2 also shows that the binding affinity of [^{3}H]*p*-MPPF in the presence of 100 μM GTP-γ-S shows no significant variation. This supports our previous conclusion that antagonist binding is independent of GTP-γ-S (see Fig. 2).

Table 2
Binding affinity of [^{3}H]OH-DPAT and [^{3}H]*p*-MPPF to 5-HT$_{1A}$ receptors from bovine hippocampal membranes[a]

Condition	Ligand	K_d (nM)
Native membrane	[^{3}H]OH-DPAT	1.68 ± 0.16
100 μM GTP-γ-S	[^{3}H]OH-DPAT	8.54 ± 0.83
1 nM GTP-γ-S	[^{3}H]OH-DPAT	1.24 ± 0.07
Native membrane	[^{3}H]*p*-MPPF	1.45 ± 0.18
100 μM GTP-γ-S	[^{3}H]*p*-MPPF	1.87 ± 0.31
1 nM GTP-γ-S	[^{3}H]*p*-MPPF	2.07 ± 0.19

[a]The binding parameters shown in this table represent the mean ± S.E.M. of duplicate points from three independent experiments while saturation binding data shown in Figs. 3 and 4 are from representative experiments. See Section 2 for other details.

In summary, we show here that the specific agonist OH-DPAT and the antagonist MPPF bind to 5-HT$_{1A}$ receptors from bovine hippocampal membranes and exhibit different sensitivities to guanine nucleotides. This difference can be potentially exploited to gain a better understanding of signal transduction processes triggered by the 5-HT$_{1A}$ receptor. These results are relevant to ongoing analyses of the overall modulation of G-protein coupling in seven transmembrane domain receptors.

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