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Ligand-Dependent Effects of Ethanol and Diethylether at Brain Benzodiazepine Receptors

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QUINLAN, J. J. AND L. L. FIRESTONE. *Ligand-dependent effects of ethanol and diethylether at brain benzodiazepine receptors.* PHARMACOL BIOCHEM BEHAV 42(4) 787–790, 1992.—The $GABA_A$ receptor chloride channel complex interacts with various categories of sedatives, including the benzodiazepines, and possibly ethanol and volatile general anesthetics. Thus, specific binding of tritiated derivatives of a benzodiazepine antagonist, flumazenil, and an agonist, flunitrazepam, to rat brain membrane fragments was monitored at equilibrium in the presence and absence of anesthetizing concentrations of ethanol and diethylether. Ethanol produced a concentration-dependent inhibition of [^{3}H]flumazenil binding, which was not reversed by the $GABA_A$ receptor competitive antagonist bicuculline, but had no effect on [^{3}H]flunitrazepam binding. Both ethanol and diethylether decreased the affinity of the benzodiazepine site for [^{3}H]flumazenil. These data indicate that ethanol and diethylether have GABA-independent effects at the benzodiazepine sites of the $GABA_A$ receptor. These findings are inconsistent with a two-state functional model of the benzodiazepine site and, instead, support a model containing a specific, antagonist-favored conformation.

GABA-benzodiazepine receptors Benzodiazepines Flumazenil Flunitrazepam Biological models Ethyl alcohol

THE $GABA_A$ receptor chloride channel macromolecule ($GABA_A$-R) is modulated by several classes of major sedatives, including benzodiazepines (BDZs). This and much in vivo and in vitro data has led to the hypothesis that the $GABA_A$-R is the important site of action for ethanol [reviewed in Dietrich et al. (5)] and the volatile general anesthetics (GAs) (2). Although it has not been technically possible to detect binding of such agents to $GABA_A$-R, studies of their physiological effects support this hypothesis. For example, both ethanol and the volatile GAs potentiate GABA-activated hyperpolarizing currents (1,12) and enhance GABA-stimulated chloride flux in rat brain synaptosomes (10,15). Ethanol has also been shown to favor the open channel conformation of the $GABA_A$-R, as monitored by binding of channel-specific ligands such as cage convulsants (*t*-butylbicyclophosphorothionate) (10).

However, equilibrium radioligand binding studies specifically testing a model in which ethanol interacts with the BDZ site yielded inconsistent results. Davis and Ticku found a small and variable increase in diazepam binding with membrane-bound receptor in the presence of ethanol (4), but Greenberg et al. found no significant effect on binding of the BDZ agonist flunitrazepam (FNP) or the BDZ antagonist flumazenil (FMZ) at intoxicating concentrations of ethanol (up to 100 mM) (6). To address this discrepancy, we extended these binding studies to and beyond anesthetizing concentrations of ethanol and diethylether. Utilizing BDZ agonist and antagonist ligands, we found ligand-dependent effects of both ethanol and diethylether on high-affinity BDZ binding.

METHOD

Brain membrane fragments were prepared from freshly dissected forebrains of adult male Wistar rats by differential centrifugation as previously reported (14). Briefly, cerebral cortices were coarsely homogenized in 0.3 M sucrose/0.1 mM phenylmethylsulfonylfluoride using a Potter apparatus. The homogenate was then centrifuged (1000 × *g*, 10 min, 4°C) and the resulting supernatant was recentrifuged (100,000 × *g*, 60 min, 4°C). The pellet was harvested quantitatively, resuspended in 50 mM Tris (pH 7.4, room temperature), and the membrane suspension analyzed for protein by the method of Hartree (9).

Either [^{3}H]FMZ (75 Ci/mmol, New England Nuclear,

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Newton, MA, 98% radiochemically pure) or [^{3}H]FNP (84 Ci/mmol, Amersham Corp., Arlington Heights, IL, 97% pure) was used without further preparation. Suspensions containing 50 μg membrane protein were incubated to equilibrium (room temperature, 20 min) with 0.5-50 nM either [^{3}H]FMZ or [^{3}H]FNP (final concentration) in Tris-buffer, with or without various concentrations of ethanol (up to 1 M) or diethylether (42 mM). Entire experiments were repeated in the presence of 50 μM bicuculline. Reaction mixtures were incubated in polyallomer tubes (250 μl, Beckman, Arlington Heights, IL) capped with Teflon plugs to reduce evaporative loss of ethanol and diethylether. Bound ligand was separated from free ligand by ultracentrifugation in a Beckman Airfuge (130,000 $\times$ g, 8 min, room temperature). Supernatants (50 μl) were sampled to determine free ligand concentration; membrane-bound ligand was measured by cutting the tip of the centrifuge tube, immersing in scintillation cocktail (Cytoscint, ICN Biomedicals, Cleveland, OH), and dispersing the pellet by vortexing. Samples were counted in a Beckman LS5000 scintillation counter using quenched samples of ^3H_2O as external standards; counting efficiency was 31-34%. Specific binding was obtained by subtracting nonspecific binding (determined when identical incubations were performed in the presence of 10 μM unlabeled FMZ) from total binding. Concentrations of ethanol and diethylether were monitored by gas chromatography (Perkin-Elmer 8500; Poropak 'P' column packing; Waters Associates, Inc., Milford, MA). Entire experiments were repeated with different membrane batches.

Free ligand concentrations were expressed in nM after radiopurity correction. Membrane-bound ligand was expressed as pmol/mg membrane protein. Binding data were fit to logistic functions by an iterative nonlinear least-squares fitting routine (17). Linear transformations of hyperbolic binding curves were fit by an unweighted linear least-squares method to yield a slope and standard error. Statistical comparisons were by two-tailed t-test using the pooled residual mean square method for comparison of slopes (18).

RESULTS

In the concentration ranges studied, both [^{3}H]FNP and [^{3}H]FMZ binding was saturable, and greater than 90% of the observed binding was specific. The apparent affinity constant for FMZ was 6.0 nM and for FNP 3.6 nM, similar to previously published values (3,6). Ethanol inhibited specific [^{3}H]FMZ binding (Fig. 1), but even at the highest concentration (1 M) had an insignificant effect on nonspecific binding (data not shown). Scatchard analysis revealed significant concentration-dependent decreases in the affinity of the BDZ site for [^{3}H]FMZ, but no change in the number of binding sites (Fig. 2 and Table 1). Similarly, diethylether at a therapeutic concentration inhibited [^{3}H]FMZ binding by decreasing the affinity of the BDZ site for [^{3}H]FMZ (Table 1). In contrast, when the agonist [^{3}H]FNP was employed ethanol failed to affect either the apparent K_d or the maximal receptor number (Table 2).

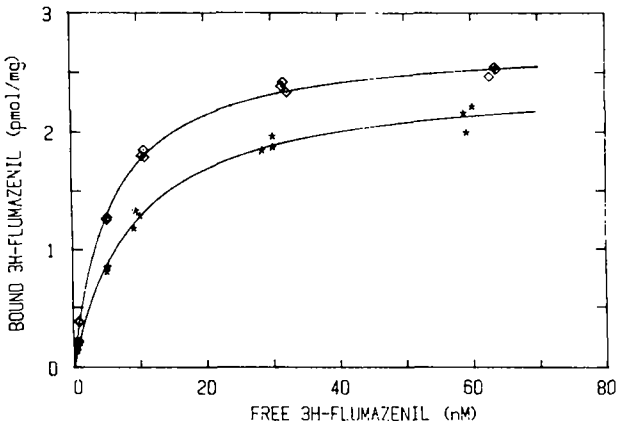

FIG. 1. Equilibrium specific binding of [^{3}H]FMZ to rat brain membranes. Each point is a single determination. A single representative experiment is illustrated in the presence (*) and absence (◇) of 500 mM ethanol. Data were fit as described in the Method section.

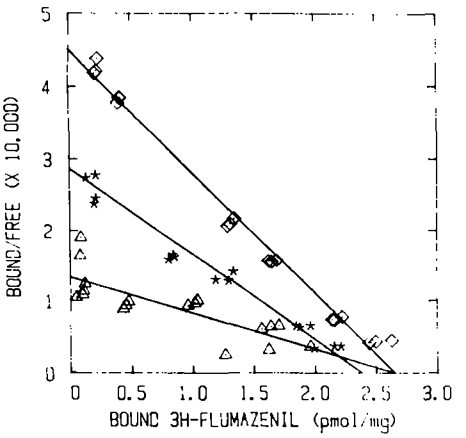

FIG. 2. Scatchard plot of [^{3}H]FMZ binding to rat brain membranes. Bound [^{3}H]FMZ is pmol/mg membrane protein measured as described in the Method section. Control values (◇) were derived from several experiments; single representative experiments are illustrated, performed in the presence of 500 mM ethanol (*) and 1 M ethanol (△). Lines were fit as described in the Method section.

DISCUSSION

Previous binding studies have revealed little effect of intoxicating concentrations of ethanol on equilibrium binding of

TABLE 1

EFFECT OF ETHANOL AND DIETHYLETHER ON EQUILIBRIUM [^{3}H]FMZ BINDING

Agent	Concentration (mM)	Apparent K_d* (nM)	B_{max}† (pmol/mg protein)
Control	0	6.0 (0.1)	2.7 (0.1)
Ethanol	250	7.4 (0.4)‡	2.4 (0.1)
	500	9.0 (0.6)‡	2.4 (0.1)
	1000	19.9 (0.4)‡	2.6 (0.3)
Diethylether	42	7.6 (0.3)‡	2.7 (0.1)

The apparent affinity constant (K_d) and maximal receptor number (B_{max}) were derived from binding data transformed by the Scatchard equation and fit by the method of least squares.

*Numbers in parentheses represent the SE of the Scatchard slope.

†Numbers in parentheses represent the range of the x-intercept derived by Scatchard analysis.

‡$p < 0.001$ compared with control.

TABLE 2

EFFECT OF ETHANOL ON EQUILIBRIUM [^{3}H]FNP BINDING

Agent	Concentration (mM)	Apparent K_d* (nM)	B_{max}† (pmol/mg protein)
Control	0	3.6 (0.2)	2.0 (0.1)
Ethanol	500	3.5 (0.2)	2.2 (0.1)

The apparent affinity constant (K_d) and maximal receptor number (B_{max}) were derived from binding data transformed by the Scatchard equation and fit by the method of least squares.

*Numbers in parentheses represent the SE of the Scatchard slope.

†Numbers in parentheses represent the range of the x-intercept derived by Scatchard analysis.

agonists to the BDZ site on the membrane-bound $GABA_A$-R (4,6). Similarly, we found no significant effect on binding of the agonist [^{3}H]FNP. However, we observed significant effects of ethanol and diethylether at obtunding concentrations with an antagonist ligand, [^{3}H]FMZ. Although FMZ may act as a partial agonist at supraphysiologic concentrations greater than 1 μM (13), nanomolar concentrations of FMZ do not affect channel gating behavior (16), justifying its classification as a BDZ antagonist.

Both anesthetic agents decreased equilibrium binding of [^{3}H]FMZ by reducing apparent receptor affinity. This effect was not mediated indirectly via effects at the GABA recognition site, since it was not blocked by bicuculline. Ethanol-induced enhancement of diazepam binding to solubilized $GABA_A$-R has been observed (4), but this effect appeared to be mediated via the GABA recognition site, since it was blocked by bicuculline. Unlike the BDZ agonists, FMZ binding is known to be unaffected by the presence of GABA (11); thus, we did not expect that ethanol-induced inhibition of [^{3}H]FMZ binding would be mediated indirectly through effects at the GABA recognition site. In a separate series of experiments not reported here, bicuculline failed to antagonize ethanol's effects on [^{3}H]FMZ binding, confirming our expectation.

The finding that ethanol's effect on equilibrium BDZ binding depends upon the ligand used has implications for the multistate models previously proposed for the functional properties of the BDZ receptor. BDZ agonists are known to allosterically modulate chloride channel gating of the $GABA_A$-R by increasing the frequency of channel openings and bursting events (16). BDZ inverse agonists decrease channel opening frequency, while antagonists have no effect on this parameter (16). These and other data have led to two alternative models (7,8). In the first model, each type of BDZ ligand binds to one of three interconvertible conformations. BDZ agonists bind to an "agonist conformation" and enhance the action of GABA on channel gating; the presence of GABA also favors this same conformation. The "inverse agonist conformation" inhibits the action of GABA and, reciprocally, GABA inhibits isomerization to this state. Antagonists bind to a resting "inactive conformation" that is unaltered by GABA and does not affect GABA's gating properties. A second model asserts that there is no specific conformation for antagonist ligands, which are proposed to interact equally well with the agonist conformation and the inverse agonist conformation (7,8). As in the first model, agonists stabilize the former state, which enhances the action of GABA, and inverse agonists stabilize the latter state, which inhibits the action of GABA. Our finding that ethanol perturbs the binding of a BDZ antagonist, but not a BDZ agonist, supports the existence of a distinct "antagonist state" and is inconsistent with any model lacking such a conformation.

In summary, ethanol and diethylether produced a concentration-dependent decrease in the affinity of the BDZ site for antagonists that was GABA independent. In contrast, ethanol had no effect on the binding of BDZ agonists. The ligand dependence of the ethanol effect on BDZ binding supports the existence of a specific, antagonist-favored conformation of the BDZ site.

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