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Effect of Mg^{2+} on guanine nucleotide sensitivity of ligand binding to serotonin$_{1A}$ receptors from bovine hippocampus[☆]

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Abstract

The serotonin$_{1A}$ (5-HT$_{1A}$) receptor is an important member of the superfamily of seven transmembrane domain G-protein coupled receptors (GPCRs). We report here that guanine nucleotide sensitivity of agonist binding to hippocampal 5-HT$_{1A}$ receptors is dependent on the concentration of Mg^{2+}. Our results show that agonist binding to 5-HT$_{1A}$ receptors is relatively insensitive to guanine nucleotides in the absence of Mg^{2+}. In contrast to this, the specific antagonist binding is insensitive to guanine nucleotides, even in the presence of Mg^{2+}. These results point out the requirement of an optimal concentration of Mg^{2+} which could be used in assays toward determining guanine nucleotide sensitivity of ligand binding to GPCRs such as the 5-HT$_{1A}$ receptor. Our results provide novel insight into the requirement and concentration dependence of Mg^{2+} in relation to guanine nucleotide sensitivity for the 5-HT$_{1A}$ receptor in particular, and GPCRs in general.

Keywords: 5-HT$_{1A}$ receptor; G-protein coupling; Mg^{2+}; 8-OH-DPAT; *p*-MPPF; Bovine hippocampus

Serotonin (5-hydroxytryptamine or 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 signaling 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 [3,4]. 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 [3,5].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [6]. Serotonin receptors are members of a superfamily of seven transmembrane domain G-protein coupled receptors [7] that couple to and transduce signals via guanine nucleotide binding regulatory proteins (G-proteins) [8]. Among the 14 subtypes of serotonin receptors, the G-protein coupled 5-HT$_{1A}$ receptor is the best characterized for a number of reasons [9,10]. We have earlier partially purified and solubilized the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form [11,12]. We have also reported the solubilization of 5-HT$_{1A}$ receptors stably expressed in Chinese hamster ovary (CHO) cells [13]. In addition, we have shown modulation of ligand binding to 5-HT$_{1A}$ receptors by metal ions [9,14], agents that perturb

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[☆] *Abbreviations:* 5-HT, 5-hydroxytryptamine; 5-HT$_{1A}$ receptor, 5-hydroxytryptamine-1A receptor; BCA, bicinchoninic acid; 8-OH-DPAT, 8-hydroxy-2(di-*N*-propylamino)tetralin; GPCR, G-protein coupled receptor; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); *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; PMSF, phenylmethylsulfonyl fluoride.

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G-proteins [15,16], local anesthetics [17], covalent modifications of the disulfide and sulfhydryl groups [10], and membrane cholesterol [18,19].

The hippocampal 5-HT_{1A} receptor is negatively coupled to adenylate cyclase through G_i-proteins [20]. Agonist binding of the 5-HT_{1A} receptor has previously been shown to be modulated by guanine nucleotides [15,21]. Activation of a G-protein coupled receptor upon binding to its ligand sets the stage for a series of events in the G-protein cycle [22] and Mg^{2+} is known to be one of the crucial components at various steps of this cycle [23,24]. In this report, we show that guanine nucleotide sensitivity of agonist binding to hippocampal 5-HT_{1A} receptors is dependent on the concentration of Mg^{2+}. Our results show that agonist binding is relatively insensitive to guanine nucleotides in the absence of Mg^{2+}. However, the antagonist binding is insensitive to guanine nucleotides, even in the presence of Mg^{2+}.

Materials and methods

Materials. EDTA, EGTA, iodoacetamide, $MgCl_2$, *p*-MPPI, PMSF, polyethylenimine, Tris, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical (St. Louis, MO, USA). GTP-γ-S was purchased from Roche Applied Science (Mannheim, Germany). [^{3}H]8-OH-DPAT (sp. activity 135.0 Ci/mmol) and [^{3}H]*p*-MPPF (sp. activity 70.5 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). BCA reagent kit for protein estimation was obtained from Pierce (Rockford, IL, USA). All other chemicals used were of the highest purity available. GF/B glass microfiber filters were from Whatman International (Kent, UK). 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.

Preparation of native hippocampal membranes. Native hippocampal membranes were prepared as described earlier [9]. Bovine hippocampal tissue (~100 g) was homogenized as 10% (w/v) with a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, and 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 cheesecloth 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 vol buffer B (50 mM Tris, 1 mM EDTA, 0.24 mM PMSF, and 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 (native membrane) was resuspended in a minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a hand-held Dounce homogenizer, flash-frozen in liquid nitrogen, and stored at −70 °C until further use. Protein concentration was determined using the BCA reagent with bovine serum albumin as standard [25].

Radioligand binding assays. Receptor binding assays for agonist and antagonist were carried out as described earlier [15] with a few modifications in the presence of increasing concentrations of Mg^{2+}. Briefly, tubes in duplicate containing 1 mg total protein in a total volume of 1 ml buffer D (50 mM Tris, 1 mM EDTA, pH 7.4) were used with increasing concentrations of $MgCl_2$. Tubes were incubated with the radiolabeled agonist [^{3}H]8-OH-DPAT (final concentration in the assay tube being 0.29 nM) or antagonist [^{3}H]*p*-MPPF (final concentration in the assay tube being 0.5 nM) for 1 h at room temperature. Non-specific binding was determined by performing the assay in the presence of 10 μM unlabeled 5-HT in case of agonist binding or 10 μM unlabeled *p*-MPPI in case of antagonist binding. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B 2.5 cm diameter (1.0 μm pore size) glass microfiber filters which were presoaked in 0.15% (w/v) polyethylenimine for 3 h [26]. The filters were then washed three times with 3 ml ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml scintillation fluid.

Table 1
Extent of inhibition in specific [^{3}H]8-OH-DPAT binding in the presence of GTP-γ-S and IC_{50} values of GTP-γ-S at different concentrations of $MgCl_2$[a]

Concentration of $MgCl_2$ (mM)	Extent of inhibition in agonist binding (%)	IC_{50} of GTP-γ-S (nM)
0	26.6	—
0.5	35.2	620
2	87.8	93
10	91.3	92

[a] The values reported are obtained from Fig. 2. See Materials and methods for other details.

Sensitivity to GTP-γ-S. Ligand binding assays at a specified concentration of $MgCl_2$ were performed in the presence of varying concentrations of GTP-γ-S as described earlier [17] with a few modifications. The concentrations of GTP-γ-S leading to 50% inhibition of specific agonist binding (IC_{50}) were calculated by non-linear regression fitting of the data to a four parameter logistic function [27]:

$$B = \frac{a}{1 + (x/I)^s} + b, \qquad (1)$$

where B is the specific binding of the agonist normalized to control binding (in the absence of GTP-γ-S), x is the concentration of GTP-γ-S, a is the range ($y_{\max} - y_{\min}$) of the fitted curve on the ordinate (y-axis), I is the IC_{50} concentration, b is the background of the fitted curve ($y_{\min}$), and s is the slope factor. The difference between inhibition in agonist binding obtained with the highest and the lowest concentrations of GTP-γ-S at a specified concentration of $MgCl_2$ is expressed as the extent of inhibition in agonist binding and shown in Table 1.

Results and discussion

We monitored the ability of Mg^{2+} to modulate the specific agonist binding to 5-HT_{1A} receptors from bovine hippocampus. Fig. 1 shows that the specific [^{3}H]8-OH-DPAT binding activity obtained in the absence of $MgCl_2$ is 17.5 fmol/mg protein while that obtained in the presence of 10 mM $MgCl_2$ is 53.1 fmol/mg protein. Thus, there is ~3-fold increase in the specific binding activity when the concentration of $MgCl_2$ is increased up to 10 mM. This indicates that the specific binding of the agonist [^{3}H]8-OH-DPAT to 5-HT_{1A} receptors is dependent on the concentration of Mg^{2+} in the system.

G-protein coupled receptors (GPCRs) transduce signals from the extracellular milieu to the inside of the cell via their interaction with heterotrimeric G-proteins located on the cytoplasmic face of the cell. The G-protein

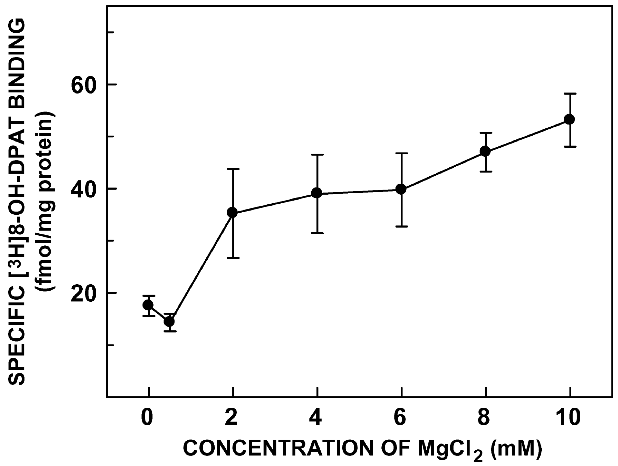

Fig. 1. Effect of increasing concentrations of $MgCl_2$ on the specific binding of the agonist $[^3H]$8-OH-DPAT to 5-HT_{1A} receptors from bovine hippocampal membranes. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and methods for other details.

heterotrimer consists of the α subunit and a pair of βγ subunits. Ligand-bound receptors activate G-proteins by facilitating GDP–GTP exchange on the α subunit where the Gα subunit bound to GTP dissociates from the βγ pair, and both α and βγ regulate their respective downstream signaling components [22,28]. Hydrolysis of GTP by Gα inactivates this process as the Gα reunites with βγ subunits to form the inactive heterotrimer. Due to receptor–G-protein interaction, guanine nucleotides are known to modulate ligand binding of G-protein coupled receptors. This has been shown to be true in case of the 5-HT_{1A} receptor [15,29]. Thus, it has been previously shown that the specific agonist 8-OH-DPAT and the antagonist *p*-MPPF differentially discriminate G-protein coupling of 5-HT_{1A} receptors from bovine hippocampus. In other words, while the agonist binds to only those receptors that are coupled to G-proteins, the antagonist binds to all receptors irrespective of their state of G-protein coupling. Non-hydrolyzable analogues such as GTP-γ-S block the G-protein cycle and thereby inhibit agonist binding to receptors (such as the 5-HT_{1A} receptor) [21]. Interestingly, Mg^{2+} has been shown to be a crucial component in various steps of the G-protein cycle such as the binding of GTP to the Gα subunit and its hydrolysis [23,27].

In order to assess the status of receptor–G-protein interaction in case of the 5-HT_{1A} receptor at varying concentrations of Mg^{2+}, we monitored the specific agonist binding of the receptor in the presence of GTP-γ-S. Fig. 2 shows the inhibition of agonist binding to 5-HT_{1A} receptors by GTP-γ-S at different concentrations of $MgCl_2$. As seen from the figure, in the absence of $MgCl_2$, agonist binding is relatively insensitive to GTP-γ-S. As the concentration of Mg^{2+} is increased up to 10 mM, the inhibition in agonist binding brought about by GTP-γ-S appears to improve significantly. This is apparent from the extent of inhibition in agonist binding caused by GTP-γ-S at various concentrations of Mg^{2+} (see Table 1). While the extent of inhibition is modest in the absence of Mg^{2+} (26.6%), it increases considerably (91.3%) in the presence of 10 mM Mg^{2+}. There is therefore ~3.5-fold increase in the inhibition in agonist binding in the presence of GTP-γ-S when the concentration of Mg^{2+} is increased from 0 to 10 mM. Table 1 also shows that the half maximal inhibition concentrations (IC_{50}) for inhibition of specific $[^3H]$8-OH-DPAT binding by GTP-γ-S decrease with increasing concentrations of Mg^{2+}. The IC_{50} value shows ~7-fold reduction when the concentration of Mg^{2+} is increased from 0.5 to 10 mM. This indicates that much less concentration of GTP-γ-S is required in the presence of Mg^{2+} to cause the same extent of inhibition in specific agonist binding. In other words, the presence of Mg^{2+} effectively makes the system more sensitive to the effect of GTP-γ-S which indicates increased coupling of the receptor to G-proteins.

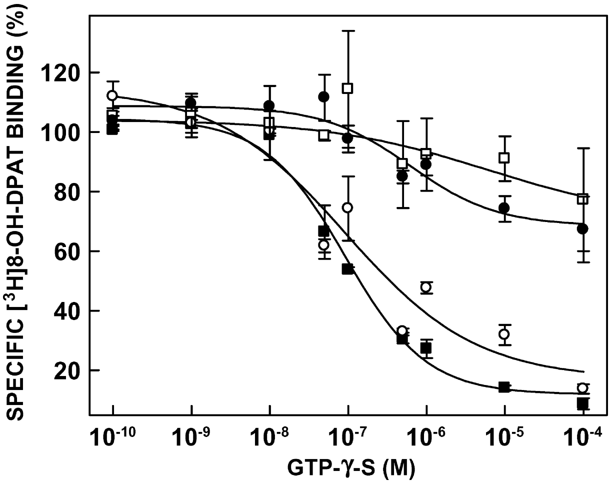

Fig. 2. Effect of increasing concentrations of GTP-γ-S on the specific binding of the agonist $[^3H]$8-OH-DPAT to 5-HT_{1A} receptors from bovine hippocampal membranes in the presence of 0 mM (□), 0.5 mM (●), 2 mM (○), and 10 mM (■) $MgCl_2$. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The curves are non-linear regression fits to the experimental data using the four parameter logistic function [27]. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and methods for other details.

The presence of divalent metal ions such as Mg^{2+} is known to inhibit antagonist *p*-MPPF binding to the 5-HT_{1A} receptor in a concentration-dependent manner [14]. This somewhat complicates monitoring the guanine nucleotide sensitivity of antagonist binding to 5-HT_{1A} receptors in the presence of Mg^{2+}. Specific $[^3H]$*p*-MPPF binding has been reported to be inhibited to a relatively low extent when the concentration of $MgCl_2$ used is 2 mM [14]. Interestingly, our results (see Fig. 2) indicate this concentration (2 mM) of $MgCl_2$ to be sufficient to

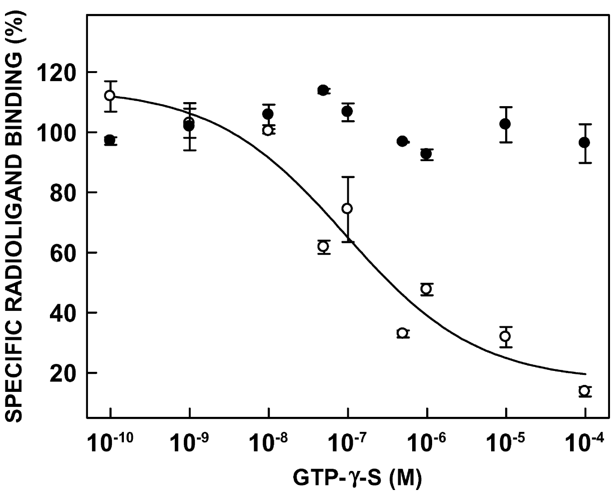

Fig. 3. Effect of increasing concentrations of GTP-γ-S on the specific binding of the antagonist [^{3}H]*p*-MPPF (●) to 5-HT$_{1A}$ receptors from bovine hippocampal membranes in the presence of 2 mM MgCl$_2$. The sensitivity of specific agonist [^{3}H]8-OH-DPAT binding (○) in the presence of 2 mM MgCl$_2$ with increasing concentrations of GTP-γ-S is shown for comparison. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The curve associated with agonist binding is a non-linear regression fit to the experimental data using the 4 parameter logistic function [27]. The data points represent means ± SE of duplicate points from three independent experiments. See Materials and methods for other details.

determine guanine nucleotide sensitivity of agonist binding. We therefore monitored the guanine nucleotide sensitivity of specific antagonist binding in the presence of 2 mM Mg^{2+} using varying concentrations of GTP-γ-S. Fig. 3 shows that the specific antagonist binding remains by and large invariant over a wide range of GTP-γ-S concentrations used in the presence of 2 mM Mg^{2+}. It has earlier been reported that agonist binding is sensitive whereas antagonist binding is insensitive to guanine nucleotides in case of several GPCRs [29]. Considering the significance of Mg^{2+} in guanine nucleotide modulation of ligand binding [23], we recommend that it is important to include Mg^{2+} while determining guanine nucleotide sensitivity of ligand (agonist or antagonist) binding.

In addition to the role of Mg^{2+} in the G-protein cycle, its presence is known to modulate both agonist and antagonist binding of the 5-HT$_{1A}$ receptor [9,14,24]. In fact, metal ion modulation of ligand binding has proved to be a characteristic feature of other important G-protein coupled receptors such as the μ-opioid receptor [30]. Our results highlight two novel aspects on the role of Mg^{2+} in guanine nucleotide sensitivity of agonist binding to serotonin$_{1A}$ receptors: (i) specific agonist binding is relatively insensitive to GTP-γ-S in the absence of Mg^{2+}; and (ii) the ability of GTP-γ-S to inhibit specific agonist binding is dependent on the concentration of Mg^{2+}, as evident from the IC$_{50}$ values of GTP-γ-S and the extent of inhibition in agonist binding caused by GTP-γ-S at various concentrations of Mg^{2+} (Table 1). These results therefore provide new insight into the concentration dependence of Mg^{2+} in relation to guanine nucleotide sensitivity. We show here that 2 mM Mg^{2+} is sufficient to determine the sensitivity of agonist binding to GTP-γ-S. Based on these results, we propose an optimum concentration of Mg^{2+} which could be used in assays toward determining guanine nucleotide sensitivity of ligand binding to GPCRs such as the 5-HT$_{1A}$ receptor. These results are relevant in ongoing analyses on the role of Mg^{2+} in the overall regulation of ligand binding and receptor activity in the 5-HT$_{1A}$ receptor in particular, and GPCRs in general, especially in the context of guanine nucleotide sensitivity.

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