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Modulation of agonist and antagonist interactions in serotonin 1A receptors by alcohols

K.G. Harikumar, Amitabha Chattopadhyay*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

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Abstract 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). Serotonergic signalling has been shown to play an important role in alcohol tolerance and dependence. We have studied the effects of alcohols on ligand (agonist and antagonist) binding to bovine hippocampal 5-HT$_{1A}$ receptor in native as well as solubilized membranes. Our results show that alcohols inhibit the specific binding of the agonist OH-DPAT and the antagonist *p*-MPPF to 5-HT$_{1A}$ receptors in a concentration-dependent manner.

Key words: Alcohol; Serotonin 1A receptor; 8-Hydroxy-2-(di-*N*-propylamino)tetralin; 4-(2′-Methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-iodobenzamido]ethyl-piperazine; 4-(2′-Methoxy)-phenyl-1-[2′-(*N*-2″-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; Bovine hippocampus

1. Introduction

Serotonin (5-hydroxytryptamine or 5-HT) is an intrinsically fluorescent [1], biogenic amine which acts as a neurotransmitter and is found in a wide variety of sites in the central and peripheral nervous systems [2]. 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 [3–12]. Disruptions in the serotonergic system have been implicated in the etiology of mental disorders such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive compulsive disorder [13–16].

Serotonin exerts its diverse actions by binding to distinct cell-surface receptors which have been classified into many groups [17]. Serotonin receptors are members of a superfamily of seven transmembrane domain receptors [18] 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 for a number of reasons [19]. We have recently partially purified and solubilized the 5-HT$_{1A}$ receptor from bovine hippocampus in a functionally active form [20] and have shown modulation of agonist binding by metal ions and guanine nucleotide [19].

Serotonergic signalling has been shown to play an important role in the regulation of alcohol intake, preference and dependence [10,21–24]. Several groups have found alterations in serotonin receptor subtypes in different regions of the rodent brain associated with alcohol tolerance and dependence [24]. Elevated alcohol consumption has recently been reported in null mutant mice lacking 5-HT$_{1B}$ receptors [10]. Alcohols have also been shown to inhibit the 5-HT$_{2A}$ receptor-induced Ca^{2+}-dependent Cl^- currents in *Xenopus laevis* oocytes [25]. Collectively, these results suggest the involvement of serotonergic neurotransmission in alcohol tolerance and dependence. However, in most of the studies reported so far, the direct effect of various alcohols on ligand binding properties of the receptor has not been examined. We report here that alcohols inhibit the binding of specific agonist and antagonist to bovine hippocampal 5-HT$_{1A}$ receptor, both in native and in solubilized membranes.

2. Materials and methods

BCA, CHAPS, EDTA, EGTA, $MgCl_2$, PMSF, Tris, iodoacetamide, polyethyleneimine, serotonin, sodium azide, and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{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). *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). All other chemicals used were of the highest available quality. GF/B glass microfiber filters were from Whatman International (Kent, UK). 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.

Crude membranes were prepared as described earlier by Chattopadhyay and Harikumar [20] with some modifications. Bovine hippocampal tissue (∼120 g) was homogenized as 10% (w/v) 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×*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 volumes 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

*Corresponding author. Fax: (91) (40) 7171195.
E-mail: amit@ccmb.ap.nic.in

Abbreviations: BCA, bicinchoninic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine; *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; OH-DPAT, 8-hydroxy-2-(di-*N*-propylamino)tetralin; PMSF, phenylmethylsulfonyl fluoride; Tris, tris-(hydroxymethyl)aminomethane

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homogenizer, flash frozen in liquid nitrogen and stored at −70°C for radioligand binding assays or solubilization.

Solubilization of native membranes using CHAPS was carried out as described previously [20] with some modifications. Crude membranes were incubated with 5 mM CHAPS either in buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) for agonist binding studies, or in buffer D (50 mM Tris, 1 mM EDTA, pH 7.4) for binding studies with the antagonist, at a final protein concentration of ~2 mg/ml for 30 min at 4°C with occasional shaking. The membranes were briefly sonicated (5 s) using a Branson model 250 sonifier, and mildly homogenized using a Dounce homogenizer (5 times) at the beginning and the end of the incubation period. After incubation for 30 min, the contents were centrifuged at $100\,000 \times g$ for 1 h. The clear supernatant was carefully removed from the pellet, and used immediately for binding assays.

Agonist binding assays were performed with varying concentrations of alcohols as follows. Tubes in triplicate containing 1–1.2 mg of total protein (for native membrane) or 500 μl of the CHAPS solubilized membrane were mixed with the appropriate amounts of any of the alcohols used (ethanol, 1-butanol, 1-hexanol or 1-octanol) and vortexed gently to allow mixing. Higher alcohols could not be used since they led to 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 concentrations of alcohols partitioned into membranes were calculated taking into account the membrane/buffer partition coefficients from the literature [26] (see Section 3) and are plotted in the abscissae of the figures. The assay tubes contained 0.29 nM [^{3}H]OH-DPAT (specific activity 127.0 Ci/mmol) in a total volume of 1 ml of buffer C (50 mM Tris, 1 mM EDTA, 10 mM $MgCl_2$, pH 7.4) and were incubated for 1 h at room temperature. Control samples were prepared in the same way except that alcohol was 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% polyethyleneimine for 3 h [27]. 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. Antagonist binding assays in presence of various alcohols were performed as above using [^{3}H]*p*-MPPF (specific activity 64.6 Ci/mmol) as the radioligand. The assay tubes contained 0.5 nM [^{3}H]*p*-MPPF in a total volume of 1 ml of buffer D. Alcohol was not added to the control samples. 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 [28].

3. Results

Mutagenesis [29,30] and molecular modelling studies [31,32] 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. It is therefore important to determine the actual concentration of alcohols partitioned into membranes while considering the effect of alcohols on ligand binding properties of the 5-HT_{1A} receptor. The partitioning of alcohols from buffer into membranes increases with increase in chain length in a given series [26]. The concentration of alcohols in membranes were determined as outlined below. The membrane/buffer partition coefficient of an alcohol is defined as:

$$P = C_M/C_B \quad (1)$$

where C_M and C_B refer to the molar concentrations of the alcohol in the membrane and buffer, respectively. The total (bulk) concentration of alcohol is given by:

$$C_T = C_M + C_B \quad (2)$$

Substituting for C_B ($C_B = C_T - C_M$) from Eq. 2 into Eq. 1, we get:

$$P = C_M/(C_T - C_M) \quad (3)$$

Upon rearrangement of Eq. 3, we get:

$$C_M = PC_T/(1 + P) \quad (4)$$

Membrane/buffer partition coefficients for various alcohols have been reported by McCreery and Hunt [26]. The membrane concentration of alcohols (C_M) can thus be calculated from the total added concentration (C_T) of the alcohol and the literature value of its membrane/buffer partition coefficient

Table 1
Membrane concentrations of alcohols from total concentrations using membrane/buffer partition coefficient

Alcohol	Membrane/buffer partition coefficient[a] (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

[a]From [26].

[26]. Table 1 shows the membrane concentrations calculated this way 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 the 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 [33]. Fig. 1 shows the inhibition of specific OH-DPAT binding to bovine hippocampal 5-HT_{1A} receptor by various alcohols in a concentration-dependent manner for native as well as solubilized membranes. The concentration of alcohol plotted here is the actual concentration of alcohol partitioned into the membrane calculated from Eq. 4. It is interesting to note that although agonist binding was found to be inhibited in all cases with increasing alcohol concentration, it was unaltered in case of ethanol for native membranes. A similar result was reported in a previous communication [34]. However, Fig. 1 shows a reduction in agonist binding for ethanol in the case of solubilized membranes, especially toward higher concentrations. This could be due to a higher water content in solubilized membranes (in which the lipids are more loosely packed) which results in increased partitioning of ethanol at a given total concentration. In general, the inhibition of binding is more pronounced for longer chain alcohols. Thus, comparable levels of inhibition of binding are achieved at lower membrane concentrations for the longer chain alcohols. The difference in the inhibition patterns for the native and the solubilized membranes probably reflects differences in lipid composition and packing in the two cases.

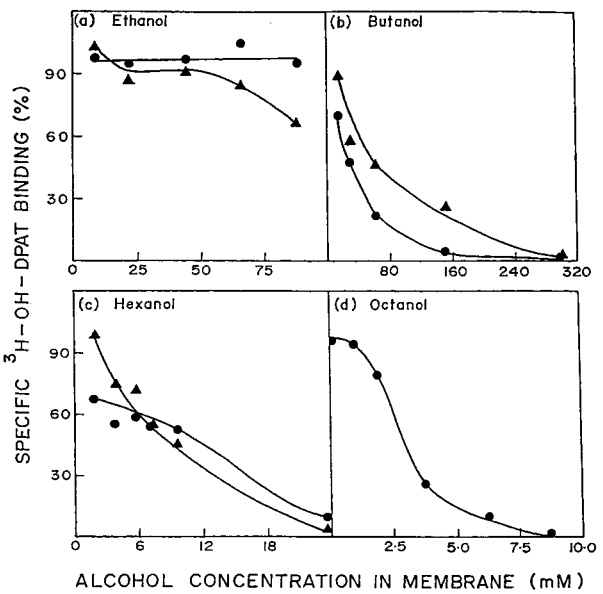

Fig. 1. Effect of increasing concentrations of alcohols on the specific binding of the agonist [^{3}H]OH-DPAT to the 5-HT_{1A} receptor from bovine hippocampal native (●) and solubilized (▲) membranes. Values are expressed as a percentage of the specific binding obtained in the absence of any alcohol. The alcohol concentration plotted here is the actual concentration of alcohol partitioned into the membrane (see Section 3). See Section 2 for other details.

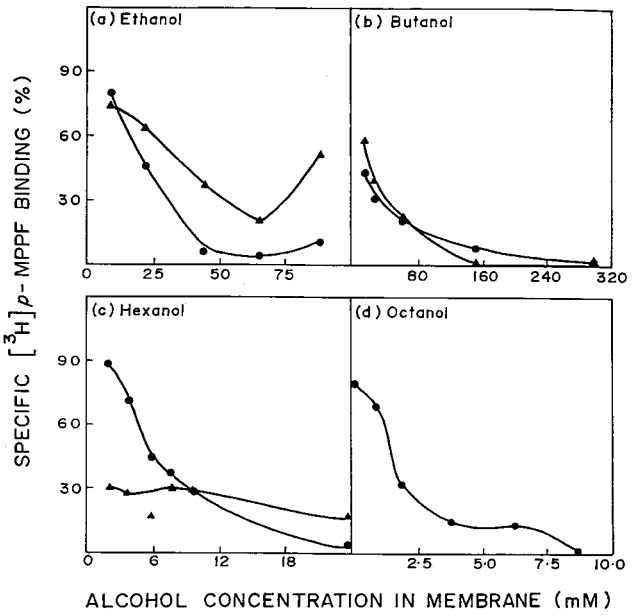

Fig. 2. Effect of increasing concentrations of alcohols on the specific binding of the antagonist [^{3}H]p-MPPF to the 5-HT_{1A} receptor from bovine hippocampal native (●) and solubilized (▲) membranes. All other conditions are as in Fig. 1. See Section 2 for other details.

We had problems performing radioligand binding assays with the solubilized membrane in the presence of octanol although assays done with native membrane gave reproducible results. The reason for this discrepancy is not clear to us. However, altered partitioning of octanol in the solubilized membrane could give rise to such artifacts. The packing of lipids is somewhat loose in the solubilized membrane giving rise to increased levels of water penetration. This would lead to an overall increase in membrane polarity which could reduce partitioning of octanol in the membrane. This effect may not be significant with other alcohols since they are more polar than octanol.

Although selective 5-HT_{1A} agonists (e.g. OH-DPAT) have been discovered more than a decade ago [33], the development of selective 5-HT_{1A} antagonists has been relatively slow and less successful. Recently, p-MPPI and p-MPPF have been introduced as selective antagonists for the 5-HT_{1A} receptor [35–38]. These compounds bind specifically to the 5-HT_{1A} receptor with high affinity. Fig. 2 shows the inhibition of specific p-MPPF binding to the 5-HT_{1A} receptor by various alcohols for native and solubilized membranes. In this case also, the inhibition of binding is more pronounced for longer chain alcohols.

4. Discussion

Both animal and human studies suggest the involvement of the serotonergic system in ethanol preference and dependence [10,22]. However, there have been very few studies, at the molecular level, on the effects of alcohols on ligand binding properties of the isolated receptors. Our results show that ligand binding in the 5-HT_{1A} receptor is altered in the presence of alcohols in a concentration-dependent manner.

Interestingly, contrary to the agonist binding results, ethanol had considerable inhibitory effects on antagonist (*p*-MPPF) binding even in the case of native membranes. This result, together with the general difference in inhibition patterns for OH-DPAT and *p*-MPPF binding observed for various alcohols, may point to overlapping but not identical binding sites for agonist and antagonist binding. Ethanol has been reported to bind in the interfacial region of the membrane, presumably hydrogen bonded to the carbonyl oxygen of the glycerol backbone [39]. Since the antagonist effects are significant in the case of native membranes at low concentrations of ethanol (see Fig. 2), it is possible that the antagonist binding pocket is more polar in nature than the agonist binding site and could even have an interfacial location, i.e. at a shallow location in the membrane compared to the agonist site.

The mechanism of action of alcohols and general anesthetics on the central nervous system is generally discussed in terms of two very different mechanisms [40]. The first mechanism (the 'lipid theory') postulates that alcohols act via some perturbation of the membrane lipids of the neurons in the central nervous system. The second mechanism (the 'protein theory') proposes that alcohols act by directly interacting with a neuronal protein site. Since neurotransmitter-gated ion channel receptors (such as the nicotinic acetylcholine receptor, $GABA_A$ receptor, the glycine receptor, and the 5-HT_3 receptor) are thought to be important targets of alcohol action in the central nervous system, many of the recent studies directed at elucidating the mechanism of action of alcohols have dealt with the effect of alcohols on ligand-gated channels [40]. The ligand binding site in these receptors lies in the extramembranous domain of the protein [41]. In contrast to this, the ligand binding sites in the superfamily of G-protein-coupled receptors (such as rhodopsin, β-adrenergic receptor, dopamine receptors, and serotonin receptors) are formed by their transmembrane domains and lie within the membrane bilayer [42]. Differentiating between the two proposed mechanisms (the 'lipid' or the 'protein' theory) for the action of alcohols is difficult, especially for members of the superfamily of G-protein-coupled receptors such as the 5-HT_{1A} receptor, for whom the ligand binding site is within the membrane. It should be mentioned here that the action of alcohols on rhodopsin, one of the best characterized members of this family, has recently been attributed to lipid-mediated mechanisms [43].

The ligand binding site of the 5-HT_{1A} receptor has stringent requirements of hydrogen bonding pattern for efficient binding [31,32]. This would require an optimal local polarity in the ligand binding pocket. Any agent and/or process that alters this parameter could induce alterations in binding. Alcohols are known to change the polarity of membrane interiors by modulating the hydration level [44,45]. In fact, it has recently been shown that alcohols can compete with water for the same hydrogen bonding sites in membranes [46]. This could explain the altered binding in the presence of alcohols. Alternatively, alcohols have also been shown to asymmetrically affect fluidity of the two leaflets of the membrane bilayer [47]. This could perturb the packing of the lipids adjacent to the membrane embedded receptor (the 'annular lipids'), giving rise to altered ligand binding characteristics. In any event, our results could be significant in the general context of the role of serotonergic signalling in alcohol tolerance and dependence.

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