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Metal Ion and Guanine Nucleotide Modulations of Agonist Interaction in G-Protein-Coupled Serotonin$_{1A}$ Receptors from Bovine Hippocampus

K. G. Harikumar[1] and Amitabha Chattopadhyay[1,2]

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SUMMARY

1. The serotonin type 1A (5-HT$_{1A}$) receptors are members of a superfamily of seven transmembrane domain receptors that couple to GTP-binding regulatory proteins (G-proteins). We have studied the modulation of agonist binding to 5-HT$_{1A}$ receptors from bovine hippocampus by metal ions and guanine nucleotide.

2. Bovine hippocampal membranes containing the 5-HT$_{1A}$ receptor were isolated. These membranes exhibited high-affinity binding sites for the specific agonist [^{3}H]OH-DPAT.

3. The agonist binding is inhibited by monovalent cations Na^+, K^+, and Li^+ in a concentration-dependent manner. Divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+}, on the other hand, show more complex behavior and induce enhancement of agonist binding up to a certain concentration. The effect of the metal ions on agonist binding is strongly modulated in the presence of GTP-γ-S, a nonhydrolyzable analogue of GTP, indicating that these receptors are coupled to G-proteins.

4. To gain further insight into the mechanisms of agonist binding to bovine hippocampal 5-HT$_{1A}$ receptors under these conditions, the binding affinities and binding sites have been analyzed by Scatchard analysis of saturation binding data. Our results are relevant to ongoing analyses of the overall regulation of receptor activity for G-protein-coupled seven transmembrane domain receptors.

KEY WORDS: 5-HT$_{1A}$ receptor; metal ions; bovine hippocampus; OH-DPAT; Scatchard analysis; guanine nucleotide.

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is an intrinsically fluorescent, biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems (Jacobs and Azmitia, 1992; Chattopadhyay *et al.*, 1996). It is present in a variety of organisms, ranging from humans to species with primitive nervous systems such as worms (Hen, 1992), and mediates a variety of physiological responses in distinct cell types. Serotonergic signaling

[1] Centre for Cellular & Molecular Biology, Uppal Road, Hyderabad 500 007, India.
[2] To whom correspondence should be addressed. Fax: +91-40-7171195. e-Mail: amit@ccmb.ap.nic.in.

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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 (Chojnacka-Wojcik *et al.,* 1991; Wilkinson and Dourish, 1991; Saudou *et al.,* 1994; Cases *et al.,* 1995; Heath and Hen, 1995; Artigas *et al.,* 1996; Bjørkum and Ursin, 1996; Crabbe *et al.,* 1996; Ohno and Watanabe, 1996; Yeh *et al.,* 1996). Disruptions in serotonergic systems have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, infantile autism, eating disorders, and obsessive compulsive disorder (Lopez-Ibor, 1988; Tecott *et al.,* 1995). Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups (Peroutka, 1993; Saudou and Hen, 1994). Serotonin receptors are members of a superfamily of receptors (Strader *et al.,* 1995) that couple to GTP-binding regulatory proteins (G-proteins).

Based on pharmacological and molecular biological techniques, a large, growing family of distinct serotonin receptor subtypes has been identified (Peroutka, 1988; Boess and Martin, 1994; Saudou and Hen, 1994). The unusually large number of receptor subtypes allows serotonin to have diverse effects in the nervous system (Saudou and Hen, 1994). Interestingly, the heterogeneity in serotonin receptors is much more than that found in other biogenic amine receptors. One of the reasons suggested for this discrepancy is that the "primordial" serotonin receptor appears to have existed prior to the evolution of muscarinic, dopaminergic, and adrenergic receptor systems (Peroutka and Howell, 1994). The fact that the early serotonin receptor evolved prior to other G-protein-coupled biogenic amine receptors could account for the large number of serotonin receptors existing today (Peroutka, 1994).

The heterogeneity of serotonin receptors was first demonstrated based on a pharmacological analysis of binding of radiolabeled serotonin, D-lysergic acid diethylamide (LSD), and spiperone to cortical membrane fragments (Peroutka and Snyder, 1979). Serotonin was found to bind to two distinct sites with a thousandfold difference in affinity. The sites that bind serotonin in the nanomolar range was designated the 5-HT_1 sites, and the sites that bind serotonin at the micromolar range, but bind spiperone at the nanomolar range, were termed the 5-HT_2 sites. The hallucinogenic drug LSD binds to both sites with a high affinity. In general, the 5-HT_1 type is characterized by a higher affinity for agonists than for antagonists. Thus, the concept of multiple serotonin receptors was introduced. Further, heterogeneity of 5-HT_1 sites was demonstrated by the ability of spiperone to compete, at low concentrations, with serotonin for some 5-HT_1 sites (Pedigo *et al.,* 1981). A subpopulation of 5-HT_1 sites, that has a high affinity for spiperone and serotonin, was designated the $5HT_{1A}$ sites.

Among the various types of serotonin receptors, the G-protein-coupled 5-HT_{1A} receptor subtype has been the most extensively studied. The reasons for this include (i) the availability of a highly selective ligand [(±)-8-hydroxy-2-(di-*N*-propylamino) tetralin (OH-DPAT)] that allows extensive biochemical, physiological, and pharmacological characterization of the receptor (Gozlan *et al.,* 1983; Middlemiss and Fozard, 1983) and (ii) the observation that certain 5-HT_{1A} agonists exert anxiolytic and antidepressant effects (Blier *et al.,* 1990). As a result, the 5-HT_{1A} receptors have become an important target in the development of therapeutic agents to treat

neuropsychiatric disorders such as anxiety and depression. Besides, the 5-HT_{1A} receptors are implicated in regulation of blood pressure, feeding, temperature regulation (Dourish *et al.,* 1987), and regulation of working memory (Ohno and Watanabe, 1996); (iii) it was the first among all the serotonin receptors to be cloned and sequenced (Kobilka *et al.,* 1987; Fargin *et al.,* 1988; Albert *et al.,* 1990). The human, rat, and mouse 5-HT_{1A} receptors have been cloned, and their amino acid sequences deduced (Fargin *et al.,* 1988; Albert *et al.,* 1990; Charest *et al.,* 1993). The cloning of the 5-HT_{1A} receptor gene has shown that it belongs to the superfamily of G-protein-coupled receptors, with 50% amino acid homology with the β_2-adrenergic receptor in the transmembrane domain. Furthermore, the receptor has been stably expressed in a number of neural and nonneural cell lines (Banerjee *et al.,* 1993); and (iv) it was the first serotonin receptor for which polyclonal antibodies were obtained (Fargin *et al.,* 1988; El Mestikawy *et al.,* 1990), allowing their visualization at the subcellular level in various regions of the brain. The 5-HT_{1A} receptor gene has also been recently implicated in Tourette's syndrome, a common hereditary motor and vocal tic disorder (Lam *et al.,* 1996).

Metal ion modulation of agonist binding is a characteristic feature of G-protein-coupled seven transmembrane domain receptors. Thus, effects of metal ions on agonist binding have been reported for the adrenergic receptors (Tsai and Lefkowitz, 1978; Ernsberger and U'Prichard, 1987; Ceresa and Limbird, 1994), opiate receptors (Pasternak *et al.,* 1975; Lee *et al.,* 1977; Blume, 1978; Sadee *et al.,* 1982; Werling *et al.,* 1985, 1986; Yabaluri and Medzihradsky, 1997), dopamine receptors (Creese *et al.,* 1978; Sibley and Creese, 1983), tachykinin receptors (Morishima *et al.,* 1989), muscarinic acetylcholine receptor (Shiozaki and Haga, 1992), and the bradykinin B_2 receptor (Quitterer *et al.,* 1996).

The 5-HT_{1A} receptor is negatively coupled to the adenylate cyclase system through G-proteins (Emerit *et al.,* 1990; Cornfield and Nelson, 1991). Since most seven transmembrane domain receptors are coupled to G-proteins (Clapham, 1996), guanine nucleotides are known to regulate agonist binding. Guanine nucleotides interact with many hormone and neurotransmitter receptor systems. For example, GTP is necessary for the activation of neurotransmitter-sensitive adenylate cyclase systems (Limbird, 1981). Guanine nucleotide sensitivity to agonist binding has been shown for adrenergic receptors (U'Prichard and Snyder, 1978; Ernsberger and U'Prichard, 1987), opiate receptors (Blume, 1978; Childers and Snyder, 1980), dopamine receptors (Creese *et al.,* 1978), tachykinin receptors (Morishima *et al.,* 1989), and muscarinic acetylcholine receptor (Shiozaki and Haga, 1992).

We have recently partially purified the 5-HT_{1A} receptor from bovine brain hippocampus and solubilized it in an active form using a premicellar concentration of the zwitterionic detergent CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} under high-salt conditions (Chattopadhyay and Harikumar, 1996). From studies of regional distribution of the 5-HT_{1A} receptor in the brain, it is known that the hippocampus has a higher density of 5-HT_{1A} receptor sites, particularly in the dentate gyrus and CA_1 regions (Marcinkiewicz *et al.,* 1984; Kia *et al.,* 1996). In this paper, we have examined the modulation of agonist ([^{3}H]OH-DPAT) binding to 5-HT_{1A} receptors in bovine hippocampal membranes by monovalent and divalent cations. In addition, since the 5-HT_{1A} receptor is coupled to G-

proteins, we have investigated the guanine nucleotide sensitivity of the agonist binding to the receptor in the presence and absence of metal ions. To gain further insight into the mechanisms of agonist binding to bovine hippocampal 5-HT_{1A} receptors under these conditions, we have analyzed the binding affinities and binding sites by Scatchard analysis of saturation binding data in all cases.

MATERIALS AND METHODS

Materials. Serotonin, iodoacetamide, polyethylenimine, sucrose, pargyline, sodium azide, Tris, EDTA, EGTA, NaCl, KCl, LiCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO). Guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) was from Boehringer Mannheim (Germany). [^{3}H]OH-DPAT (147.2 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, U.K.). A bicinchoninic acid (BCA) reagent kit for protein estimation was obtained from Pierce (Rockford, IL). 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 till further use.

Preparation of Crude Hippocampal Membranes. Bovine hippocampal tissue (~120 g) was homogenized as 10% (wt/vol) in a polytron homogenizer in buffer A (2.5 m*M* Tris, 0.32 *M* sucrose, 5 m*M* EDTA, 5 m*M* EGTA, 0.02% sodium azide, 0.24 m*M* PMSF, 10 m*M* 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 of buffer B (50 m*M* Tris, 1 m*M* EDTA, 0.24 m*M* PMSF, 10 m*M* iodoacetamide, pH 7.4) using a Dounce homogenizer and centrifuged at 50,000*g* for 20 min at 4°C. This procedure was repeated till the supernatant was clear. The final pellet (crude membrane) was resuspended in a minimum volume of 50 m*M* Tris buffer (pH 7.4), homogenized using a Dounce homogenizer, flash-frozen in liquid nitrogen, and stored at −70°C. The homogenate thus obtained was either used directly for receptor binding or incubated with various concentrations of monovalent (Na^+, K^+, Li^+) or divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+}) in the presence and absence of GTP-γ-S, a nonhydrolyzable analogue of GTP, before radioligand binding assay was performed.

Receptor Binding Assay. Tubes in triplicate containing 1–1.2 mg of total protein in a total volume of 1 ml of buffer C (50 m*M* Tris, 2 m*M* $MgCl_2$, 20 μ*M* pargyline, 0.1% ascorbic acid, pH 7.4; it should be noted that $MgCl_2$ was not used in experiments where Mg^{2+} effects on agonist binding were examined) were incubated with 0.29 n*M* [^{3}H]OH-DPAT (sp. act. 147.2 Ci/mmol) for 1 hr at room temperature in the presence and absence of 10 μ*M* unlabeled 5-HT. 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, which were presoaked in 0.3% polyethylenimine for 3 hr (Bruns *et al.,* 1983). The filters were then washed three times with 3 ml of ice-cold water

Table I. Affinity and B_{max} Values of [^{3}H]OH-DPAT Binding of 5-HT$_{1A}$ Receptor from Bovine Hippocampal Membranes[a]

Metal ion	K_d (nM)	B_{max} (fmol/mg protein)
None (native membrane)	1.91 ± 0.06	120.01 ± 27.70
	(2.77 ± 0.56)[b]	(34.57 ± 3.63)[b]
Na^+	3.42 ± 0.18	71.60 ± 14.39
	(2.10 ± 0.64)[b]	(14.65 ± 0.76)[b]
K^+	1.51 ± 0.43	82.93 ± 5.11
Li^+	3.72 ± 0.30	86.59 ± 23.31
	(2.45 ± 0.52)[b]	(31.81 ± 5.97)[b]
Ca^{2+}	1.21 ± 0.05	94.21 ± 0.62
Mg^{2+}	1.38 ± 0.33	83.63 ± 11.58
	(2.54 ± 0.67)[b]	(32.94 ± 2.99)[b]
Mn^{2+}	1.02 ± 0.10	91.16 ± 9.34

[a] The binding parameters shown in this table represent the mean ± standard deviation of duplicate points from three independent experiments, while saturation binding data shown in Figs. 2, 4, and 6 are from representative experiments. See Materials and Methods for other details.
[b] In the presence of 50 μM GTP-γ-S.

and dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid.

Saturation Binding Assay. Saturation binding assays were carried out using different concentrations of radiolabeled ligand (sp. act. 147.2 Ci/mmol) with varying concentrations of [^{3}H]OH-DPAT (0.1–7.5 nM) using 1.2 mg of crude membrane. Nonspecific binding was measured in the presence of 10 μM unlabeled 5-HT. Binding assays were carried out at room temperature as mentioned above (see receptor binding assay) in the presence of different metal ions at a specific concentration. The specific metal ion concentration at which the assay was done was the half-maximal inhibition concentration (IC_{50} value) for monovalent cations, determined using the LIGAND program (McPherson, 1985), obtained from Biosoft, Cambridge, U.K. For divalent cations, the concentrations at which maximum stimulation in binding were observed were used for saturation binding assays. Binding data were analyzed as described by Hulme (1990). The concentration of bound ligand was calculated from the equation

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

where B is the bound radioactivity in dpm (i.e., total dpm − nonspecific 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^* vs L^*) 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 = 0.93–0.99). The B_{max} values were obtained from the intercept on the abscissa. The B_{max} values reported in Table I have been normalized with respect to the amount of crude membrane used. The binding parameters shown in Table I were obtained by averaging the results of three independent experiments, while saturation binding data shown in Figs. 2, 4, and 6 are from representative experiments. The extent of

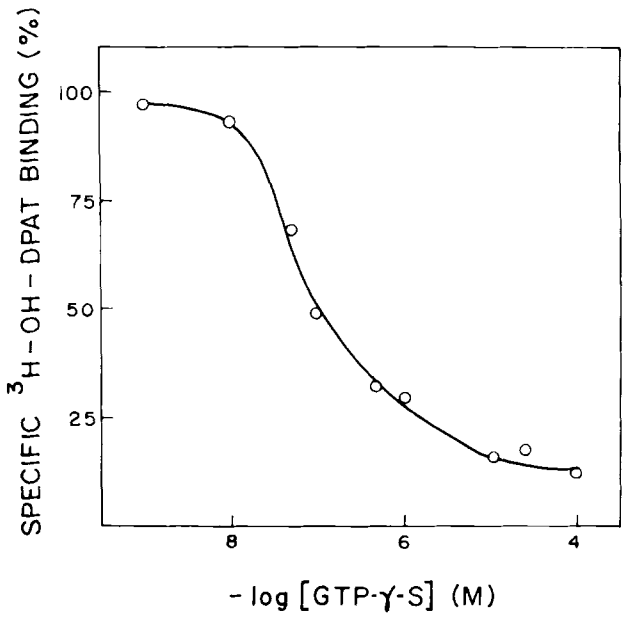

Fig. 1. Effect of increasing concentrations of GTP-γ-S on the specific binding of [^{3}H]OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. Values are expressed as a percentage of the specific binding obtained in the absence of GTP-γ-S. The data points are the means of duplicate points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

G-protein coupling was monitored by saturation binding assays done with and without 50 μM GTP-γ-S. Protein concentration was determined using BCA reagent (Smith *et al.*, 1985).

RESULTS

Ligand binding assay using the 5-HT$_{1A}$ agonist [^{3}H]OH-DPAT shows the presence of high-affinity binding sites in bovine hippocampal membranes with apparent dissociation constant of 1.91 nM obtained from saturation binding studies (see Table I). We found the ligand binding to be optimal with 1.2 mg of the crude membrane. The crude membrane prepared from bovine hippocampus typically showed a 10- to 12-fold enrichment in the receptor content as found by receptor binding assay (data not shown).

Figure 1 shows the inhibition of OH-DPAT binding to the 5-HT$_{1A}$ receptor by GTP-γ-S, a nonhydrolyzable analogue of GTP, in a characteristic concentration-dependent manner. This shows that the 5-HT$_{1A}$ receptor in these membranes is coupled to G-proteins, as suggested by previous reports for 5-HT$_{1A}$ receptors from other sources (Schlegel and Peroutka, 1986; Emerit *et al.*, 1990; Cornfield and Nelson, 1991; Harrington and Peroutka, 1990). In fact, OH-DPAT has previously

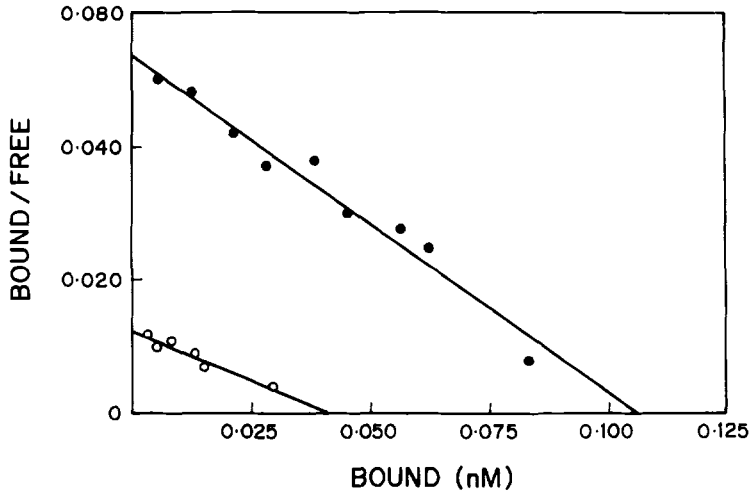

Fig. 2. Scatchard analysis of the specific binding of [^{3}H]OH-DPAT to the 5-HT_{1A} receptor from bovine hippocampal membranes in the presence (○) and absence (●) of 50 μM GTP-γ-S. The concentration of [^{3}H]OH-DPAT ranged from 0.1 to 7.5 nM. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Materials and Methods for other details.

been shown to bind to the high-affinity binding sites of only that population of 5-HT_{1A} receptors that is G-protein coupled (Newman-Tancredi *et al.*, 1992; Sundaram *et al.*, 1993, 1995).

Figure 2 shows the Scatchard analysis of the specific binding of [^{3}H]OH-DPAT to the high-affinity 5-HT_{1A} receptor in bovine hippocampal membranes. Analysis of the Scatchard plot shows tight binding (K_d = 1.91 nM) with a maximum number of binding sites (B_{max}) of about 120 fmol/mg protein (see Table I). Table I shows that in the presence of GTP-γ-S, both the binding affinity and the number of binding sites are reduced. Thus, while the binding affinity is reduced, corresponding to a K_d of 2.77 nM, the number of binding sites is reduced by 71%. GTP-γ-S appears to induce a transition of the 5-HT_{1A} receptor from the high-affinity to a low-affinity state (Schlegel and Peroutka, 1986). Such affinity transition induced by guanine nucleotides has been observed previously for the adrenergic receptors (Hoffman and Lefkowitz, 1980).

Figure 3 shows the inhibition of [^{3}H]OH-DPAT binding to 5-HT_{1A} receptors by monovalent cations such as Na^+, K^+, and Li^+. There is a concentration-dependent inhibition of binding in all cases, with the potency decreasing in the order $Li^+ > K^+ > Na^+$. Figure 3 also shows that the agonist binding is further inhibited in the presence of GTP-γ-S in all cases, implying that the receptor is coupled to G-proteins. Closer inspection of Figs. 1 and 3 show that the inhibition to agonist binding by monovalent ions and GTP-γ-S is additive to a large extent. Such additive inhibition by guanine nucleotide and monovalent ions was previously observed in the case of opiate receptors (Childers and Snyder, 1980).

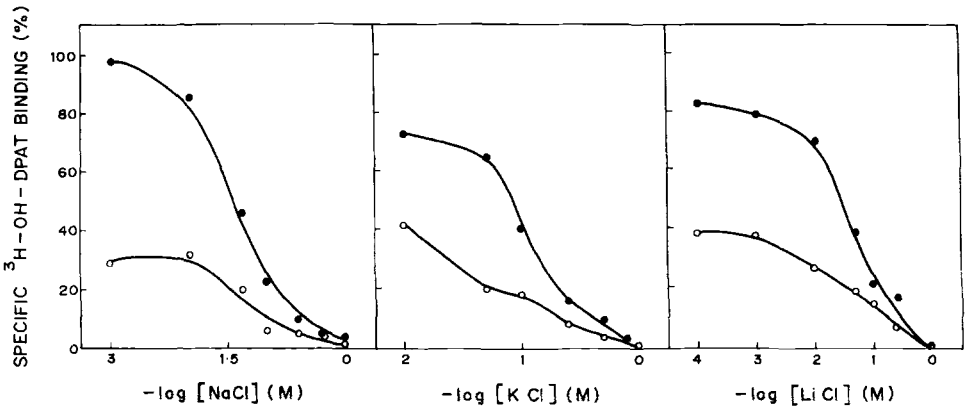

Fig. 3. Effect of monovalent cations on [^{3}H]OH-DPAT specific binding to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence (○) and absence (●) of 50 μM GTP-γ-S. The data points are the means of duplicate points from three independent experiments. See Materials and Methods for other details.

Scatchard analysis of the saturation binding assay (Fig. 4) shows that in the case of Na^+ and Li^+, there is a reduction in the specific binding affinity (K_d = 3.42 and 3.72 nM, in the case of Na^+ and Li^+, respectively) of the agonist (see Table I). This is accompanied by a concomitant decrease in the number of binding sites. There is a 40% decrease in the number of binding sites in presence of Na^+ ions, while the decrease in binding sites is about 28% for Li^+ (Table I). However, these results are somewhat different in the case of K^+ ions. There is an increase in binding affinity (corresponding to a K_d of 1.51 nM) with an accompanying 31% reduction in the number of binding sites (Table I). The interaction of monovalent cations with 5-HT$_{1A}$ receptors is thus characterized by an altered agonist binding affinity and a reduction in number of binding sites.

Table I also shows that when GTP-γ-S is present along with monovalent cations such as Na^+ and Li^+, there is a further reduction in agonist binding sites. Thus, there is a 88% decrease in binding sites when GTP-γ-S is present along with Na^+ (compared to native membranes). The corresponding figure for Li^+, when present with GTP-γ-S, is 73%. The agonist binding affinity (K_d = 2.10 and 2.45 nM for Na^+ and Li^+, respectively), on the other hand, is slightly lowered compared to that of the native membrane (K_d = 1.91 nM).

Figure 5 shows the modulation of [^{3}H]OH-DPAT binding to 5-HT$_{1A}$ receptors by divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+}. The effect of divalent cations on agonist binding appears to be more complex. There is a concentration-dependent enhancement in binding up to a certain concentration in all cases. The concentration at which maximum enhancement of binding is observed is specific to the metal ion used. Thus, while agonist binding is maximally enhanced in the presence of 10 mM Ca^{2+} or Mg^{2+}, the corresponding concentration for Mn^{2+} is 1 mM. The sensitivity of divalent cations for stimulating agonist binding therefore decreases in the order $Mn^{2+} > Ca^{2+} \approx Mg^{2+}$. At higher concentrations of divalent cations, inhibition to

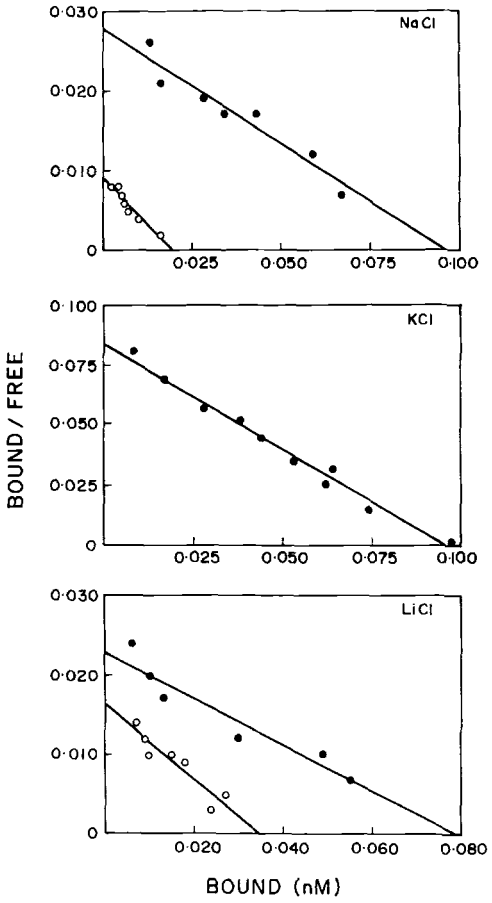

Fig. 4. Scatchard analysis of the specific binding of [^{3}H]OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence of monovalent cations with (○) and without (●) 50 μM GTP-γ-S. The concentration of [^{3}H]OH-DPAT ranged from 0.1 to 7.5 nM. The concentrations of monovalent cations used were their respective IC_{50} values (Na^+, 33.00 mM; Li^+, 5.53 mM; K^+, 31.00 mM). Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Materials and Methods for other details.

agonist binding is observed similar to results obtained with monovalent cations. These results are in agreement with previous reports in which divalent cations have been shown to enhance agonist binding in 5-HT receptors (Hall *et al.*, 1986), opiate receptors (Pasternak *et al.*, 1975; Sadee *et al.*, 1982), and dopamine receptors (Creese *et al.*, 1978). Figure 5 also shows that the agonist binding is further inhibited in the presence of GTP-γ-S in all cases, implying that the receptor is coupled to G-proteins. Interestingly, the enhancement of agonist binding up to a certain concentration

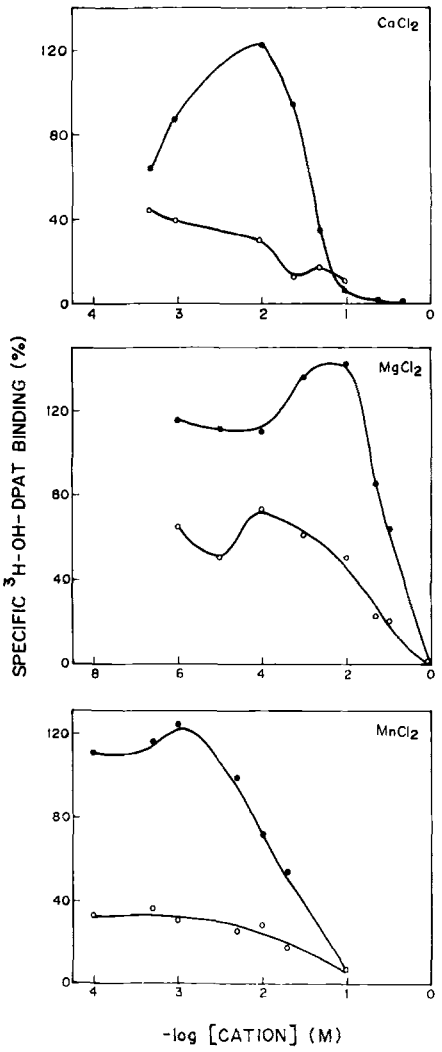

Fig. 5. Effect of divalent cations on [^{3}H]OH-DPAT specific binding to the 5-HT_{1A} receptor from bovine hippocampal membranes in the presence (○) and absence (●) of 50 μM GTP-γ-S. The data points are the means of duplicate points from three independent experiments. See Materials and Methods for other details.

observed when divalent cations are present alone (discussed above) is masked in the presence of GTP-γ-S for Ca^{2+} and Mn^{2+}. In the case of Mg^{2+}, the enhancement in binding is still observed in the presence of GTP-γ-S, although the concentration at which maximum binding takes place is about two orders of magnitude lower (0.1 mM) than observed in the absence of GTP-γ-S (Figure 5). This could be due to the specific effects of Mg^{2+} on the interaction of G-proteins with GTP-γ-S (Higashijima *et al.*, 1987).

Figure 6 shows the Scatchard analysis of the saturation binding assay in the presence of divalent cations. There is an increase in binding affinity of the agonist in all cases (see Table I). The apparent dissociation constants (K_d) in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+} are 1.21, 1.38, and 1.02 nM, respectively. This is accompanied

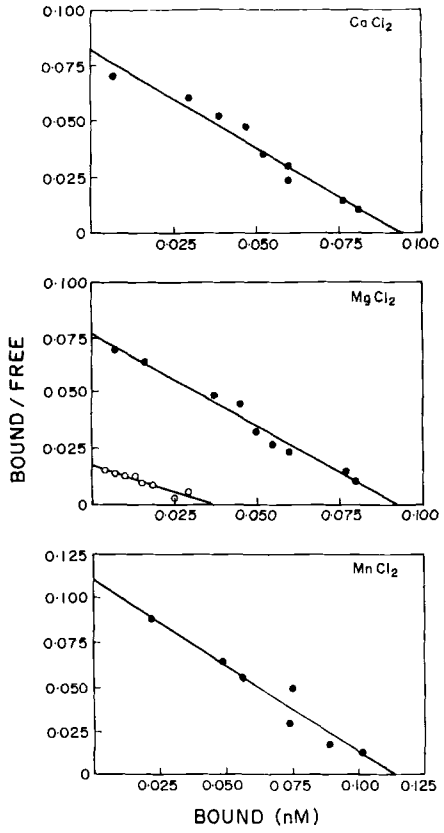

Fig. 6. Scatchard analysis of specific [^{3}H]OH-DPAT binding to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes in the presence of divalent cations with (○) and without (●) 50 μM GTP-γ-S. The concentration of [^{3}H]OH-DPAT ranged from 0.1 to 7.5 nM. The concentrations of divalent cations used were 10 mM in the case of Ca^{2+} and Mg^{2+} and 1 mM for Mn^{2+}. Data shown are from a representative experiment and each point is the mean of duplicate determinations. See Materials and Methods for other details.

by a concomitant decrease in the number of binding sites. The reduction in binding sites is 22, 30, and 24% in the presence of Ca^{2+}, Mg^{2+}, and Mn^{2+}, respectively (see Table I). The interaction of divalent cations with 5-HT$_{1A}$ receptors then is characterized by an increase in agonist binding affinity and a reduction in number of binding sites.

It is interesting to note from Table I that the presence of GTP-γ-S along with Mg^{2+} lowers the agonist binding affinity corresponding to a K_d of 2.54 nM. This represents a significantly lower binding affinity compared to that with Mg^{2+} alone (K_d = 1.38 nM) as well as compared to that of the control membranes (K_d = 1.91 nM). Such affinity transitions of G-protein-coupled seven transmembrane domain receptors induced by divalent cations have been reported previously (Sadee *et al.*, 1982; DeVinney and Wang, 1995). This is accompanied by a 73% decrease in binding sites when GTP-γ-S is present along with Mg^{2+} (compared to native membranes).

DISCUSSION

There is heterogeneity of the [^{3}H]OH-DPAT binding sites in different regions of the brain. Low-affinity binding sites are present in the striatum and cerebral

cortex, while the CA_1 and dentate gyrus regions of the hippocampus show high-affinity binding sites (Nenonene *et al.*, 1994; Gozlan *et al.*, 1995). The hippocampal 5-HT_{1A} receptors are localized postsynaptically (Verge *et al.*, 1986; Palacios *et al.*, 1990). These are the sites that are apparently labeled by [^{3}H]OH-DPAT (Hall *et al.*, 1986; Dumuis *et al.*, 1988). Using the 5-HT_{1A} agonist [^{3}H]OH-DPAT, we show here that high-affinity agonist binding sites are present in bovine hippocampus and are coupled to G-proteins. The modulation of agonist binding to 5-HT_{1A} receptors from bovine hippocampus by metal ions and guanine nucleotide is the focus of this report. Metal ion modulation and guanine nucleotide sensitivity of agonist binding are important characteristics of G-protein-coupled receptors. In this manuscript, we show that these characteristics are exhibited by the 5-HT_{1A} receptor from bovine hippocampus. Although metal ion modulation and guanine nucleotide sensitivity of agonist binding for other serotonin receptors have been reported previously (Battaglia *et al.*, 1984; Hall *et al.*, 1986), the present results are the first detailed report describing these effects for the 5-HT_{1A} subtype receptor, especially from bovine hippocampus. In addition, in most earlier reports the accompanying changes in binding affinities and binding sites have not been characterized. We have analyzed the changes in binding affinities and binding sites under these conditions by Scatchard analysis of saturation binding data.

Our results show that GTP-γ-S, a nonhydrolyzable GTP analogue, induces transition of the bovine hippocampal 5-HT_{1A} receptor from the high-affinity to a low-affinity state (Schlegel and Peroutka, 1986). The latter state of the receptor is sometimes referred to as the "nonbinding state" (Harrington and Peroutka, 1990). The affinity transition is accompanied by a decrease in the number of binding sites. These observations can be rationalized in terms of the "ternary complex model" (De Lean *et al.*, 1980; Wregget and De Lean, 1984), which assumes that the agonist affinity for its binding site and the fraction of total receptors in any given conformation will depend on the extent of receptor–G-protein coupling. The G-protein-coupled form of the receptor will exhibit a high-affinity agonist state, whereas the uncoupled receptor will display a lower affinity for the agonist. Receptor isomerization between high- and low-affinity states can be reversibly shifted by guanyl nucleotides. Similar findings have been reported previously by other groups (Schlegel and Peroutka, 1986; Harrington and Peroutka, 1990; Emerit *et al.*, 1990, 1991; Khawaja *et al.*, 1995).

Rhodopsin and the β-adrenergic receptor serve as representative members for the study of the structure and function of the G-protein-coupled receptor family (Strosberg, 1991; Donnelly and Findlay, 1994; Strader *et al.*, 1995). The alighnment of G-protein-coupled receptor sequences indicates that the hydrophilic components of the transmembrane segments are the most likely site of agonist–receptor interactions. It is known that the agonist binding site for the β-adrenergic receptor is in the membrane-embedded region of the receptor, similar to the retinal binding site in rhodopsin (Dixon *et al.*, 1987; Strader *et al.*, 1987). Subsequent studies using the β-adrenergic, α-adrenergic, and muscaranic receptors have demonstrated that the location of the binding site inside the membrane is a common feature of all these receptors (Ostroski *et al.*, 1992). The agonists for these receptors contain an amine group that is believed to form a complex with the negatively charged aspartate

residue in the third transmembrane domain. This is believed to constitute one of the epitopes necessary for high-affinity binding. The primary structures of the members of the G-protein-coupled receptor family indicate that this binding mechanism is conserved in all the receptors that have a charged amine group as a feature of their activating ligand. All cloned 5-HT receptors also contain the aspartic acid residue in the third transmembrane domain at a comparable position (Wang *et al.*, 1993).

For serotonin receptors, mutagenesis and molecular modeling studies have shown that the ligand binding site is located in a transmembrane domain (Ho *et al.*, 1992; Chanda *et al.*, 1993; Peroutka, 1993; Wang *et al.*, 1993; Sylte *et al.*, 1993, 1996; Bremner *et al.*, 1997). In the case of 5-HT$_{1A}$ receptors, molecular dynamics (Sylte *et al.*, 1996) and site-specific and random mutagenesis studies (Ho *et al.*, 1992; Chanda *et al.*, 1993) have provided vital insights into receptor structure and function. The 5-HT$_{1A}$ receptor agonists belong to diverse chemical classes but have in common a basic amino group and an aromatic ring that usually carries groups such as an hydroxyl or a methoxy which has the potential for hydrogen bond formation (Ho *et al.*, 1992). The involvement of the conserved residues Asp^{82}, Asp^{116}, Ser^{199}, and Thr^{200} for agonist binding to the 5-HT$_{1A}$ receptor has been pointed out by site-directed mutation (Ho *et al.*, 1992) as well as molecular modeling studies (Sylte *et al.*, 1996). Monovalent cations such as Na^+ and Li^+ could induce conformational change of the agonist binding site in such a way as to alter the binding affinity and sites. Interestingly, a conserved aspartate residue in the second transmembrane helix of many G-protein-coupled receptors has been implicated in agonist binding and modulation by sodium ions (Horstman *et al.*, 1990). Thus, in the case of the α_2-adrenergic receptor, Asp^{79} has been shown to be directly involved in regulation of the interaction of Na^+ with the receptor (Horstman *et al.*, 1990; Kong *et al.*, 1993). This is achieved by binding of Na^+ to the carboxylate form of the residue. A conserved aspartate (Asp^{82}) in a similar region of the 5-HT$_{1A}$ receptor has been shown to be essential for agonist binding (Ho *et al.*, 1992). The monovalent ions could interact with Asp^{82} of the 5-HT$_{1A}$ receptor, thereby altering the agonist binding site and affinity.

The 5-HT$_{1A}$ receptors are known to exist in different conformational states with varying affinities (Mongeau *et al.*, 1992). It has been proposed that the G-protein-coupled seven transmembrane domain receptors are in equilibrium between the inactive conformation (R) and a spontaneously active conformation (R*) that can couple to G-proteins in the absence of ligand (Bond *et al.*, 1995). Agonists such as OH-DPAT have a high affinity for the active-state receptor (R*) and thus increase the concentration of R*. The inactive state (R) is not coupled to G-proteins and is favored in the presence of monovalent cations such as Na^+ and Li^+. In the presence of GTP-γ-S, the equilibrium is shifted further toward the inactive state. The agonist-dependent alteration of the balance between receptor activation and inactivation serves as the basis of receptor function. Disregulation of the balance results in constitutive receptor activity leading to various diseases (Lefkowitz, 1993).

Lithium as a salt is used in the treatment of mania, in the augmentation treatment of depression, and in the prophylaxis of manic-depressive illness (Belmaker *et al.*, 1996). It has been proposed that the 5-HT$_{1A}$ receptors could be the

site of action of antidepressants (Nenonene *et al.*, 1994). It is known that Li^+ affects the enhanced binding of GTP to G-proteins caused by neurotransmitter stimulation of receptors (Belmaker *et al.*, 1996). Short-term lithium treatment has been reported to enhance responsiveness of postsynaptic but not of presynaptic 5-HT_{1A} receptors (Blier *et al.*, 1987). Our results show that Li^+ can induce affinity transition in bovine hippocampal 5-HT_{1A} receptors, which are located postsynaptically (Verge *et al.*, 1986; Palacios *et al.*, 1990), resulting in low-affinity receptors with an accompanying reduction in number of binding sites.

Our results show that divalent cations have a complex effect on the binding of [^{3}H]OH-DPAT to the 5-HT_{1A} receptor. Divalent cations showed enhancement of binding at a certain concentration in all cases, with an accompanying increase in binding affinity. It is known that divalent cations are required for the stabilization of an agonist-specific high-affinity state at the expense of the low-affinity state by promoting the formation of 5-HT_{1A} receptor–ligand–G-protein ternary complexes (Khawaja *et al.*, 1995).

The interactions of receptors with G-proteins are known to be regulated by Mg^{2+} (Higashijima *et al.*, 1987). The 5-HT_{1A} receptor is negatively coupled to the adenylate cyclase system through pertussis toxin-sensitive G_i-protein (Harrington *et al.*, 1988; Cornfield and Nelson, 1991). Mg^{2+} has the ability to interact with GTP-γ-S (Higashijima *et al.*, 1987) to form a complex along with G-protein ($G_\alpha \cdot$ GTP-γ-S$\cdot Mg^{2+}$). This complex, upon interaction with the receptor, could induce the high-affinity state of the receptor (Shiozaki and Haga, 1992).

In summary, our results show that the interaction of monovalent cations with 5-HT_{1A} receptors is characterized by a concentration-dependent inhibition of agonist binding, an altered agonist binding affinity, and a reduction in the number of binding sites. Divalent cations display more complex behavior. There is an enhancement of agonist binding up to a certain concentration, followed by inhibition in agonist binding at higher concentrations. This is accompanied by an increase in binding affinity with a concomitant reduction in binding sites. The effect of the metal ions on agonist binding is strongly modulated in the presence of GTP-γ-S, a nonhydrolyzable analogue of GTP, indicating that these receptors are coupled to G-proteins. The 5-HT_{1A} receptor activity in bovine hippocampus is therefore very well regulated by the ionic condition of the environment. Multiple-affinity states of the 5-HT_{1A} receptor induced by metal ions and guanine nucleotides could be physiologically significant. For example, the effect of Na^+ on 5-HT_{1A} receptor affinity states may be relevant in hypertension since excess dietary Na^+ may exert its pressor effect in part by potentiating 5-HT_{1A} receptor function (Insel and Motulsky, 1984). In view of the multiple roles played by the serotonergic systems in the central and peripheral nervous systems, the results presented here could be significant in the overall regulation of receptor activity for G-protein-coupled seven-transmembrane domain receptors.

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