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Effect of alcohols on G-protein coupling of serotonin$_{1A}$ receptors from bovine hippocampus

K. G. Harikumar and Amitabha Chattopadhyay*

Centre for Cellular and Molecular Biology, Hyderabad, India

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ABSTRACT: The serotonin$_{1A}$ (5-hydroxytryptamine [5-HT]$_{1A}$) receptors are members of a superfamily of seven transmembrane domain receptors that couple to G-proteins. Serotonergic signalling has been shown to play an important role in alcohol intake, preference and dependence. G-protein coupling of the 5-HT$_{1A}$ receptor serves as an important determinant for serotonergic signalling. We have studied the effect of alcohols on G-protein coupling of bovine hippocampal 5-HT$_{1A}$ receptors in native membranes. This was done by monitoring the modulation of ligand (agonist and antagonist) binding in presence of alcohols by guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolyzable analogue of GTP. Our results show that alcohols inhibit the specific binding of the agonist 8-hydroxy-2-(di-*N*-propylamino)tetralin (except in case of ethanol) and the antagonist 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine to 5-HT$_{1A}$ receptors in a concentration-dependent manner. Further, we show here that the action of alcohols on the bovine hippocampal 5-HT$_{1A}$ receptors could be modulated by guanine nucleotides and that the mode of action of ethanol on the 5-HT$_{1A}$ receptor may be quite different than that of other alcohols. The effect of GTP-γ-S on the agonist and the antagonist binding is found to be markedly different. Our results could be significant in the overall context of the role of G-protein coupling in serotonergic neurotransmission and its role in alcohol tolerance and dependence. © 2000 Elsevier Science Inc.

KEY WORDS: Alcohols, 5-HT$_{1A}$ receptor, OH-DPAT, *p*-MPPF, Bovine hippocampus, GTP-γ-S.

INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [5], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems [18]. It mediates a variety of physiological responses in distinct cell types. Serotonergic signalling appears to play a key role in the generation and modulation of various cognitive and behavioral functions including sleep, mood, pain, addiction, locomotion, sexual activity, depression, anxiety, alcohol abuse, aggression and learning [1,30,31]. 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 [16,28,30].

Serotonin exerts its diverse actions by binding to distinct cell surface receptors which have been classified into many groups [29]. Serotonin receptors are members of a superfamily of seven transmembrane domain receptors [34] that couple to guanosine 5′-triphosphate (GTP)-binding regulatory proteins (G-proteins). Among the various types of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor subtype has been the most extensively studied for a number of reasons [13]. We have earlier partially purified and solubilized the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form [4] and have shown modulation of ligand binding by metal ions, guanine nucleotide, chemical modifications, and alcohols [12–15].

Recent work in pathophysiology of alcoholism at a cellular and molecular level indicate that alcohol affects hormone- and neurotransmitter-activated signal transduction leading to short term changes in regulation of cellular functions and long term changes in gene expression [8]. Serotonergic signalling has been shown to play an important role in the regulation of alcohol intake, tolerance, and dependence [7,26,27,32]. In particular, chronic alcohol administration has been shown to alter expression levels of the 5-HT$_{1A}$ receptor in rat brain [24]. We have earlier monitored the effects of alcohols on ligand binding to bovine hippocampal 5-HT$_{1A}$ receptors [14]. Our results showed that alcohols inhibit the specific binding of the agonist 8-hydroxy-2-(di-*N*-propylamino) tetralin (OH-DPAT) and the antagonist 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine (*p*-MPPF) to 5-HT$_{1A}$ receptors in a concentration dependent manner. Since most seven transmembrane domain receptors are coupled to G-proteins [6], guanine nucleotides are known to regulate ligand binding giving rise to variations in serotonergic signalling. The 5-HT$_{1A}$ receptor is negatively coupled to the adenylate cyclase system through G-proteins [9]. We have previously studied the effect of guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolyzable analogue of GTP, on agonist and antagonist binding to bovine hippocampal 5-HT$_{1A}$ receptors [12]. Our results showed that agonist binding is sensitive to guanine nucleotides and the specific binding of the agonist is inhibited with increasing concentrations of GTP-γ-S. On the other hand, antagonist binding was found to be insensitive to guanine nucleotides and exhibited no significant reduction over a large range of GTP-γ-S concentration. In the present study, we have investigated the effect of alcohols on G-protein coupling of bovine hippocampal 5-HT$_{1A}$ receptors.

* Address for correspondence: Amitabha Chattopadhyay, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India. E-mail: amit@ccmb.ap.nic.in

MATERIALS AND METHODS

Materials

EDTA, EGTA, $MgCl_2$, $MnCl_2$, phenylmethylsulfonyl fluoride (PMSF), Tris, iodoacetamide, polyethylenimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). GTP-γ-S was purchased from Boehringer Mannheim (Germany). [^{3}H]OH-DPAT (127.0 Ci/mmol) and [^{3}H]*p*-MPPF (64.6 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA, USA). The unlabeled antagonist 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido-]ethyl-piperazine (*p*-MPPI) was from Research Biochemicals International (Natick, MA, USA) and was a kind gift from Dr. V. Bakthavachalam (National Institute of Mental Health Chemical Synthesis Program, Natick, MA, USA). All other chemicals used were of the highest available quality. GF/B glass microfiber filters were from Whatman International (Kent, UK). A Bicinchoninic acid (BCA) reagent kit for protein estimation was obtained from Pierce (Rockford, IL, USA). 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 by Harikumar and Chattopadhyay [13]. In short, bovine hippocampal tissue (~100 g) was homogenized as 10% (wt./vol.) in a polytron homogenizer in buffer A (2.5 mM Tris, 0.32 M sucrose, 5 mM EDTA, 5 mM EGTA, 0.02% sodium azide, 0.24 mM PMSF, 10 mM iodoacetamide, pH 7.4). The homogenate was centrifuged at 900 × *g* for 10 min at 4°C. The supernatant was filtered through four layers of cheese cloth and the pellet was discarded. The supernatant was further centrifuged at 50,000 × *g* for 20 min at 4°C. The resulting pellet was suspended in 10 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 × *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 mM Tris buffer (pH 7.4), homogenized using a Dounce homogenizer, flash frozen in liquid nitrogen and stored at −70°C for radioligand binding assays.

Radioligand Binding Assays

Agonist binding assays were performed with varying concentrations of alcohols (in the presence or absence of GTP-γ-S) as follows: Tubes in duplicate containing 1 mg of total protein was mixed with appropriate amounts of any of the alcohols used (ethanol, 1-butanol, 1-hexanol or 1-octanol) and vortexed gently to allow mixing in the presence (or absence) of 50 μM GTP-γ-S. Higher alcohols could not be used since they resulted in insolubility. Bulk (total) alcohol concentrations varied as follows: ethanol: 0.1–1 M; butanol: 25–500 mM; hexanol: 2–25 mM; octanol: 0.06–8.81 mM. The actual concentrations of alcohols partitioned into membranes were calculated taking into account the membrane/buffer partition coefficients from the literature [23] and are plotted in the abscissae of the figures. The assay tubes contained 0.29 nM [^{3}H]OH-DPAT (sp. activity 127.0 Ci/mmol) in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.4) and were incubated for 1 h at room temperature. Control samples were prepared the same way except that alcohol and GTP-γ-S were not added to them. Non-specific binding was determined by performing the assay in the presence of 10 μM unlabeled serotonin. The incubation was terminated by rapid filtration under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0-μm pore size) 2.5-cm diameter glass microfiber filters which were presoaked in 0.3% polyethylenimine for 3 h [2]. The filters were then washed three times with 3 ml of ice-cold water, dried, and the retained radioactivity was measured in a Packard Tri-Carb 1500 scintillation counter using 5 ml of scintillation fluid. Antagonist binding assays with varying concentrations of alcohols and in the presence or absence of 50 μM GTP-γ-S were performed as above using [^{3}H]*p*-MPPF (sp. activity 64.6 Ci/mmol) as the radioligand. The assay tubes contained 0.5 nM [^{3}H]*p*-MPPF in a total vol. of 1 ml of buffer D (50 mM Tris, 1 mM EDTA, pH 7.4). Control samples were prepared the same way except that alcohol and GTP-γ-S were not added to them. Non-specific binding was determined by performing the assay in the presence of 10 μM unlabeled *p*-MPPI. Protein concentration was determined using BCA reagent [33].

TABLE 1.

MEMBRANE CONCENTRATIONS OF ALCOHOLS FROM TOTAL CONCENTRATIONS USING MEMBRANE/BUFFER PARTITION COEFFICIENT

Alcohol	Membrane/buffer partition coefficient* (P)	Total concentration C_T (mM)	Membrane concentration C_M (mM)
Ethanol	0.096	100	8.76
		250	21.90
		500	43.80
		750	65.7
		1000	87.6
Butanol	1.52	25	15.08
		50	30.16
		100	60.32
		250	150.79
		500	301.59
Hexanol	21.4	2	1.91
		4	3.82
		6	5.73
		8	7.64
		10	9.55
		25	23.88
Octanol	189	0.06	0.06
		0.94	0.93
		1.89	1.88
		3.77	3.75
		6.29	6.26
		8.81	8.76

* From [23].

RESULTS AND DISCUSSION

Since the bulk of the G-protein-coupled seven transmembrane domain receptors are embedded in the membrane, it is important to determine the actual concentration of alcohols partitioned into the membrane while considering the effect of alcohols on ligand binding properties. This assumes special significance in case of the 5-HT_{1A} receptor since mutagenesis [3,17] and molecular modelling studies [19,35] have shown that the ligand binding site in serotonin receptors in general, and in 5-HT_{1A} receptor in particular, is located in a transmembrane domain. The partitioning of alcohols from buffer into membranes increases with increase in chain length in a given series [23]. The concentrations of alcohols in membranes were determined from the literature values [23] of

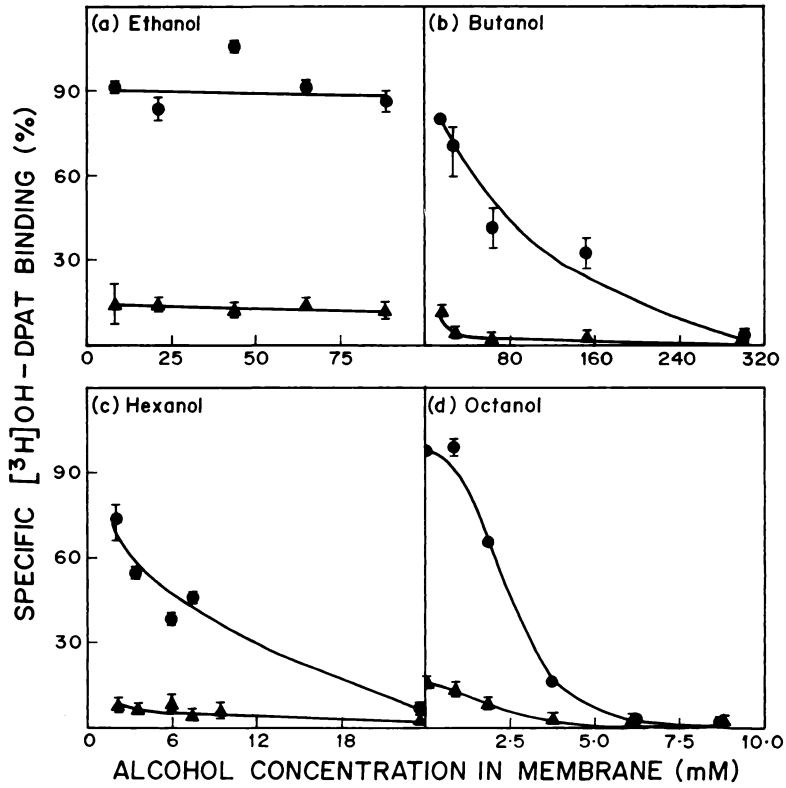

FIG. 1. Effect of increasing concentrations of alcohols on the specific binding of the agonist [^{3}H]8-hydroxy-2-(di-*N*-propylamino) tetralin (OH-DPAT) to the 5-hydroxytryptamine$_{1A}$ receptor from bovine hippocampal membranes in the presence (▲) and absence (●) of 50 μM guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a non-hydrolyzable analogue of guanosine 5′-triphosphate. Values are expressed as a percentage of the specific binding obtained in the absence of any alcohol and GTP-γ-S. The alcohol concentrations plotted here are the actual concentrations of alcohol partitioned into the membrane (see Results, Discussion, and Table 1). The data points are the means ± SEM of duplicate points from three independent experiments. See Materials and Methods for other details.

membrane/buffer partition coefficients of alcohols. The membrane/buffer partition coefficient (P) of an alcohol is defined as $P = C_M/C_B$, where C_M and C_B refer to the molar concentration of the alcohol in the membrane and buffer, respectively. As described earlier [14], the concentrations of alcohols actually partitioned into membranes (C_M) can be calculated from a knowledge of the total added concentration (C_T) using the partition coefficient (P). Table 1 shows the concentrations of alcohols partitioned into membranes for various alcohols used in this study. It is apparent from the table that the difference between the total concentration (C_T) and membrane concentration (C_M) decreases from ethanol to octanol because of increased partitioning into membranes as shown by the increasing value of partition coefficient (P). The concentrations of alcohols plotted in Figs. 1 and 2 are actual membrane concentrations calculated this way and as shown in Table 1.

Among the various types of serotonin receptors, the G-protein-coupled 5-HT$_{1A}$ receptor subtype has been the most extensively studied. One of the major reasons for this is the early availability of a highly selective agonist, OH-DPAT, that allows extensive biochemical, physiological, and pharmacological characterization of the receptor [10]. The hippocampal 5-HT$_{1A}$ receptors are localized postsynaptically [25] and display high affinity for OH-DPAT [11]. Figure 1 shows the inhibition of specific OH-DPAT binding to bovine hippocampal 5-HT$_{1A}$ receptor in native membranes by various alcohols in the presence and absence of 50 μM GTP-γ-S in a concentration dependent manner. The concentration of alcohols plotted here is the actual concentration of alcohol partitioned into the membrane (see Materials and Methods and Table 1). An interesting feature is that although agonist binding was found to be inhibited in all cases with increasing alcohol concentration, it was predominantly unaltered in case of ethanol for native membranes. However, agonist binding was found to be further inhibited in all cases in presence of 50 μM GTP-γ-S and was almost abolished for longer chain alcohols, especially at higher concentrations of alcohols. We have previously shown that agonist binding to the 5-HT$_{1A}$ receptor is sensitive to guanine nucleotides [12,13]. Thus, the specific binding of the agonist is inhibited with increasing concentrations of GTP-γ-S along with a reduction in the binding affinity. In other words, GTP-γ-S induces an affinity transition in the 5-HT$_{1A}$ receptor. Our previous results show that 50 μM GTP-γ-S is capable of inducing a low affinity state of the 5-HT$_{1A}$ receptor which corresponds to ~15% agonist binding when compared to samples without GTP-γ-S [12,13]. Keeping this in mind, a close examination of Fig. 1 shows that the inhibition in agonist binding observed in case of ethanol in presence of GTP-γ-S is more or less independent of the added ethanol. This is perhaps not surprising since ethanol alone does not induce any significant change in agonist binding in the range of concen-

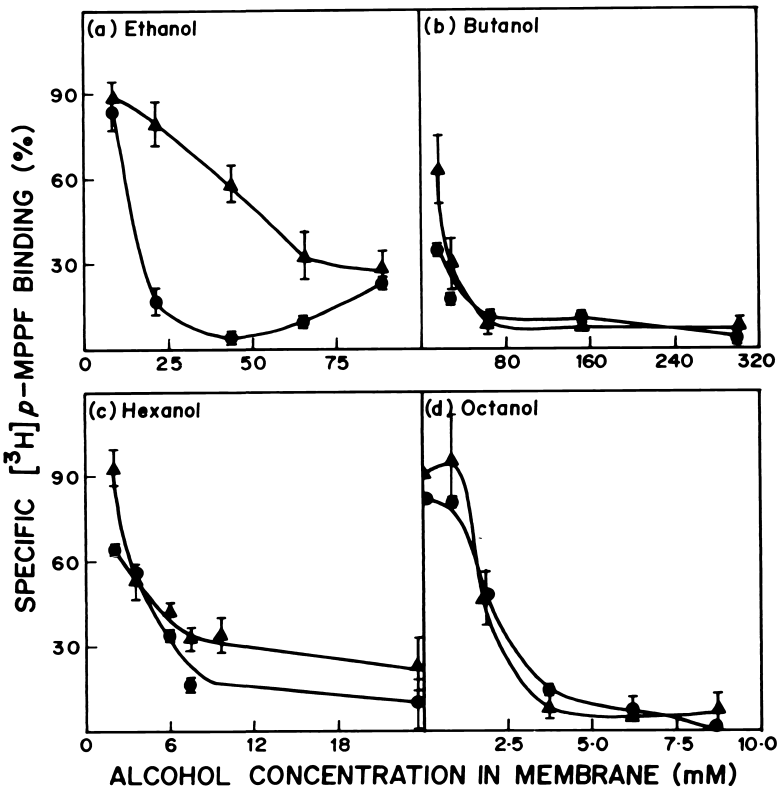

FIG. 2. Effect of increasing concentrations of alcohols on the specific binding of the antagonist [^{3}H] 4-(2′-methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine (*p*-MPPF) to the 5-hydroxytryptamine$_{1A}$ receptor from bovine hippocampal membranes in the presence (▲) and absence (●) of 50 μM guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S). Values are expressed as a percentage of the specific binding obtained in the absence of any alcohol and GTP-γ-S. The data points are the means ± SEM of duplicate points from three independent experiments. All other conditions are as in Fig. 1. See Materials and Methods for other details.

tration studied (see Fig. 1a). However, in case of longer chain alcohols the observed decrease in agonist binding in presence of GTP-γ-S is more than what is expected from the effect of GTP-γ-S alone [12,13] resulting in complete abolition of agonist binding especially at higher concentrations of alcohols (see Figs. 1b and d). This suggests that in these cases the effects of alcohols and GTP-γ-S could be additive and that the inhibition in agonist binding observed with longer alcohols (in absence of GTP-γ-S) could be due to the ability of the alcohols to induce conformational transition in the receptor from a G-protein-coupled high affinity state to a low affinity state that is not coupled to G-proteins. Thus, ethanol and other alcohols have different effects on the agonist binding activity of the bovine hippocampal 5-HT$_{1A}$ receptor and this could be due to differences in their mechanism of action.

Although selective 5-HT$_{1A}$ agonists such as OH-DPAT have been discovered more than a decade back [10], 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 [20–22,36]. These compounds bind specifically to 5-HT$_{1A}$ receptor with high affinity. Figure 2 shows the inhibition of specific *p*-MPPF binding to bovine hippocampal 5-HT$_{1A}$ receptor in native membranes by various alcohols in the presence or absence of GTP-γ-S in a concentration dependent manner. The inhibition in antagonist binding (in the absence of GTP-γ-S) is also found to be dependent on the chain length of alcohols with longer chain alcohols giving rise to more pronounced inhibition in antagonist binding. We have earlier shown that antagonist binding to bovine hippocampal 5-HT$_{1A}$ receptor is insensitive to guanine nucleotides and shows no significant reduction over a large range of GTP-γ-S concentrations [12]. Figure 2 shows that for longer chain alcohols (but not ethanol), the difference in the inhibition of specific antagonist binding observed in the presence and absence of GTP-γ-S is not significant. The slight increase in antagonist binding found in some cases (e.g., in case of hexanol) may be due to the fact that GTP-γ-S has been found to increase antagonist binding by a small amount (10–20%) [12].

As noted in case of agonist binding (see above), the effect of ethanol appears to be different than the effects induced by other alcohols. In the case of antagonist also, we observe a pronounced difference in the pattern of inhibition in binding in the case of ethanol. Thus, the extent of inhibition in antagonist binding for ethanol is reduced considerably in presence of GTP-γ-S. Taken together, these results point out that the mode of action of ethanol on the bovine hippocampal 5-HT$_{1A}$ receptor may be quite different than that of other alcohols. Our results point out that the action of alcohols on the bovine hippocampal 5-HT$_{1A}$ receptor could be modulated by guanine nucleotides. Further, the effect of GTP-γ-S, a non-hydrolyzable analogue of GTP, on the agonist and the antagonist binding is markedly different. It is worth mentioning here that we have earlier proposed that the agonist and antagonist

binding sites may be overlapping but not identical in the 5-HT$_{1A}$ receptor [14]. These results could be significant in the overall context of the role of G-protein coupling in serotonergic neurotransmission and its role in alcohol tolerance and dependence.

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