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EFFECT OF ETHANOL ON THE BINDING OF CONFORMATIONALLY RIGID AND LABILE LIGANDS OF OPIOID RECEPTORS TO RAT BRAIN MEMBRANES

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Abstract—The effect of ethanol on the binding of conformationally rigid and labile ligands for μ and δ opioid receptors to rat brain membranes was determined. The μ ligands used for the studies were [^{3}H]naltrexone and [^{3}H]Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol (DAGO), and δ ligands used were [^{3}H]Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSTLE) and [^{3}H]Tyr-D-Ala-Gly-Phe-Leu (DADLE). The binding of all the opioid ligands was inhibited by ethanol in a concentration-dependent manner. For μ ligands the inhibition was greater for [^{3}H]DAGO binding than for the binding of [^{3}H]naltrexone. For δ ligands, the inhibition by ethanol of the binding of [^{3}H]DADLE was greater than that of [^{3}H]DSTLE. Fourier-transform infrared (FT-IR) spectroscopy was used to determine the conformation of opioid peptides. The data indicated that the conformation of peptides was altered in the presence of ethanol. The results suggest that ethanol inhibits the binding of both μ and δ opioid ligands with greater inhibition observed with conformationally labile ligands. Finally, the alteration in the conformation of the peptide ligands by ethanol, in addition to denaturation of the receptor protein, may also account for the observed inhibitory effect of ethanol on brain opioid receptors.

Several lines of evidence suggest that ethanol interacts with brain opioid systems. Naloxone and maltrexone, antagonists of opioid receptors, have been shown to modify symptoms of ethanol intoxication in both animals and humans. For example, in a double-blind, cross-over study of male volunteers, intravenous injection of naloxone prevented the psychomotor performance impairment induced by low levels of blood ethanol [1]. Naloxone also caused rapid regaining of normal consciousness in human subjects with severe ethanol intoxication [2–4]. In mice, naloxone was inactive, whereas naltrexone was active in reducing the duration of loss of righting reflux induced by mice but in the rat both naloxone and naltrexone were inactive [5]. Khanna *et al.* [6] also could not observe antagonism of ethanol narcosis by naloxone given subcutaneously in either single or repeated doses. Since these effects have been confirmed by some investigators and not by others, strain-dependent effects of naltrexone on ethanol actions suggested a genetic component in the interaction of ethanol and opioid systems [7].

Naloxone has also been shown to antagonize ethanol-induced antinociception measured by the tail-flick test [8] and to block the development of physical dependence on ethanol in mice [9]. Further evidence for ethanol–opioid interaction was based on the observation that methionine-enkephalin, an endogenous opioid, suppresses the consumption of ethanol in rats [10]. Finally, chronic administration of ethanol to mice selectively decreases the number of μ receptors in the frontal cortex [11].

Whether ethanol has a direct action on brain opioid systems, the effect of *in vitro* addition of ethanol on the binding of various opioid ligands has also been studied with varying results. For example, Hiller *et al.* [12] reported that ethanol (200 mM) *in vitro* selectively inhibits the binding of [^{3}H]D-Ala^2-D-Leu-enkephalin (DADLE) to rat brain δ receptors since there was no effect on the binding of [^{3}H]dihydromorphine (DHM) and [^{3}H]naloxone. Both naloxone and DHM bind to μ opioid receptors. The selective change in the binding of DADLE was attributed to changes in the affinity of the ligand [12]. On the other hand, Gianoulakis [13] reported a stimulation in the binding of DHM, an inhibition of DADLE binding, and a lack of effect of ethanol (200 mM) on the binding of [^{3}H]naloxone to rat brain membranes. The inhibition in DADLE binding was shown to be due to changes in the receptor density. Tabakoff and Hoffman [14] reported that at low concentrations (below 100 mM) ethanol significantly increases the binding of DHM to mouse striatal membranes, whereas at higher concentrations ethanol inhibits it. The binding of enkephalin was reported to be inhibited by ethanol at all concentrations used. Thus, it appears that ethanol inhibits the binding of DADLE to mouse and rat brain opioid receptors, but the effects on the binding of μ agonist (DHM) and antagonist (naloxone) are less consistent.

In view of the above observations, the present studies have examined the effect of ethanol on the

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binding of opioid ligands, both conformationally rigid and labile, to rat brain membranes. It is believed that the conformation of both the ligand and the receptor is important in the binding process. The μ ligands used in the present studies included [^{3}H]naltrexone as the rigid analog and [^{3}H]Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol (DAGO) as the labile analog, whereas the δ ligands included [^{3}H]Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSTLE) (a more selective agonist) and [^{3}H]Tyr-D-Ala-Gly-Phe-Leu (DADLE) as a non-selective agonist [15]. In addition, since our earlier studies have demonstrated significant changes in the conformation of Met^5-enkephalinamide by ethanol [16], the conformation of opioid peptide ligands in the presence of ethanol has also been investigated using Fourier-transform infra-red (FT-IR) spectroscopy to determine the possible role of conformation of the peptides in overall binding to the opioid receptors.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (200–250 g), obtained from the King Animal Co., Oregon, WI, were housed three to a cage in a room with controlled temperature (23 ± 1°), humidity (50 ± 10%) and light (6:00 a.m. to 6:00 p.m.) for at least 4 days before being used. Food and water were made available continuously.

Chemicals

[^{3}H]Naltrexone (sp. act. 26.7 Ci/mmol) was a gift from the National Institute on Drug Abuse, Rockville, MD; [^{3}H]DADLE (sp. act. 43.6 Ci/mmol) and [^{3}H]DSTLE (sp. act. 30.5 Ci/mmol) were purchased from New England Nuclear-DuPont, Boston, MA; and [^{3}H]DAGO (sp. act. 57.5 Ci/mmol) was purchased from Amersham, Arlington Heights, IL. Unlabeled levorphanol was supplied by Hoffmann-LaRoche, Nutley, NJ. All other compounds were purchased from commercial sources.

Membrane preparation

The rats were decapitated, and their brains were quickly removed and set on ice. The cerebellum which has negligible amounts of opioid receptors was separated and discarded. The remainder of the brain was used for binding studies. The brain devoid of cerebellum was homogenized in 30 vol. of ice-cold Tris–HCl buffer (0.05 M, pH 7.4) using a Polytron homogenizer (setting 5 for 20 sec). The homogenate was centrifuged at 49,000 *g* for 15 min, and the pellet was resuspended in the same buffer and incubated at 37° for 15 min to remove the endogenous opioids from the receptor sites. After a second centrifugation at 49,000 *g* for 15 min, the pellet was resuspended in Tris–HCl buffer and used for binding studies.

Binding assay

The binding of [^{3}H]DSTLE to brain membranes was carried out essentially according to the method described previously [17, 18]. The binding was carried out in a final volume of 0.5 ml containing 0.05 M Tris–HCl buffer and 0.1 ml of the brain membranes (500–600 μg protein). Similarly, the binding of [^{3}H]-naltrexone, [^{3}H]DAGO, and [^{3}H]DADLE was carried out as described previously [18, 19]. The end concentration of the ^{3}H-ligands in the incubation tubes was 1 nM.

All binding assays were carried out in triplicate at 37° for 30 min. The specific binding was defined as the difference in binding observed in the absence and presence of 1 μM unlabeled ligand [12]. After incubations the contents of the incubation tubes were filtered rapidly under reduced pressure using Millipore manifold filtration units and Whatman GF/F glass fiber filters. It was followed by two 5-ml washes of the same ice-cold buffer used for the assay. The filters were transferred to liquid scintillation vials containing 10 ml of 3a70 scintillant (Research Products International Corp., Mount Prospect, IL). After an overnight equilibration period, the radioactivity in the samples was determined in a Packard Liquid Scintillation Counter (model 4640) with a 54% counting efficiency. The concentration of protein in the samples was determined by the method of Lowry *et al.* [20].

To determine the effect of ethanol on the binding of ^{3}H-ligands, the final concentration of ethanol ranged from 0.1 to 5.0% (v/v) which was prepared by dilution from 95% ethanol. The binding data are expressed as percent of control. Statistical significance was determined using analysis of variance followed by Student's *t*-test.

Table 1. Effect of ethanol on the binding of [^{3}H]DAGO and [^{3}H]naltrexone ligands to μ opioid receptors on the rat brain membranes

Concentration of ethanol (%, v/v)	Binding of μ opioid ligands (% control)	
	[^{3}N]Naltrexone	[^{3}H]DAGO
	100	100
0.1	95.45 ± 2.41	71.37 ± 2.12*
0.5	95.32 ± 3.76	67.93 ± 2.66*
1.0	93.29 ± 1.65*	63.10 ± 4.84*
2.0	84.78 ± 2.26*	58.96 ± 2.71*
3.0	80.40 ± 2.83*	54.56 ± 2.44*
4.0	67.23 ± 1.06*	51.92 ± 6.57*
5.0	60.60 ± 1.83*	43.06 ± 3.69*

Values are means ± SEM, N = 4. Control values (fmol/mg protein): [^{3}H]naltrexone, 88.77 ± 4.79; and [^{3}H]DAGO, 12.43 ± 0.59.
* $P < 0.05$ vs controls.

Table 2. Effect of ethanol on the binding of [^{3}H]DADLE and [^{3}H]DSTLE to δ opioid receptors on rat brain membranes

Concentration of ethanol (% v/v)	Binding of δ opioid ligands (% control)	
	[^{3}H]DADLE	[^{3}H]DSTLE
	100	100
0.1	86.07 ± 13.60	96.22 ± 8.75
0.5	68.27 ± 10.71*	87.27 ± 3.29*
1.0	64.67 ± 15.98*	89.63 ± 6.99*
2.0	34.10 ± 3.23*	82.95 ± 6.52*
3.0	28.78 ± 3.81*	70.82 ± 5.42*
4.0	21.68 ± 0.61*	62.85 ± 5.16*
5.0	9.33 ± 0.22*	48.54 ± 3.94*

Values are means ± SEM, N = 4. Control values (fmol/mg protein): [^{3}H]DADLE, 24.22 ± 1.67; and [^{3}H]DSTLE, 20.89 ± 0.38.
* $P < 0.05$ vs controls.

FT-IR spectroscopy

FT-IR spectra of DSTLE and DADLE in aqueous solution and in the presence of ethanol were recorded as previously described for DAGO and Met^5-enkephalinamide [16, 21]. The spectra were recorded as thin films on an Analect FT-IR spectrometer at ambient temperatures. Resolution enhancement was performed using the algorithm proposed by Kauppinen [22].

RESULTS

Effect of ethanol on the binding of opioid ligands to rat brain membranes

Addition of ethanol to the incubation medium inhibited the binding of tritiated ligands for both μ and δ opioid receptors to rat brain membranes. The specific binding of each ligand at 1 nM concentration in fmol/mg protein was as follows: [^{3}H]DSTLE (20.89 ± 0.38), [^{3}H]DAGO (12.43 ± 0.59), [^{3}H]DADLE (24.22 ± 1.67) and [^{3}H]naltrexone (88.77 ± 4.79) (mean ± SEM, N = 4). The effect of ethanol on the binding of μ ligands to rat brain membranes is shown in Table 1. At 0.1% (v/v) concentration, the binding of [^{3}H]naltrexone was unaffected but that of [^{3}H]DAGO was decreased by almost 30%. Significant inhibition in the binding of [^{3}H]naltrexone was observed at 1.0% (v/v) and 40% inhibition was seen at 5.0% (v/v) concentration. In contrast, significant inhibition in the binding of [^{3}H]DAGO was evident at 0.1% (v/v) concentration, and a 57% inhibition could be seen at 5.0% (v/v) concentration. At each concentration of ethanol used, the inhibition in binding of [^{3}H]DAGO was significantly greater than that of [^{3}H]naltrexone binding.

The binding of [^{3}H]DSTLE and [^{3}H]DADLE, δ opioid receptor ligands, to rat brain membranes was also inhibited by ethanol in a concentration-dependent manner (Table 2). Significant inhibition in the binding of [^{3}H]DADLE (32%) and [^{3}H]DSTLE (13%) could be seen at 0.5% (v/v) concentration of ethanol. At 5% (v/v) concentration of ethanol, the inhibition in the binding of [^{3}H]DADLE and [^{3}H]DSTLE amounted to 91 and 52% respectively. At each concentration of ethanol used, the inhibition in the binding of [^{3}H]DADLE was much greater compared to the binding of [^{3}H]DSTLE to rat brain membranes.

Effect of ethanol on the conformation of peptide ligands for the opioid receptors

Since at 2 and 5%, ethanol produced a significant inhibition in the binding of opioid ligands, the effects of these concentrations of ethanol on the conformation of DADLE and DSTLE were determined using FT-IR spectroscopy.

DSTLE. DSTLE in aqueous solution manifested two intense amide I bands centered at 1637 cm^{-1} and 1651 cm^{-1} (Fig. 1A). In the presence of 2% ethanol, conformational equilibrium did not seem to be influenced as evidenced by the appearance of amide I bands of identical frequency, with a slight diminution of intensities, and a very weak shoulder appeared at 1667 cm^{-1} (Fig. 1B). In contrast, 5% ethanol drastically altered the conformation as evidenced by the disappearance of the 1651 cm^{-1} amide I band and the appearance of an intense 1660 cm^{-1} band (Fig. 1C). The 1637 cm^{-1} band shifted to 1635 cm^{-1} and increased in intensity. In addition, two new weak shoulders also appear at 1648 cm^{-1} and 1688 cm^{-1} respectively.

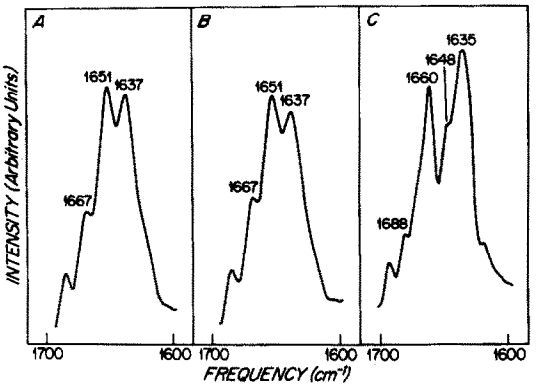

Fig. 1. (A) Resolution-enhanced amide I region of the FT-IR spectrum of DSTLE in aqueous solution; (B) in the presence of 2% (v/v) ethanol; and (C) in the presence of 5% (v/v) ethanol.

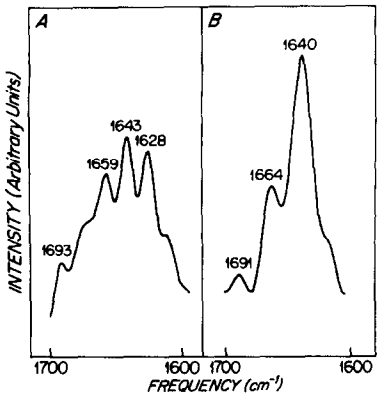

Fig. 2. (A) Resolution-enhanced amide I region of the FT-IR spectrum of DADLE in aqueous solution; and (B) in the presence of 2% (v/v) ethanol.

DADLE. DADLE, in contrast, exhibited a multiplet amide I structure with three bands centered at 1628 cm^{-1}, 1643 cm^{-1}, and 1659 cm^{-1} respectively (Fig. 2A). In sharp contrast, the addition of 2% ethanol resulted in a simplified spectrum with a very intense band at 1640 cm^{-1} and a new band of low intensity at 1664 cm^{-1}. In addition, a very weak shoulder appeared at 1691 cm^{-1} when 2% ethanol was added (Fig. 2B). Since 2% ethanol itself significantly influenced the conformational features of DADLE, effects of 5% ethanol were not investigated. Effects of addition of ethanol to an aqueous solution of DAGO have been reported earlier [21].

DISCUSSION

The present studies indicate that ethanol inhibits the binding of ligands to μ and δ opioid receptors. The binding of both peptide (DAGO) and non-peptide (naltrexone) ligands for μ receptors was decreased by ethanol, although the effect was much greater on the binding of DAGO than of naltrexone. If one assumes that ethanol alters the conformation of the receptor protein only, then the binding of DAGO and naltrexone should have been inhibited to a similar degree by ethanol.

The difference in chemical structures of the two ligands is the conformation. The conformation of naltrexone is fairly rigid, whereas that of DAGO is flexible. If ethanol can alter the conformation of DAGO, then its binding at the receptor site would obviously be expected to change and that can account for the difference in the binding of DAGO and naltrexone. Preliminary studies from our laboratories [21] showed that conformation of DAGO was altered in the presence of 2 and 5% ethanol. The background subtracted FT-IR spectra of DAGO in water exhibited a doublet amide I band with an intense band at 1651 cm^{-1}, a less intense band at 1636 cm^{-1} and a shoulder around 1670–1680 cm^{-1}. From the conformation dependence of IR amide bands, the intense band at 1651 cm^{-1} was assigned either an α-helical or "unordered" structure, the 1636 cm^{-1} band a β structure, and the broad shoulder at 1670–1680 cm^{-1} a β-turn structure [23]. The occurrence of α-helical structure seemed less likely because of the short peptide backbone of DAGO. Hence, DAGO is expected to exist in a conformational equilibrium involving β-turn, β-sheet, and "unordered" structures. In the presence of ethanol, the resolution-enhanced amide I region was simplified by the appearance of two bands, an intense band at 1635 cm^{-1} and a less intense band at 1660 cm^{-1}.

The binding of δ opioid ligands DADLE and DSTLE to brain membranes was also inhibited by ethanol with greater action in DADLE. It should be pointed out that DADLE has been shown to be a rather non-specific ligand for δ receptors since it cross-reacts with μ opioid sites, whereas DSTLE is more specific to δ sites [15].

From the FT-IR studies on DSTLE and DADLE, it seems clear that the two peptides which differ in sequence and in length (number of residues) assume different conformations. Of the two, in the absence of ethanol, DADLE manifested a greater conformational lability, although it is a pentapeptide in contrast to DSTLE which is a hexapeptide. The 1637 cm^{-1} and 1651 cm^{-1} bands in the FT-IR spectra suggest that DSTLE conformation is a mixture of β-sheet and possibly "unordered structures". These conformational assignments have been based on previously described studies from our laboratory [24, 25]. On the other hand, DADLE may contain some proportion of β-turn, if the credibility of the weak 1693 cm^{-1} shoulder is taken into account.

The absence of β-turn conformation in DSTLE is somewhat surprising since D-residues contained in peptides generally induce β-turns. DSTLE seemed to be little influenced by ethanol, even at a 5% level. DADLE in the presence of 2% ethanol, on the other hand, clearly showed the onset of β-turn conformation by the appearance of the low intensity band at 1664 cm^{-1}. The more striking effect of ethanol on DADLE is in line with the results of the binding assay.

In summary, the present studies suggest that ethanol can inhibit the binding of conformationally rigid and labile ligands of opioid receptors to rat brain membranes. The intensity of effect of ethanol on the μ receptor system was greater on conformationally labile ligands, indicating that the alteration in ligand conformation may also be important. The inhibition in binding of μ and δ opioid ligands by ethanol, thus, involves modification of the conformation of both the receptors and the ligands.

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