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Modulation of Antagonist Binding to Serotonin$_{1A}$ Receptors From Bovine Hippocampus by Metal Ions

K. G. Harikumar[1,2] and Amitabha Chattopadhyay[1,3]

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SUMMARY

1. The serotonin$_{1A}$ (5-HT$_{1A}$) receptors are members of a superfamily of seven transmembrane domain receptors that couple to G-proteins. They appear to be involved in various behavioral and cognitive functions. Although specific 5-HT$_{1A}$ agonists have been discovered more than a decade back, the development of selective 5-HT$_{1A}$ antagonists has been achieved only recently.

2. We have examined the modulation of the specific antagonist [^{3}H]*p*-MPPF binding to 5-HT$_{1A}$ receptors from bovine hippocampal membranes by monovalent and divalent metal ions. Our results show that the antagonist binding to 5-HT$_{1A}$ receptors is inhibited by both monovalent and divalent cations in a concentration-dependent manner. This is accompanied by a concomitant reduction in binding affinity.

3. Our results also show that the specific antagonist *p*-MPPF binds to all available receptors in the bovine hippocampal membrane irrespective of their state of G-protein coupling and other serotonergic ligands such as 5-HT and OH-DPAT effectively compete with the specific antagonist [^{3}H]*p*-MPPF.

4. These results are relevant to ongoing analyses of the overall modulation of ligand binding in G-protein-coupled seven transmembrane domain receptors.

KEY WORDS: 5-HT$_{1A}$ receptors; *p*-MPPI; *p*-MPPF; metal ions; Scatchard analysis; bovine hippocampus.

INTRODUCTION

The neurotransmitter serotonin (5-hydroxytryptamine or 5-HT) exerts its diverse actions by binding to distinct cell surface receptors, which have been classified into many groups (Peroutka, 1993). Serotonin receptors are members of a superfamily of seven transmembrane domain G-protein-coupled receptors (Strader *et al.*, 1995). The natural endogenous ligand serotonin is an intrinsically fluorescent (Chattopadhyay *et al.*, 1996), biogenic amine and is found in a wide variety of sites in the central and peripheral nervous systems (Jacobs and Azmitia, 1992). Serotonergic signaling

[1] Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India.
[2] Present address: Center for Basic Research in Digestive Diseases, Mayo Clinic and Foundation, Rochester, Minnesota 55905.
[3] To whom correspondence should be addressed at Centre for Cellular and Molecular Biology, Hyderabad 500 007, India; e-mail: amit@ccmb.ap.nic.in.

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appears to play an important role in the generation and modulation of various cognitive and behavioral functions such as sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression, and learning (Artigas *et al*., 1996; Casadio *et al*., 1999; Ramboz *et al*., 1998; Rocha *et al*., 1998). 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 (Heisler *et al*., 1998; Ramboz *et al*., 1998; Tecott *et al*., 1995).

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 (Harikumar *et al*., 2000). We have earlier partially purified and solubilized the 5-HT_{1A} receptor from bovine hippocampus in a functionally active form (Chattopadhyay and Harikumar, 1996) and have shown modulation of ligand binding by alcohols (Harikumar and Chattopadhyay, 1998b, 2000), guanine nucleotide (Harikumar and Chattopadhyay, 1999), and chemical modifications (Harikumar *et al*., 2000).

Metal ion modulation of ligand binding is a characteristic feature of G-protein-coupled seven transmembrane domain receptors. We have previously examined the metal ion sensitivity of agonist binding to the 5-HT_{1A} receptor from bovine hippocampus (Harikumar and Chattopadhyay, 1998a). Our results showed that the interaction of metal ions with the 5-HT_{1A} receptor is characterized by modulation of agonist binding accompanied by an altered binding affinity and sites. However, such an analysis for antagonist binding is lacking mainly due to the nonavailability of suitable 5-HT_{1A} antagonists. Although selective 5-HT_{1A} agonists such as OH-DPAT have been discovered more than a decade back (Gozlan *et al*., 1983), the development of selective 5-HT_{1A} antagonists has been relatively slow and less successful. Recently, two selective antagonists for the 5-HT_{1A} receptor, *p*-MPPI and *p*-MPPF, have been introduced (Allen *et al*., 1997; Kung *et al*., 1994a,b, 1995; Thielen and Frazer, 1995; Zhuang *et al*., 1995). These compounds bind specifically to the 5-HT_{1A} receptor with high affinity. It is interesting to note here that findings from a variety of animal model studies suggest that selective 5-HT_{1A} receptor antagonists such as *p*-MPPI may have therapeutic effects in anxiety or stress-related disorders (Griebel, 1999; Ramboz *et al*., 1998). We have earlier shown that while the agonist OH-DPAT binding to the 5-HT_{1A} receptor is sensitive to guanine nucleotides (Harikumar and Chattopadhyay, 1998a,b, 1999), the antagonist (*p*-MPPF) binding is found to be insensitive to guanine nucleotides (Harikumar and Chattopadhyay, 1999). This could be due to the binding of the agonist only to the population of receptors which are coupled to G-proteins while the antagonist binds to all receptors irrespective of their state of G-protein coupling.

In this report, we have examined the modulation of the specific antagonist ([^{3}H]*p*-MPPF) binding to 5-HT_{1A} receptors from bovine hippocampal membranes by monovalent and divalent cations. To gain further insight into the mechanism of antagonist binding to the bovine hippocampal 5-HT_{1A} receptor under these conditions, we have analyzed the binding affinities and binding sites by Scatchard analysis of saturation binding data. Our results show that the antagonist binding to 5-HT_{1A} receptors is inhibited by both monovalent and divalent cations in a concentration-dependent

manner. In addition, we show here that serotonergic ligands such as 5-HT and OH-DPAT effectively compete with the specific antagonist *p*-MPPF.

MATERIALS AND METHODS

Materials

EDTA, EGTA, KCl, $MgCl_2$, $MnCl_2$, NaCl, PMSF, Tris, iodoacetamide, polyethylenimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]OH-DPAT (specific activity 127.0 Ci/mmol) and [^{3}H]*p*-MPPF (specific activity 78.3 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). The unlabeled agonist OH-DPAT and antagonist *p*-MPPI were from Research Biochemicals International (Natick, MA). All other chemicals used were of the highest available quality. GF/B glass microfiber filters were from Whatman International (Kent, U.K.). 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 Native Hippocampal Membranes

Native hippocampal membranes were prepared as described earlier (Harikumar and Chattopadhyay, 1998a). Bovine hippocampal tissue ($\sim$100 g) was homogenized as 10% (wt/vol) in 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, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at $900 \times g$ 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 $50{,}000 \times g$ for 20 min at 4°C. The resulting pellet was suspended in 10 vol 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 $50{,}000 \times g$ for 20 min at 4°C. This procedure was repeated till the supernatant was clear. The final pellet was resuspended in minimum volume of buffer C (50 mM Tris, pH 7.4), homogenized using a Dounce homogenizer, flash frozen in liquid nitrogen and stored at -70°C for radioligand binding assays.

Radioligand Binding Assays

Antagonist binding assays were performed with varying concentrations of metal ions as follows: Tubes in duplicate containing 1 mg of total protein were incubated for 1 h at room temperature with 0.5 nM [^{3}H]*p*-MPPF (specific activity 78.3 Ci/mmol) in a total volume of 1 mL of buffer D (50 mM Tris, 1 mM EDTA, pH 7.4). Nonspecific binding was determined by performing the assay in the presence of 10 μM unlabeled *p*-MPPI. 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 h (Bruns *et al.*, 1983). The filters were then washed 3 times with 3 mL of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 mL of scintillation fluid.

Agonist binding assays were performed as above, using [^{3}H]OH-DPAT (specific activity 127.0 Ci/mmol) as the radioligand. The assay tubes contained 0.29 nM [^{3}H]OH-DPAT in a total volume of 1 ml of buffer E (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4). Nonspecific binding was determined by performing the assay in the presence of 10 μM unlabeled serotonin.

Saturation Binding Assays

Saturation binding assays were carried out using varying concentrations (0.1–7.5 nM) of radiolabeled antagonist [^{3}H]*p*-MPPF using native membranes containing 1 mg of total protein. Nonspecific binding was measured in the presence of 10 μM unlabeled *p*-MPPI. Binding assays were carried out at room temperature as mentioned above. The specific metal ion concentrations at which the assays were done were the respective half maximal inhibition concentrations (IC_{50} values) taken from

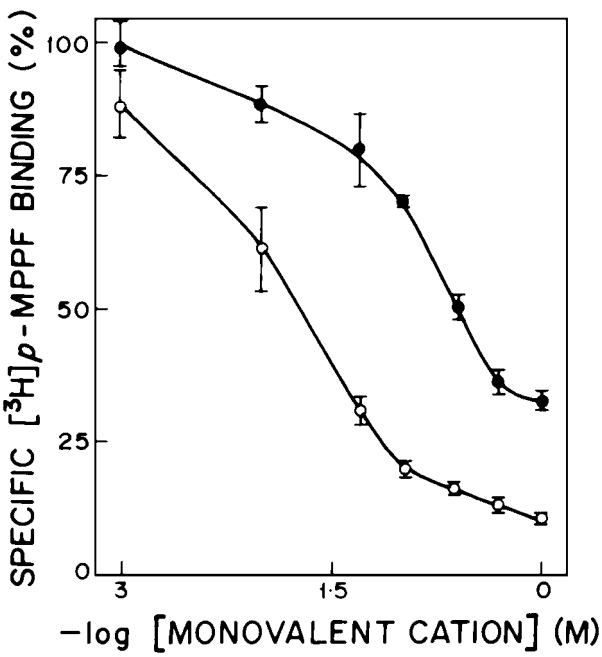

Fig. 1. Effect of monovalent cations Na^+ (○) and K^+ (●) on [^{3}H]*p*-MPPF specific binding to the 5-HT_{1A} receptor from bovine hippocampal membranes. The concentration of [^{3}H]*p*-MPPF used was 0.5 nM. Values are expressed as a percentage of the specific binding obtained in the absence of any monovalent cations. The data points are the means ± SE of duplicate points from three independent experiments (see Materials and Methods for other details).

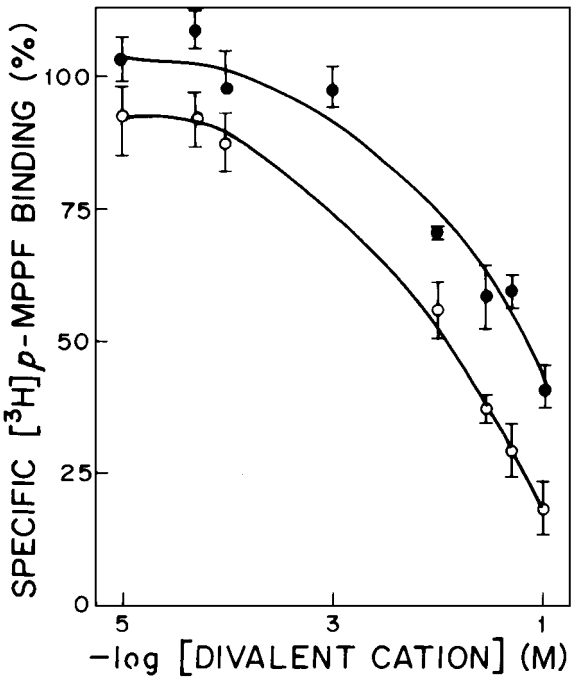

Fig. 2. Effect of divalent cations Mg^{2+} (○) and Mn^{2+} (●) on [^{3}H]*p*-MPPF specific binding to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. The concentration of [^{3}H]*p*-MPPF used was 0.5 nM. Values are expressed as a percentage of the specific binding obtained in the absence of any divalent cations. The data points are the means ± SE of duplicate points from three independent experiments (see Materials and Methods for other details).

Figs. 1 and 2. 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 disintegrations per minute (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. 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 ($r = 0.90$–0.99). The B_{max} values were obtained from the intercept on the abscissa. The B_{max} values reported in Table II have been normalized with respect to the amount of native membrane used. The binding parameters shown in Table II were obtained by averaging the results of three independent experiments while saturation binding data shown in Fig. 3 are from representative experiments. Protein concentration was determined using BCA reagent (Smith *et al.*, 1985).

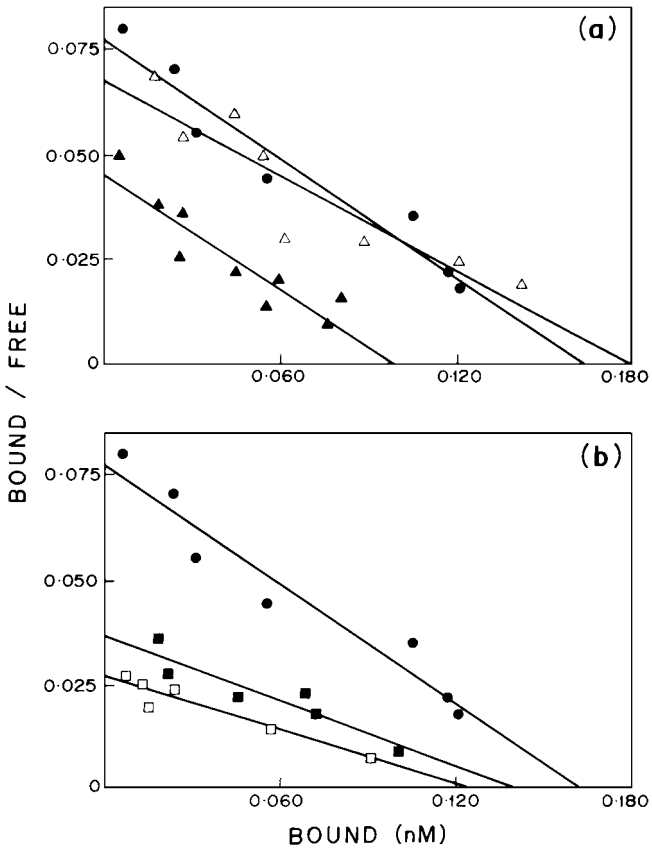

Fig. 3. Scatchard analysis of specific binding of [^{3}H]*p*-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes (a) in the presence of monovalent cations Na^+ (△), K^+ (▲) and (b) in the presence of divalent cations Mg^{2+} (□), Mn^{2+} (■). The data for control membranes (i.e. native membranes without any added metal ions) are also shown in both panels for comparison (●). The concentration of [^{3}H]*p*-MPPF ranged from 0.1–7.5 nM. The concentrations of metal ions used were 20 and 125 mM in case of Na^+ and K^+ respectively, and 10 mM each for Mg^{2+} and 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).

Competition Binding Assays

The competition binding assays were carried out as follows. Tubes in duplicate containing 1 mg of membrane protein were incubated in presence of 0.5 nM [^{3}H]*p*-MPPF in a total volume of 1 ml of buffer D. Nonspecific binding was determined by performing the assay in the presence of 10^{-4} M of unlabeled *p*-MPPI. The final concentrations of the competitive ligands (unlabeled OH-DPAT and serotonin) in the assay tubes ranged from 10^{-12} to 10^{-4} M. The radioligand binding assay was carried out at room temperature for 1 h as mentioned above. The affinity values

of the displacing ligands are expressed as the apparent dissociation constants (K_i) for the competing ligands, where K_i is calculated from IC_{50} (concentration of ligand which inhibit 50% of binding) value using the equation (Cheng and Prusoff, 1973):

$$K_i = IC_{50}/(1 + [L]/K_d)\,M$$

where IC_{50} is the concentration of the competing ligand leading to 50% inhibition of specific binding and [L] and K_d are the concentration and dissociation constant of the labeled ligand. K_d value for [^{3}H]*p*-MPPF binding to the 5-HT$_{1A}$ receptor (1.95 nM) was taken from Table II. The average of the K_i values for the competitive ligands are shown in Table III.

RESULTS

Table I shows that the specific activity obtained using the agonist [^{3}H]OH-DPAT is 71.4 fmol/mg protein while that obtained using the antagonist [^{3}H]*p*-MPPF is 113.9 fmol/mg protein. There is thus ~60% increase in specific activity when the antagonist [^{3}H]*p*-MPPF is used. This 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 (Harikumar and Chattopadhyay, 1999), the antagonist [^{3}H]*p*-MPPF binds to both G-protein-coupled and free receptor giving rise to 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. Figure 1 shows the concentration-dependent inhibition of specific [^{3}H]*p*-MPPF binding to 5-HT$_{1A}$ receptors by monovalent ions Na^+ and K^+ in bovine hippocampal native membranes with the potency of inhibition being greater for Na^+ than K^+. This is apparent from the respective IC_{50} values of 20 and 125 mM obtained for inhibition in antagonist binding by Na^+ and K^+ respectively (see Fig. 1). Figure 2 shows the inhibition of specific [^{3}H]*p*-MPPF binding to hippocampal 5-HT$_{1A}$ receptors by increasing concentrations of divalent ions Mg^{2+} and Mn^{2+}. As was observed in the case of monovalent cations, the extent of inhibition in antagonist binding is concentration-dependent and increases at higher concentrations of the metal ions with an IC_{50} value of 10 mM in both cases.

The above results indicate that the monovalent and divalent metal ions induce an affinity transition of the receptor from a high affinity to a low affinity state. Figure 3 shows the Scatchard analysis of the specific binding of [^{3}H]*p*-MPPF to the bovine hippocampal 5-HT$_{1A}$ receptor in the presence of monovalent and divalent cations at their respective IC_{50} values. The binding parameters under these conditions are

Table I. 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)	71.4 ± 4.5
[^{3}H]*p*-MPPF (antagonist)	113.9 ± 3.8

[a] See Materials and Methods for other details.
[b] Data reported are mean ± SE of four independent experiments.

Table II. Affinity and B_{max} Values of [^{3}H]*p*-MPPF Binding to 5-HT$_{1A}$ Receptors From Bovine Hippocampal Membranes[a]

Metal ion	K_d (nM)	B_{max} (fmol/mg of protein)
None (native membrane)	1.95 ± 0.17	135.2 ± 4.54
Na^+	3.04 ± 0.19	137.6 ± 35.60
K^+	2.25 ± 0.10	70.2 ± 11.00
Mg^{2+}	5.16 ± 0.33	127.6 ± 27.40
Mn^{2+}	3.48 ± 0.19	116.9 ± 4.06

[a]The specific metal ion concentrations at which the assays were done were the respective half maximal inhibition concentrations (IC_{50} values in mM: Na^+, 20; K^+, 125; Mg^{2+}, 10; and Mn^{2+}, 10) taken from Figs. 1 and 2. The binding parameters shown in this table represent the means ± SE of duplicate points from three independent experiments while saturation binding data shown in Fig. 3 are from representative experiments. See Materials and Methods for other details.

summarized in Table II. As can be seen from the table, the binding affinity of [^{3}H]*p*-MPPF to the 5-HT$_{1A}$ receptor shows a considerable reduction in the presence of both monovalent and divalent cations confirming that the receptor is in a low affinity state especially at high metal ion concentrations. These results are in agreement with Figs. 1 and 2 which show that at high metal ion concentrations, the low affinity form of the receptor predominates. The accompanying changes in the number of binding sites are not significant except in the case of K^+ where there is ~48% decrease in number of binding sites (see Table II). The reason for such a large reduction in the number of binding sites in case of K^+ is not clear.

Competition binding analysis of serotonergic ligands to the bovine hippocampal 5-HT$_{1A}$ receptors was performed by determining their ability to compete for binding to the receptor labeled with the radiolabeled 5-HT$_{1A}$ receptor specific antagonist [^{3}H]*p*-MPPF. The displacement curves of [^{3}H]*p*-MPPF by the competing ligands serotonin and OH-DPAT are shown in Fig. 4. Figure 4 shows that the these serotonergic ligands competitively displace the labeled antagonist [^{3}H]*p*-MPPF and exhibit typical displacement patterns. The half maximal inhibition concentrations (IC_{50}) and K_i values for the competing ligands are reported in Table III. While both the ligands,

Table III. IC_{50} and K_i Values of Serotonergic Ligands for Binding to 5-HT$_{1A}$ Receptors From Bovine Hippocampal Membranes[a]

Ligands	IC_{50} (nM)	K_i (nM)
5-HT	63.92 ± 5.90	50.87 ± 0.36
OH-DPAT	27.29 ± 2.18	21.72 ± 1.74

[a]The IC_{50} values and the apparent dissociation constants (K_i) shown in the table represent the means ± SEM of duplicate points from three independent experiments. The apparent dissociation constants (K_i) for the competing ligands were calculated by using the following relation: $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the concentration of the competing ligand leading to 50% inhibition of specific total binding and [L] and K_d are the concentration and dissociation constant of the labeled ligand (see Hulme, 1990). See Materials and Methods for other details.

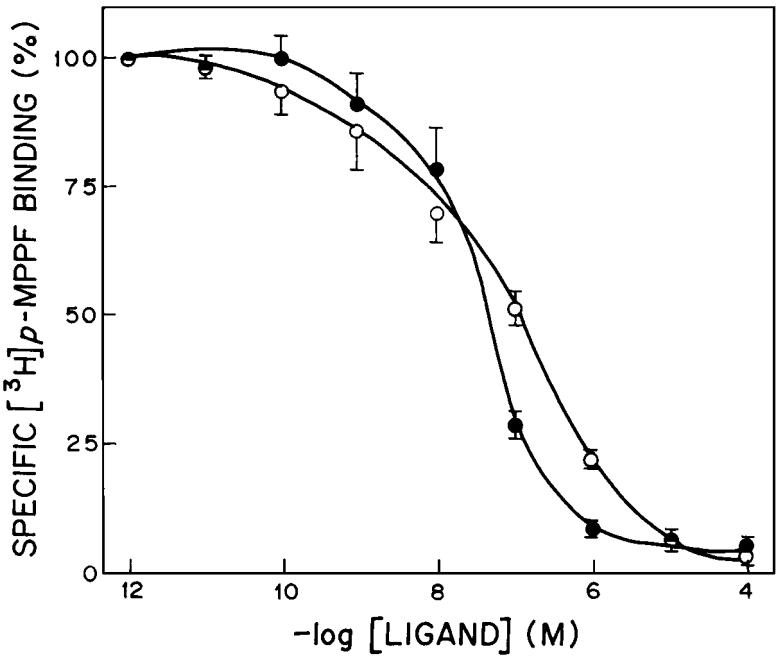

Fig. 4. Competition binding analysis of serotonergic ligands to the 5-HT$_{1A}$ receptor from bovine hippocampal membranes. The concentration range used was from 10^{-12} to 10^{-4} M for serotonin (○) and OH-DPAT (●). Values of specific binding measured in the presence of the competing ligands are expressed as a percentage of total binding. The data points are the means ± SE of duplicate points from three independent experiments. Nonspecific binding was determined in the presence of 10^{-4} M unlabeled *p*-MPPI. K_i values were determined according to Cheng and Prusoff (1973) and average values are listed in Table III (see Materials and Methods for other details).

serotonin and OH-DPAT, show similar displacement patterns, OH-DPAT appears to be more effective in competing with bound [^{3}H]*p*-MPPF, as judged by its IC_{50} and K_i values (see Table III).

DISCUSSION

Metal ion modulation of ligand binding has proved to be a characteristic feature of G-protein-coupled seven transmembrane domain receptors (Yabaluri and Medzihradsky, 1997). We have previously examined the metal ion sensitivity of agonist binding to the 5-HT$_{1A}$ receptor from bovine hippocampus (Harikumar and Chattopadhyay, 1998a). Our results show that the interaction of monovalent cations with 5-HT$_{1A}$ receptors is characterized by concentration-dependent inhibition of agonist binding, an altered agonist binding affinity and a reduction in 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 presence of GTP-γ-S, a nonhydrolyzable analogue of GTP, indicating that these receptors are coupled to G-proteins. Such an analysis for antagonist binding to the 5-HT_{1A} receptor is lacking mainly due to the non-availability of suitable 5-HT_{1A} antagonists till a few years back (Allen *et al*., 1997; Kung *et al*., 1994a,b, 1995; Thielen and Frazer, 1995; Zhuang *et al*., 1995). Our present results show that the specific antagonist *p*-MPPF binds to all available receptors irrespective of their state of G-protein coupling. More importantly, the antagonist binding to 5-HT_{1A} receptors is inhibited by both monovalent and divalent cations in a concentration-dependent manner. This is accompanied by a concomitant reduction in binding affinity. We also show that serotonergic ligands such as 5-HT and OH-DPAT effectively compete with the specific antagonist [^{3}H]*p*-MPPF in bovine hippocampal membranes.

We have previously postulated, based on ethanol effects on the agonist and antagonist binding, that the antagonist binding site is more polar in nature than the agonist binding site and may even have an interfacial location, that is, at a shallow location in the membrane compared to the agonist site (Harikumar and Chattopadhyay, 1998b). This was also apparent from the pattern of inhibition to specific antagonist binding to the 5-HT_{1A} receptor following treatment with DTT and NEM which covalently modify the disulfides and the sulfhydryl groups of native and solubilized membranes (Harikumar *et al*., 2000). Keeping in mind these observations, we have earlier postulated that the agonist and antagonist binding sites may be overlapping but not identical in the bovine hippocampal 5-HT_{1A} receptor (Harikumar *et al*., 2000; Harikumar and Chattopadhyay, 1998b, 2000). It should be noted that different binding domains for agonists and antagonists have earlier been shown for kappa opioid receptor (Kong *et al*., 1994). In the light of this, the mechanism of inhibition by metal ions could be different for agonists and antagonists.

The 5-HT_{1A} receptors are known to exist in different conformational states with varying affinity (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 could be favored by antagonists and metal ions. In other words, according to this model (termed the reciprocal model), there exists at least two interconvertable forms of the receptor, which are reciprocally favored by agonists and antagonists (Schütz and Freissmuth, 1992). It is important to note here that we have earlier reported that antagonist binding to the 5-HT_{1A} receptor is insensitive to guanine nucleotides (Harikumar and Chattopadhyay, 1999).

In summary, our results show that the binding of the specific antagonist *p*-MPPF to bovine hippocampal 5-HT_{1A} receptors is inhibited by both monovalent and divalent cations in a concentration-dependent manner. This is accompanied by a concomitant reduction in binding affinity. The antagonist binding activity in hippocampal 5-HT_{1A} receptors is therefore very well-regulated by the ionic environment. Multiple affinity states of the 5-HT_{1A} receptor induced by metal ions could be physiologically

significant. For example, 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). These results are relevant to ongoing analyses of the overall regulation of ligand binding and receptor activity in G-protein-coupled seven transmembrane domain receptors.

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