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# Role of Disulfides and Sulfhydryl Groups in Agonist and Antagonist Binding in Serotonin$_{1A}$ Receptors from Bovine Hippocampus

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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. Mutagenesis and modeling studies point out that the ligand-binding sites in serotonin receptors are located in the transmembrane domain. However, these binding sites are not very well characterized. Since disulfide bonds and sulfhydryl groups have been shown to play vital roles in the assembly, organization, and function of various G-protein-coupled receptors, we report here the effect of disulfide and sulfhydryl group modifications on the agonist and antagonist binding activity of 5-HT$_{1A}$ receptors from bovine hippocampus.

2. DTT or NEM treatment caused a concentration-dependent reduction in specific binding of the agonist and antagonist in 5-HT$_{1A}$ receptors from bovine hippocampal native and solubilized membranes. This is supported by a concomitant reduction in binding affinity.

3. Pretreatment of the receptor with unlabeled ligands prior to chemical modifications indicate that the majority of disulfides or sulfhydryl groups that undergo modification giving rise to inhibition in binding activity could be at the vicinity of the ligand-binding sites.

4. In addition, ligand-binding studies in presence of GTP-$\gamma$-S, a nonhydrolyzable analogue of GTP, indicate that sulfhydryl groups (and disulfide bonds to a lesser extent) are vital for efficient coupling between the 5-HT$_{1A}$ receptor and the G-protein.

5. Our results point out that disulfide bonds and sulfhydryl groups could play an important role in ligand binding in 5-HT$_{1A}$ receptors.

**KEY WORDS:** 5-HT$_{1A}$ receptor; DTT; NEM; 8-OH-DPAT; MPPF; bovine hippocampus.

## INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT)[3] is an intrinsically fluorescent (Chattopadhay *et al.*, 1996), biogenic amine which acts as a neurotransmitter and is

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[3] Abbreviations: BCA, bicinchoninic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; L-DTT, L-dithiothreitol; GTP-$\gamma$-S, guanosine-5′-*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; NEM, N-ethylmaleimide; 8-OH-DPAT, 8-hydroxy-2-(di-N-propylamino)tetralin; PMSF, phenylmethylsulfonyl fluoride; *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.



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found in a wide variety of sites in the central and peripheral nervous systems (Jacobs and Azmitia, 1992). It mediates a variety of physiological responses in distinct cell types. 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, learning, and memory (Artigas *et al.*, 1996; Ramboz *et al.*, 1998; Rocha *et al.*, 1998; Casadio *et al.*, 1999). 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; Parks *et al.*, 1998; Ramboz *et al.*, 1998).

Serotonin exerts its diverse actions by binding to distinct cell-surface receptors which have been classified into many groups (Zifa and Fillion, 1992; Peroutka, 1993). Serotonin receptors are members of a superfamily of seven transmembrane domain receptors (Strader *et al.*, 1995) 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. The reasons for this include:

1. The availability of a selective ligand (8-OH-DPAT) allows extensive biochemical, physiological, and pharmacological characterization of the receptor (Gozlan *et al.*, 1983).

2. 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. The 5-$HT_{1A}$ receptors are also implicated in regulation of blood pressure, feeding, temperature regulation (Dourish *et al.*, 1987), and regulation of working memory (Ohno and Watanabe, 1996).

3. It was the first serotonin receptor 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 $\beta_2$-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).

4. 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).

We earlier partially purified and solubilized the 5-$HT_{1A}$ receptor from bovine hippocampus in a functionally active form (Chattopadhyay and Harikumar, 1996) and showed modulation of ligand binding by metal ions, guanine nucleotide, and alcohols (Harikumar and Chattopadhyay, 1998a,b, 1999, 2000). Evidence from mutagenesis (Ho *et al.*, 1992; Chanda *et al.*, 1993; Kuipers *et al.*, 1997) and molecular modeling studies (Sylte *et al.*, 1996; Bremner *et al.*, 1997; Kuipers *et al.*, 1997) have shown that the ligand-binding sites in serotonin receptors in general, and 5-$HT_{1A}$

receptors in particular, are located in the transmembrane domain. However, since the 5-HT$_{1A}$ receptor has not been purified to homogeneity and high-resolution structural data are lacking, these binding sites are not very well characterized. Disulfide bonds and sulfhydryl groups have been shown to play vital roles in the assembly, organization, and function of various G-protein-coupled receptors (Kamikubo *et al.*, 1988; Karnik *et al.*, 1988; Emerit *et al.*, 1991; Javitch *et al.*, 1994; Lin *et al.*, 1996; Nenonene *et al.*, 1996; Gaibelet *et al.*, 1997; Martini *et al.*, 1997; Brandt *et al.*, 1999). In this paper, we examine the role of disulfide bonds and sulfhydryl groups in the binding activity of agonist and antagonist to 5-HT$_{1A}$ receptors from bovine hippocampus by chemical modification of these groups.

## MATERIALS AND METHODS

### Materials

Serotonin, iodoacetamide, polyethylenimine, sucrose, sodium azide, Tris, $MgCl_2$, CHAPS, EDTA, EGTA, L-DTT, NEM, and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO). GTP-$\gamma$-S was from Boehringer Mannheim (Germany). *p*-MPPI was from Research Biochemicals International (Natick, MA) and was a kind gift from Dr. V. Bakthavachalam (National Institute of Mental Health Chemical Synthesis Program). [$^3$H]8-OH-DPAT (specific activity 127.0 Ci/mmol) and [$^3$H]*p*-MPPF (specific activity 64.6 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA). GF/B glass microfiber filters were from Whatman International (Kent, UK). 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. Stock solution of DTT was in water. Stock solution of NEM was prepared in water with traces of ethanol. The ethanol content in the assay tubes (for NEM-treated samples) was negligible (generally less than 1% v/v) and control studies showed that this did not cause any significant change in binding activity of the receptor (Harikumar and Chattopadhyay, 1998b).

### Preparation of Native Hippocampal Membranes

Native hippocampal membranes were prepared as described earlier (Harikumar and Chattopadhyay, 1998a). In short, bovine hippocampal tissue (~100g) was homogenized as 10% (w/v) 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 $\times$ *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 m*M* Tris, 1 m*M* EDTA, 0.24 m*M* PMSF, 10 m*M* 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 until the supernatant was clear. The final pellet (native 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 membrane preparation thus obtained was used either directly for receptor binding after preincubation with DTT or NEM or for solubilization.

### Solubilization of Native Membranes

Solubilization of native membranes using CHAPS was carried out as described previously (Harikumar and Chattopadhyay, 1998b). Native membranes (~2 mg/ml total protein) were incubated with 5 m*M* CHAPS either in buffer C (50 m*M* Tris, 1 m*M* EDTA, 10 m*M* $MgCl_2$, pH 7.4) for agonist-binding studies, or in buffer D (50 m*M* Tris, 1 m*M* EDTA, pH 7.4) for antagonist-binding studies for 30 min at 4°C with occasional shaking. The membranes were sonicated (5 sec) using a Branson model 250 sonifier and mildly homogenized using a hand-held Dounce homogenizer (five times) at the begining and the end of the incubation period. After incubation for 30 min, the contents were centrifuged at 100,000 × *g* for 1 hr. The clear supernatant was carefully removed from the pellet and used immediately for receptor binding after preincubation with DTT or NEM.

### Chemical Modifications and Receptor-Binding-Assays

Receptor-Binding-Assays for agonist and antagonist were carried out as described earlier (Harikumar and Chattopadhyay, 1998b). Briefly, tubes in duplicate containing 1 mg of total protein (in case of native membranes) or 500 μl of the CHAPS-solubilized membrane in a total volume of 1 ml of buffer C for agonist and buffer D for antagonist was used for the assays. The tubes were preincubated for 30 min at room temperature with various concentrations of DTT or NEM. The final concentration of DTT or NEM in the assay tubes ranged from 1 n*M* to 1 *M*. After 30 min the tubes were further incubated with the radiolabeled agonist [$^3$H]8-OH-DPAT (final concentration in the assay tube 0.29 n*M*) for 1 hr at room temperature. The modifying agents (DTT or NEM) were not added to the control samples. Nonspecific binding was determined by performing the assay in the presence 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 and dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid.

Antagonist-binding assays in presence of various concentrations of DTT or NEM were performed as above using [$^3$H]*p*-MPPF as the radioligand. The assay tubes contained 0.5 nM [$^3$H]*p*-MPPF in a total volume of 1 ml of buffer D. The modifying agents (DTT or NEM) were not added to the control samples. Nonspecific binding was determined by performing the assay in the presence of 10 μ*M* unlabeled *p*-MPPI.

In some experiments (Table III) the native membrane was pretreated with unlabeled agonist (5-HT) or antagonist (*p*-MPPI) by incubation with 1 $\mu M$ of 5-HT (in buffer C) or *p*-MPPI (in buffer D) for 30 min at room temperature. After the incubation the membrane suspension was centrifuged at 20,000 × g for 10 min at 4°C. The pellet was resuspended in the respective buffer and incubated with DTT or NEM (at their respective $IC_{50}$ values) for 30 min at room temperature and ligand-binding assays were carried out as above.

For experiments in which GTP-γ-S was used (Table IV) the ligand-binding assays were performed either in presence of GTP-γ-S alone as described earlier (Harikumar and Chattopadhyay, 1999) or after preincubation with DTT or NEM (at their respective $IC_{50}$ values) for 30 min at room temperature in presence of GTP-γ-S. Protein concentration was determined using BCA reagent (Smith *et al.*, 1985).

## Saturation Binding Assays

Saturation binding assays were carried out using varying concentrations (0.1–7.5 n*M*) of radiolabeled agonist ([$^3$H]8-OH-DPAT) or antagonist ([$^3$H]*p*-MPPF) using native membranes or chemically modified (DTT- or NEM-treated) membranes containing 1 mg of total protein. Nonspecific binding was measured in the presence of 10 $\mu M$ unlabeled 5-HT (for agonist) or *p*-MPPI (for antagonist). Binding assays were carried out at room temperature as mentioned above. The specific DTT or NEM concentration at which the assays were done was the half maximal inhibition concentration ($IC_{50}$ value) taken from Table I. Binding data were analyzed as described by Hulme (1990). The concentration of bound ligand was calculated from the equation

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

where $B$ is 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) on an IBM PC. The dissociation constants $K_d$ were obtained from the negative inverse of the slopes, determined by linear regression

**Table I.** $IC_{50}$ for Inhibiton of Specific [$^3$H]8-OH-DPAT and [$^3$H]*p*-MPPF Binding to 5-$HT_{1A}$ Receptors from Bovine Hippocampal Native and Solubilized Membrane by DTT and NEM[a]

| | $IC_{50}$ (m*M*) | |
|---|---|---|
| | DTT | NEM |
| Native membrane | | |
| [$^3$H]8-OH-DPAT | 10 ± 1.10 | 0.1 ± 0.01 |
| [$^3$H]*p*-MPPF | 20 ± 2.80 | 40 ± 0.63 |
| Solubilized membrane | | |
| [$^3$H]8-OH-DPAT | 0.82 ± 0.03 | 0.03 ± 0.002 |
| [$^3$H]*p*-MPPF | 1.20 ± 0.08 | 0.32 ± 0.042 |

[a] All values are the average of three independent experiments. See Materials and Methods for other details.

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 Figs. 5 and 6 are from representative experiments.

## RESULTS

### Inhibition of Specific Agonist-Binding Activity Following DTT and NEM Treatment of Native and Solubilized Membranes

Figure 1 shows the effect of increasing concentrations of DTT and NEM on the specific binding of the agonist [$^3$H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal native membranes. As shown in the figure, treatment with DTT or NEM results in a concentration-dependent inhibition of agonist binding. However, NEM appears to be more effective than DTT in producing the inhibition in agonist binding since more inhibition is observed at lower concentrations of NEM. This is supported by the respective half maximal inhibition concentration ($IC_{50}$) values shown in Table I. The $IC_{50}$ value obtained for NEM (0.1 m$M$) is about 100 times less than that for DTT (10 m$M$) in the native membrane. This points out that the agonist-binding site, possibly localized in the hydrophobic integral membrane portion of the receptor, is relatively easily accessed by NEM, which would partition better into the membrane because of its relatively nonpolar nature compared to DTT (also see below).

Figure 2 shows the concentration-dependent inhibition of specific [$^3$H]8-OH-DPAT binding in hippocampal membranes solubilized by the zwitterionic detergent CHAPS. Interestingly, while both DTT and NEM treatment show inhibition in specific [$^3$H]8-OH-DPAT binding in solubilized membranes, the concentrations of the modifying agents required to produce a given magnitude of inhibition were found to be lower than what were needed in case of the native membrane. Accord-

**Table II.** Affinity and $B_{max}$ Values of [$^3$H]8-OH-DPAT and [$^3$H]$p$-MPPF Binding to 5-HT$_{1A}$ Receptors from Bovine Hippocampal Native Membrane Following DTT and NEM Treatment[a]

| | [$^3$H]8-OH-DPAT | | [$^3$H]$p$-MPPF | |
|---|---|---|---|---|
| | $K_d$ (n$M$) | $B_{max}$ (fmol/mg of protein) | $K_d$ (n$M$) | $B_{max}$ (fmol/mg of protein) |
| Native membrane | 1.18 ± 0.19 | 108.0 ± 13.10 | 0.67 ± 0.04 | 220.3 ± 13.80 |
| DTT | 3.18 ± 0.77 | 106.0 ± 11.20 | 0.83 ± 0.06 | 162.0 ± 14.10 |
| NEM | 2.60 ± 0.26 | 89.0 ± 4.22 | 1.13 ± 0.13 | 66.0 ± 9.70 |

[a] The binding parameters represent the mean ±SE of duplicate points from three independent experiments, while saturation binding data shown in Figs. 5 and 6 are from representative experiments. The concentrations of DTT and NEM used were 10 and 0.1 m$M$ for [$^3$H]8-OH-DPAT binding and 20 and 40 m$M$ for [$^3$H]$p$-MPPF binding, which correspond to the respective $IC_{50}$ values (see Table I). See Materials and Methods for other details.

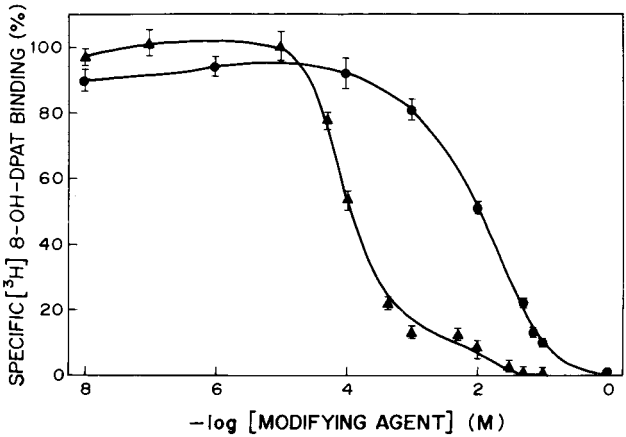


**Fig. 1.** Effect of increasing concentrations of DTT (●) and NEM (▲) on the specific binding of the agonist [$^3$H]8-OH-DPAT to the 5-HT$^{1A}$ receptor from bovine hippocampal native membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any modifying agent (DTT or NEM). The data points are the means ± SE of duplicate points from three independent experiments. See Materials and Methods for other details.

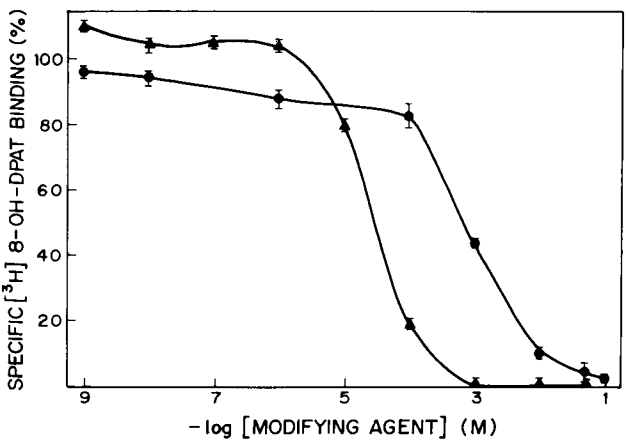


**Fig. 2.** Effect of increasing concentrations of DTT (●) and NEM (▲) on the specific binding of the agonist [$^3$H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal solubilized membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any modifying agent (DTT or NEM). The data points are the means ± SE of duplicate points from three independent experiments. See Materials and Methods for other details.

ingly, the corresponding $IC_{50}$ values (see Table I) in case of the solubilized membrane showed a marked reduction (0.82 and 0.03 m*M* for DTT and NEM, respectively). This could be due to the general difference in organization and packing pattern in the native and solubilized membranes. The lipids in the solubilized membrane are loosely packed, resulting in increased partitioning of the externally added modifying agents (DTT or NEM). In addition, the solubilized membrane has a higher water content (Harikumar and Chattopadhyay, 1998b; Rukmini, R., Shanti, K., and Chattopadhyay, A., unpublished observations) and this also helps in partitioning of relatively polar molecules such as DTT. It is perhaps for this reason that the reduction in $IC_{50}$ value obtained with the solubilized membrane (when compared to native membrane $IC_{50}$ values) in case of DTT treatment is higher (12-fold reduction) than in case of NEM treatment (3-fold).

## Inhibition of Specific Antagonist-Binding Activity Following DTT and NEM Treatment of Native and Solubilized Membranes

Although selective 5-$HT_{1A}$ agonists such as 8-OH-DPAT were discovered some time ago (Gozlan *et al.*, 1983), the development of selective 5-$HT_{1A}$ antagonists have been relatively slow and less successful. Recently two specific antagonists for the 5-$HT_{1A}$ receptor, *p*-MPPI and *p*-MPPF, have been introduced (H. F. Kung *et al.*, 1994; M.-P. Kung *et al.*, 1994, 1995; Thielen and Frazer, 1995). These compounds bind specifically to 5-$HT_{1A}$ receptor with high affinity. The effect of increasing concentrations of DTT and NEM on the specific binding of the antagonist [$^3$H]*p*-MPPF to the 5-$HT_{1A}$ receptor in native hippocampal membranes is shown in Fig. 3. DTT or NEM treatment results in a concentration-dependent inhibition of antagonist binding similar to the inhibition in agonist binding described above. However, a major difference observed in the case of inhibition of specific [$^3$H]*p*-MPPF binding is that both DTT and NEM appear to have comparable efficiency in inhibiting antagonist binding. This is evident from the respective $IC_{50}$ values (see Table I), which differ only by a factor of two. This supports our earlier observation (Harikumar and Chattopadhyay, 1998b) of overlapping, but not identical binding sites for agonist and antagonist in the 5-$HT_{1A}$ receptor. We 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, i.e., at a shallow location in the membrane compared to the agonist site. This is consistent with our present observation (see Fig. 1 and Table I) of a large difference in $IC_{50}$ values for DTT and NEM treatment for agonist inhibition in the native membrane. It should be noted here that different binding domains for agonists and antagonists were shown for the kappa opioid receptor (Kong *et al.*, 1994).

The concentration-dependent inhibition of specific [$^3$H]*p*-MPPF binding in hippocampal CHAPS-solubilized membranes is shown in Fig. 4. The $IC_{50}$ values in the case of antagonist binding also (see Table I) showed a marked reduction (1.20 and 0.32 m*M* for DTT and NEM, respectively) probably because of altered packing of lipids in solubilized membranes giving rise to increased accessibility of the modifying agents to the binding site.

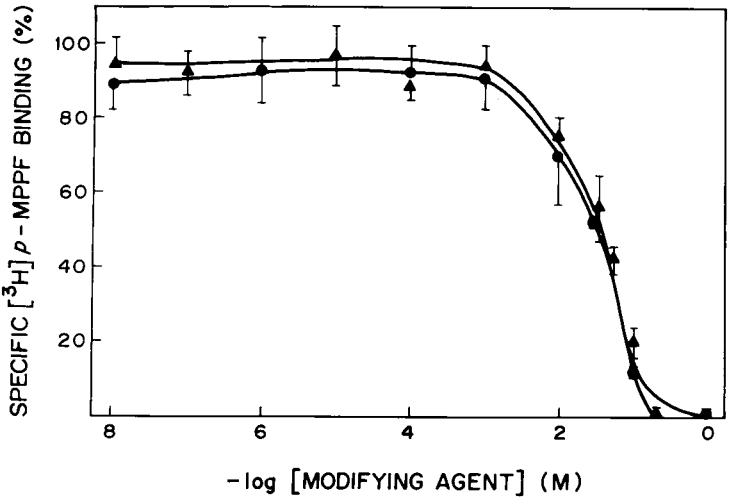


**Fig. 3.** Effect of increasing concentrations of DTT (●) and NEM (▲) on the specific binding of the antagonist [$^3$H]$p$-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal native membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any modifying agent (DTT or NEM). The data points are the means ± SE of duplicate points from three independent experiments. See Materials and Methods for other details.

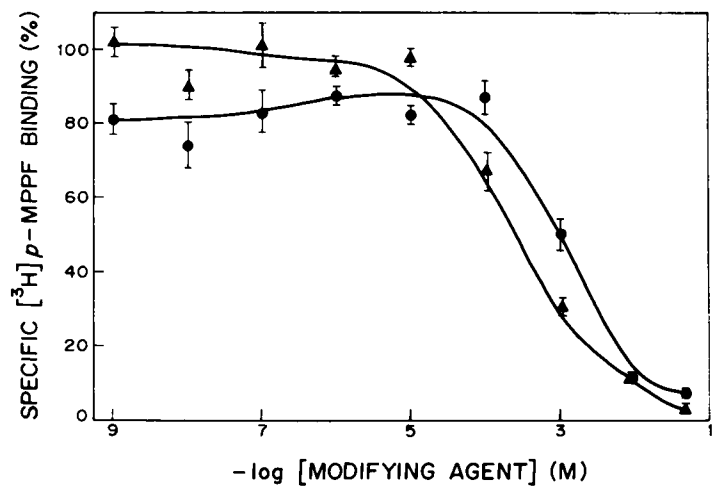


**Fig. 4.** Effect of increasing concentrations of DTT (●) and NEM (▲) on the specific binding of the antagonist [$^3$H]$p$-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal solubilized membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any modifying agent (DTT or NEM). The data points are the means ± SE of duplicate points from three independent experiments. See Materials and Methods for other details.

## Changes in Binding Affinity and Sites upon DTT and NEM Treatment by Saturation Binding Analysis

Figures 5 and 6 show Scatchard analyses of the specific binding of [$^3$H]8-OH-DPAT and [$^3$H]*p*-MPPF to the 5-HT$_{1A}$ receptors in bovine hippocampal membranes following DTT and NEM treatment. The binding parameters under these conditions are summarized in Table II. The specific binding affinity of [$^3$H]8-OH-DPAT shows considerable reduction upon chemical modification by DTT or NEM. This change in binding affinity supports the inhibition in agonist binding observed upon DTT or NEM treatment (Fig. 1). However, the accompanying reduction in the number of maximum binding sites $B_{max}$ is rather small. Table II also shows a reduction in binding affinity of [$^3$H]*p*-MPPF when the native membrane was treated with DTT or NEM, which is in agreement with Fig. 3. Interestingly, there is a concomitant decrease in the number of maximum binding sites $B_{max}$ in this case.

## Effect of Pretreatment with Unlabeled Ligands Prior to Chemical Modifications on Specific Agonist- and Antagonist-Binding Activity

The inhibition in ligand binding due to chemical modifications of the receptor could have its origin in the chemical modification of the disulfides or sulfhydryl groups in the vicinity of the ligand-binding sites which would alter the conformation and hence binding of the receptor. Alternatively, DTT and NEM could affect the disulfides or sulfhydryl groups in other regions of the protein (not close to the

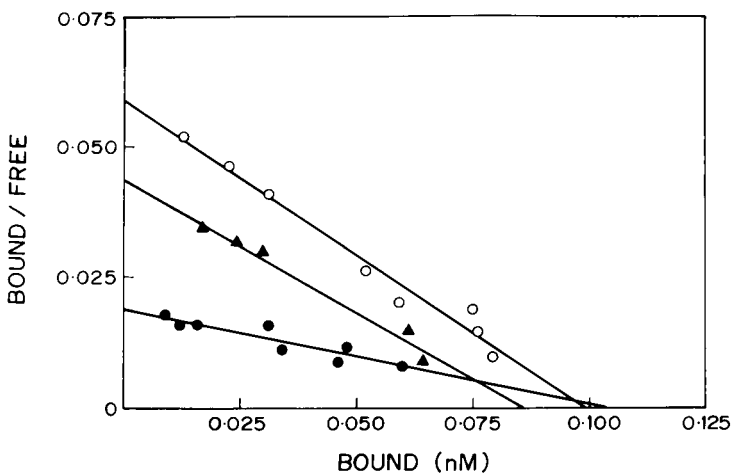


**Fig. 5.** Scatchard analysis of specific binding of the agonist [$^3$H]8-OH-DPAT to the 5-HT$_{1A}$ receptor from bovine hippocampal native membranes (○) and in DTT-treated (●) and NEM-treated (▲) membranes. The concentrations of DTT and NEM used were 10 and 0.1 m*M*, which correspond to the respective IC$_{50}$ values (see Table I). Data shown are from a representative experiment and each point is the mean of duplicate determinations. The concentration of [$^3$H]8-OH-DPAT ranged from 0.1 to 7.5 n*M*. See Materials and Methods for other details.

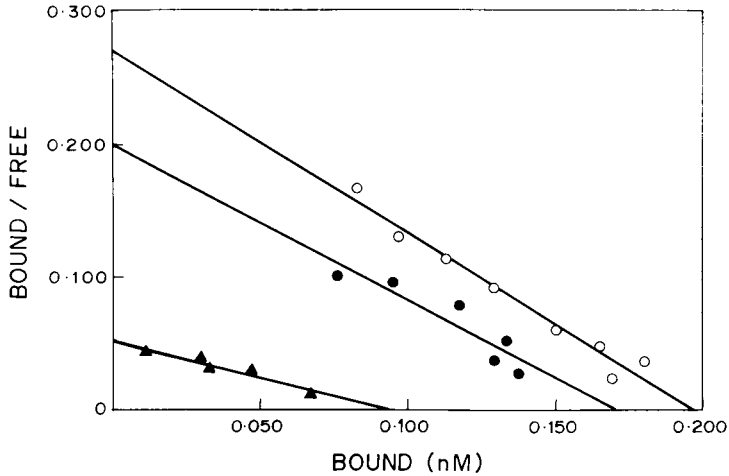


**Fig. 6.** Scatchard analysis of specific binding of the antagonist [$^3$H]*p*-MPPF to the 5-HT$_{1A}$ receptor from bovine hippocampal native membranes (○) and in DTT-treated (●) and NEM-treated (▲) membranes. The concentrations of DTT and NEM used were 20 and 40 m*M*, which correspond to the respective IC$_{50}$ values (see Table I). Data shown are from a representative experiment and each point is the mean of duplicate determinations. The concentration of [$^3$H]*p*-MPPF ranged from 0.1 to 7.5 n*M*. See Materials and Methods for other details.

binding sites), which in turn could induce a conformation that inhibits ligand binding. In order to distinguish between these two possible mechanisms, we preincubated the native membrane containing the 5-HT$_{1A}$ receptor with excess unlabeled ligand (serotonin or *p*-MPPI) before DTT or NEM treatment. The concentrations of the modifying agents were such that in the absence of any preincubation with unlabeled ligand, 50% of specific binding (normalized to the control native membrane arbitrarily taken as 100%) would have been expected since IC$_{50}$ values of the modifying agents were used (see Table I). Table III shows that in all cases the specific binding obtained was higher than 50%, implying that the presence of the unlabeled ligand at the binding site protected the inhibition of ligand binding to a considerable extent. While this indicates that the majority of disulfides or sulfhydryl groups that undergo modification giving rise to inhibition in binding activity could be at the vicinity of the ligand-binding sites, the involvement of disulfides or sulfhydryl groups from other regions of the receptor cannot be ruled out (see Discussion).

## Effect of GTP-$\gamma$-S Addition Following Chemical Modifications on Specific Agonist- and Antagonist-Binding Activity

Since most seven-transmembrane-domain receptors are coupled to G-proteins (Clapham, 1996), guanine nucleotides are known to regulate agonist binding. The 5-HT$_{1A}$ receptor is negatively coupled to the adenylate cyclase system through G-proteins (Emerit *et al.*, 1990). We reported (Harikumar and Chattopadhyay, 1999)

**Table III.** Specific Binding of [$^3$H]8-OH-DPAT and [$^3$H]*p*-MPPF to 5-HT$_{1A}$ Receptors from Bovine Hippocampal Native Membrane Preincubated with 5-HT and *p*-MPPI Followed by DTT and NEM Treatment[a]

| Modifying agent | Specific binding (%) |
|---|---|
| Control (native membrane) | 100 |
| Control + 5-HT + DTT | 84 ± 0.6 |
| Control + 5-HT + NEM | 67 ± 1.8 |
| Control + *p*-MPPI + DTT | 78 ± 4.4 |
| Control + *p*-MPPI + NEM | 88 ± 1.8 |

[a] The binding parameters represent the mean ± SE of duplicate points from three independent experiments. The concentration of 5-HT or *p*-MPPI used was 1 $\mu M$ in all cases. The concentrations of DTT and NEM used were 10 and 0.1 m*M* for [$^3$H]8-OH-DPAT binding, and 20 and 40 m*M* for [$^3$H]*p*-MPPF binding, which correspond to the respective $IC_{50}$ values (see Table I). See Materials and Methods for other details.

that agonist binding to the 5-HT$_{1A}$ receptor in bovine hippocampal native membranes is sensitive to guanine nucleotides, while antagonist binding to the 5-HT$_{1A}$ receptor was found to be insensitive to guanine nucleotides. This was monitored by studying the effect of GTP-$\gamma$-S, a nonhydrolyzable analogue of GTP, on agonist and antagonist binding to the receptor. Our results showed that the specific binding of the agonist was inhibited with increasing concentrations of GTP-$\gamma$-S and antagonist binding was unaltered and remained invariant over a large range of GTP-$\gamma$-S concentrations. In order to investigate the effect of chemical modifications on G-protein-coupling in 5-HT$_{1A}$ receptors, we checked whether there was any change in specific binding of [$^3$H]8-OH-DPAT and [$^3$H]*p*-MPPF to 5-HT$_{1A}$ receptors in presence of GTP-$\gamma$-S following treatment with DTT or NEM. The results of these experiments are shown in Table IV. The specific [$^3$H]8-OH-DPAT binding shows

**Table IV.** Specific Binding of [$^3$H]8-OH-DPAT and [$^3$H]*p*-MPPF to 5-HT$_{1A}$ Receptors in Presence of GTP-$\gamma$-S from Bovine Hippocampal Native Membrane Following DTT and NEM Treatment[a]

| | Specific binding (%) | |
|---|---|---|
| Contition | [$^3$H]8-OH-DPAT | [$^3$H]*p*-MPPF |
| GTP-$\gamma$-S (55 n*M*) | 48.0 ± 0.6 | 98.7 ± 5.1 |
| DTT | 49.7 ± 1.1 | 43.7 ± 0.8 |
| DTT + GTP-V-S | 20.1 ±2.0 | 45.8 ± 1.1 |
| NEM | 49.3 ± 4.1 | 45.7 ± 1.0 |
| NEM + GTP-$\gamma$-S | 12.8 ± 2.2 | 38.7 ± 3.2 |

[a] The binding parameters represent the mean ± SE of duplicate points from four independent experiments. The concentrations of DTT and NEM used were 10 and 0.1 m*M* for [$^3$H]8-OH-DPAT binding and 20 and 40 m*M* for [$^3$H]*p*-MPPF binding, which correspond to the respective $IC_{50}$ values (see Table I). The concentration of GTP-$\gamma$-S used was 55 n*M*, which corresponds to ~50% inhibition in [$^3$H]8-OH-DPAT binding (Harikumar and Chattopadhyay, 1999). See Materials and Methods for other details.

further reduction in presence of GTP-$\gamma$-S following chemical modifcations (DTT or NEM treatment). This indicates that sulfhydryl groups (and disulfide bonds to a lesser extent since the reduction in activity is less with DTT treatment) are important for efficient coupling between the receptor and the G-protein as shown for the $\alpha_2$-adrenoceptors (Kitamura and Nomura, 1987). Table IV also shows that in accordance with our earlier results (Harikumar and Chattopadhyay, 1999), specific [$^3$H]*p*-MPPF binding was not altered in the presence ofGTP-$\gamma$-S and there was no significant decrease in binding in the presence of GTP-$\gamma$-S following DTT or NEM treatment.

## DISCUSSION

The overall goal of this paper has been to evaluate the role of disulfide and sulfhydryl groups in ligand binding in 5-HT$_{1A}$ receptors from bovine hippocampus by chemical modification of these groups. As mentioned earlier, disulfide bonds and sulfhydryl groups have been shown to play vital roles in the assembly, organization, and function of various G-protein-coupled receptors (Kamikubo *et al.*, 1988; Karnik *et al.*, 1988; Emerit *et al.*, 1991; Javitch *et al.*, 1994; Lin *et al.*, 1996; Nenonene *et al.*, 1996; Gaibelet *et al.*, 1997; Martini *et al.*, 1997; Brandt *et al.*, 1999). Our results show concentration-dependent inhibition of agonist and antagonist binding to 5-HT$_{1A}$ receptors when the membrane was treated with DTT or NEM either in its native state or when solubilized using the zwitterionic detergent CHAPS. This inhibition in ligand binding is supported by a reduction in binding affinity. Further, experiments in which the receptor was pretreated with the unlabeled ligand prior to chemical modifications indicate that while the majority of disulfides or sulfhydryl groups that undergo modification giving rise to inhibition in binding activity could be at the vicinity of the ligand-binding sites, the involvement of disulfides or sulfhydryl groups from other regions of the receptor cannot be ruled out. In addition, ligand-binding studies in the presence of GTP-$\gamma$-S, a nonhydrolyzable analogue of GTP, indicate that sulfhydryl groups (and disulfide bonds to a lesser extent) are vital for efficient coupling between the 5-HT$_{1A}$ receptor and the G-protein.

It has been reported recently that the ligand 8-OH-DPAT also binds to 5-HT$_7$ receptors in addition to binding to the 5-HT$_{1A}$ receptor (Vanhoenacker *et al.*, 2000). However, the specificity of this interaction is relatively modest and the affinity is found to be almost two orders of magnitude lower than what is known for the 5-HT$_{1A}$ receptor (Ruat *et al.*, 1993). In addition, analysis of the distribution pattern of the 5-HT$_7$ receptor in the brain show that the receptor is mainly localized in the hypothalamus, thalamus, and cortical regions (Vanhoenacker *et al.*, 2000), as opposed to the 5-HT$_{1A}$ receptor, which displays a predominant hippocampal localization (Palacios *et al.*, 1990).

The 5-HT$_{1A}$ receptors are members of a superfamily of seven-transmembrane-domain receptors that couple to G-proteins. Mutagenesis studies have revealed that several residues which are highly conserved among members of the G-protein-coupled receptor family possess important structural and functional roles (Kobilka, 1992; Strader *et al.*, 1994). Interestingly, one of the common structural features

shared by many G-protein-coupled seven-transmembrane-domain receptors is a pair of conserved cysteine residues in the first and second extracellular loops which are often linked as a disulfide bond (Dohlman *et al.*, 1990; Savarese *et al.*, 1992; Lin *et al.*, 1996; Ji *et al.*, 1998). The decrease in ligand binding observed here could probably be due to the destabilization of the disulfide linkage which may hold the active conformation of the receptor together as shown earlier in the case of the $\beta_2$-adrenergic receptor (Lin *et al.*, 1996).

The alignment of seven-transmembrane-domain G-protein-coupled receptor sequences indicates that the hydrophilic components of the transmembrane segments are the most likely site of agonist–receptor interactions. Studies using the $\beta$-adrenergic, $\alpha$-adrenergic, and muscaranic acetylcholine receptors have demonstrated that the location of the binding site inside the membrane is a common feature of all these receptors (Ostrowski *et al.*, 1992). The agonists for these receptors contain an amine group that is proposed 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). It is of interest to note here that the putative topological model for the 5-$HT_{1A}$ receptor shows a total of eight cysteine residues in the transmembrane domain of which the maximum number (four) of cysteine residues are localized in the third transmembrane domain where the conserved aspartic acid residue is also located (Boess and Martin, 1994). Site-directed mutation studies of these transmembrane cysteine residues could provide vital information about the possible role of these residues in ligand binding.

In summary, our results point out that disulfide and sulfhydryl groups play an important role in the agonist and antagonist binding to 5-$HT_{1A}$ receptors and efficient G-protein coupling. These results are relevant to ongoing analyses of the structure–function relationships for G-protein-coupled receptors in ligand-binding processes in general, and the importance of disulfide and sulfhydryl groups in ligand binding in case of the 5-$HT_{1A}$ receptors in particular.

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