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Ethanol Effects on Synaptic Glutamate Receptors and on Liposomal Membrane Structure

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MICHAELIS, E. K., J. D. ZIMBRICK, J. A. McFAUL, R. A. LAMPE AND M. L. MICHAELIS. *Ethanol effects on synaptic glutamate receptors and on liposomal membrane structure.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 197–202, 1980.—Exposure of synaptic plasma membranes to 50 mM ethanol *in vitro* brought about a 3.5°C decrease in the transition temperature of the high affinity glutamate binding process in these membranes. Ethanol had no effect on the energy of activation of glutamate binding below the transition temperature but decreased the energy of activation above the transition temperature. Electron paramagnetic resonance (EPR) studies of lipid organization of egg lecithin and bovine brain phospholipid liposomes indicated that ethanol at low concentrations (0.04–2 mM) caused small increases in the rigidity of the membrane near the surface. At higher concentrations (0.04–2 M) ethanol brought about increasing fluidization of both the surface and inner areas of the bilayer. Even at 4 mM concentration ethanol enhanced the ordered to fluid state transition of liposome membranes as shown by a 5.2°C and 1.9°C decrease in the transition temperatures of the membrane determined with the cholestane EPR probe.

Ethanol Glutamate receptors Liposomes Membrane lipids Membrane structure
Transition temperature EPR Synaptic membranes

THE chronic oral administration of ethanol to rats was previously shown to cause increases in the maximum L-glutamic acid binding capacity of brain synaptic membranes [11,13]. These ethanol-induced increases in glutamate receptor binding capacity were stable for 24 to 72 hours following ethanol withdrawal and were correlated with an apparent hypersensitivity of glutamate receptors during the period when post-ethanol withdrawal seizures occurred [6,11]. These changes in glutamate receptor density and sensitivity were thought to represent an adaptive response of neurons to the acute effects of ethanol on these cells.

Exposure of the synaptic membranes to ethanol *in vitro* was found to produce biphasic effects on the L-(^{3}H) glutamate binding activity of these membranes: ethanol concentrations in the range of 2.5 to 50 mM causes a substantial enhancement in glutamate binding whereas ethanol concentrations of 50–200 mM brought about a return of the synaptic membrane binding activity to baseline levels [10,13]. The increases in L-(^{3}H) glutamate binding produced by incubation of the synaptic membranes with ethanol were thought to be due in part to ethanol's disruption of the interaction of the receptor glycoprotein with its surrounding membrane gangliosides [10]. The latter membrane macromolecules were shown to modulate glutamate binding activity downwards while the addition of 5 mM ethanol was found to counteract the ganglioside inhibition of glutamate binding to synaptic membranes [10]. These observations were suggestive of perturbations in the receptor microenvironment in synaptic membranes brought about by low concentrations of ethanol. Such perturbations might be distinguishable from the more remarkable and well-described membrane "fluidization" produced by higher concentrations of ethanol [3, 4, 14]. The present study represents an attempt to document further the influence of low concentrations of ethanol on the glutamate receptor microenvironment in synaptic membranes and to explore the possibility that ethanol may produce differential effects on the membrane bilayer when it is present at low as opposed to high concentrations.

METHOD

Synaptic Membrane Preparation and L-(^{3}H) Glutamate Binding Determination

Rat brain synaptic plasma membranes were isolated and stored under the conditions described previously [2,12]. L-(^{3}H) Glutamic acid (40 Ci/mmol, New England Nuclear Corp.) binding to the synaptic membranes was measured by the microfuge centrifugation procedure [9]. All samples were incubated for 25 min in the presence or in the absence of 50 mM ethanol at the temperatures indicated in the Results section. Non-specific L-glutamic acid binding at each tempera-

ture was determined by simultaneously incubating synaptic membranes with L-(^{3}H) glutamic acid in the presence of excess unlabeled L-glutamate (0.1 mM). The protein concentration of all samples was measured by the Lowry procedure [8].

Electron Paramagnetic Resonance (EPR) Spectroscopy of Liposomal Membranes

The EPR studies with model membranes were performed using vesicles prepared from chloroform:hexane solutions of egg lecithin (primarily phosphatidyl-choline), or Type III Bovine Brain Extract (primarily phosphatidylserine), both obtained from the Sigma Chemical Company and used without further purification. The lipids were mixed with solutions which contained one of the EPR probes, 5-, 7-, or 12-doxyl stearic acid or the cholestane spin label, in a mole ratio of about 130 lipid:1 spin label. The samples were dried in a boiling flask using a rotary evaporator. All traces of solvent were removed by drying the samples in a vacuum for at least 6 hours. Vesicles were formed by first vortexing the spin labeled lipids with the desired aqueous solvent followed by sonication of the solution in a Branson model B 220 ultrasonicator for three sixty-second periods. The samples were flame sealed in 100 μl glass capillary tubes and their EPR spectra were measured in a Varian model E-109 EPR Spectrometer. The spectrometer was equipped with a Varian model B-4556-9 variable temperature accessory and was interfaced to a Nicolet model 1180 minicomputer system.

Data Analysis

Line fitting of all Arrhenius-type plots was performed by regression analysis. Repeated iterations from both the high and low temperature range were used in order to determine the best line fit (highest r coefficient) of the experimental points.

RESULTS

Ethanol Effects on the Temperature Dependence of Glutamate Binding

The high affinity glutamate binding activity of isolated synaptic plasma membranes was dependent on the temperature of the incubation medium. A semilogarithmic plot of the glutamate binding activity vs. the inverse of the incubation temperature (Arrhenius-type plot) revealed the presence of two different rates of activation of glutamate binding caused by increases in the incubation temperature (Fig. 1A). The transition temperature (T_t) for these two states of activation of the synaptic membrane glutamate receptors was found to be 16°C (Fig. 1A). When the glutamate binding assays were conducted in the presence of 50 mM ethanol, a similar temperature dependence of glutamate binding activity was observed (Fig. 1B). However, the introduction of ethanol at this concentration resulted in a substantial decrease in the T_t of activation of L-glutamate binding (T_t=12.5°C) (Fig. 1B). Despite the marked decrease in the T_t of glutamate binding activity of synaptic membrane receptors in the presence of ethanol, this agent had only a very small effect on the energy of activation of this process below the transition temperature. The energy of activation of glutamate binding was determined from the slope of the linear segments in Fig. 1 and it was found to be 14.4 kcal/mol for the range of 2–16°C in the control membranes. This value was almost equal to that cal-

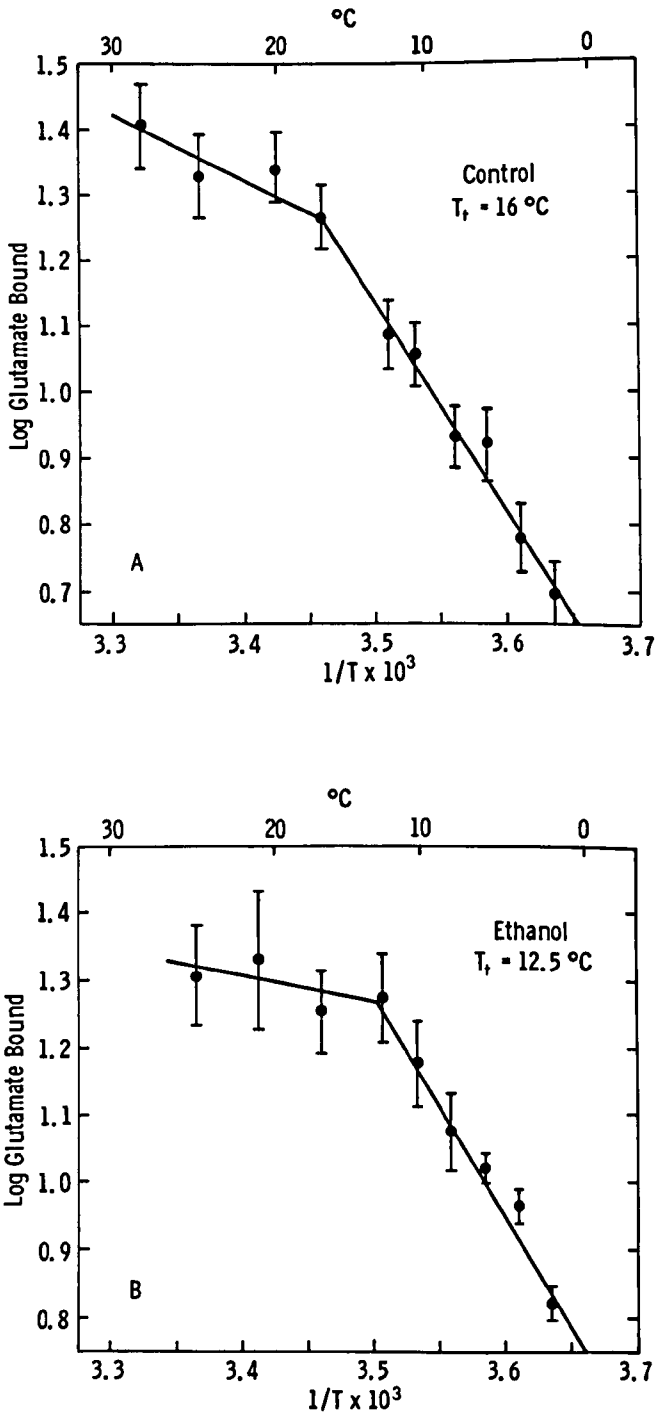

FIG. 1. Arrhenius-type plot of L-(^{3}H) glutamate binding to brain synaptic membranes. L-Glutamic acid binding was measured over the temperature range of 2–28°C according to the procedures described in the text. Each point is the mean (±SEM) of 18 determinations from 6 membrane preparations for the control membranes (A) and of 6 determinations from 2 membrane preparations for the membranes which were incubated in the presence of 50 mM ethanol (B).

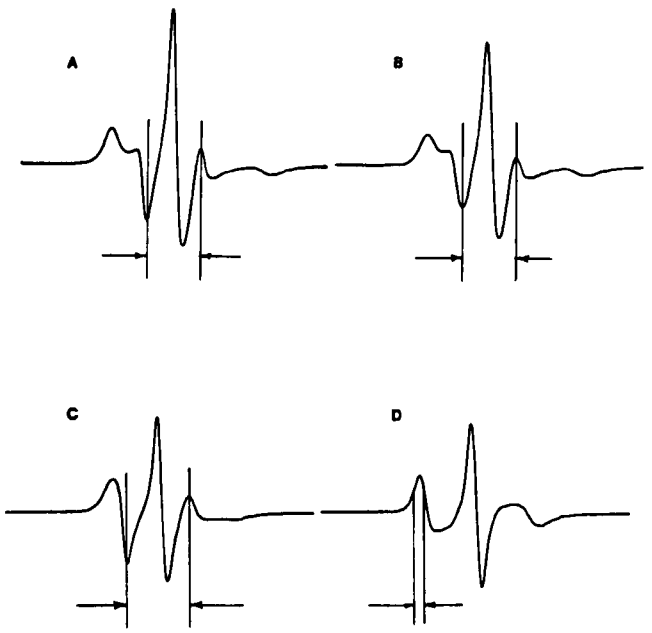

FIG. 2. Sample spectra of the four spin labels used in the EPR studies. (A) 5-doxyl stearic acid, (B) 7-doxyl stearic acid, (C) 12-doxyl stearic acid, and (D) cholestane. The spectral parameters $2T_\perp$ (in the case of a, b, c) and the low field line width at half maximum amplitude (in the case of d) are indicated on each spectrum.

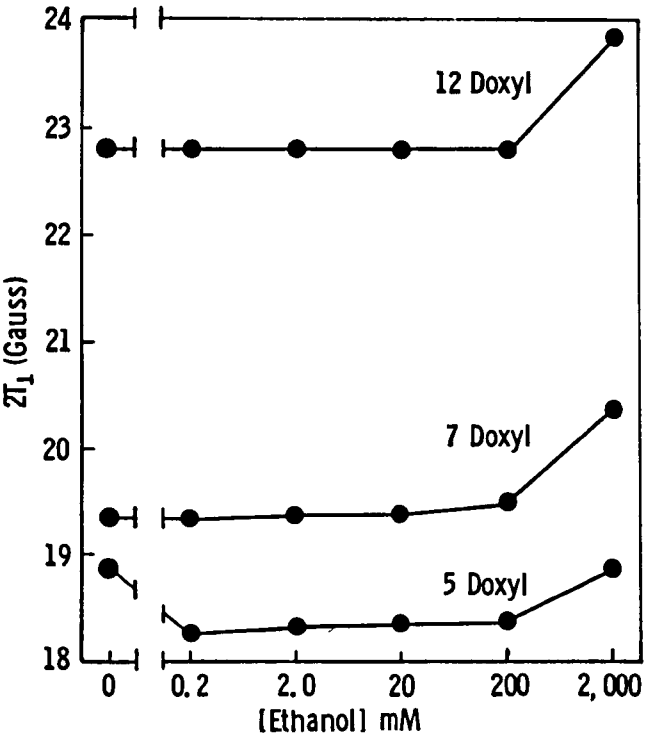

FIG. 3. Effects of ethanol on membrane organization of egg lecithin vesicles. Changes in the $2T_\perp$ parameter with increasing ethanol concentrations were recorded for egg lecithin vesicles labeled with 5-, 7-, and 12-doxyl stearic acid probes. Each point is the mean of two determinations performed at room temperature.

culated for the ethanol-treated membranes (2–12.5°C) which was 15.1 kcal/mol. However, the energy of activation of the binding process above the transition temperature was significantly lower in the presence of 50 mM ethanol (1.7 kcal/mol) as compared to the control membranes (3.9 kcal/mol).

The appearance of a transition in the temperature-induced activation of a membrane protein's function can be considered as evidence of phase changes in the membrane lipid environment of this protein [5]. It would appear, then, that the binding activity of glutamate receptors was affected by the state of their lipid microenvironment and that exposure of the membranes to 50 mM ethanol brought about a perturbation of the receptor microenvironment. Such a lipid perturbation was revealed by the marked lowering of the transition temperature for glutamate binding caused by the presence of this alcohol in the incubation medium.

The lowering of the transition temperature of glutamate binding by ethanol may be indicative of a process which promotes the transition of membrane lipids from an ordered to a fluid phase. The effects of ethanol on the organization of lipids in model membranes was explored in subsequent studies in order to gain some understanding of the types of perturbation which may be produced by ethanol.

Ethanol Effects on Model Membranes (Liposomes)

In studies designed to evaluate the effects of ethanol at various depths within the membrane bilayer two types of EPR probes were employed: (1) 5-, 7-, and 12-doxyl stearate, and (2) cholestane. The cholestane probe is known to be incorporated near the surface of the bilayer with its long axis perpendicular to the bilayer plane [14]. On the other hand, in the case of the 5-, 7-, and 12-doxyl stearic acid probes the nitroxide group would be situated at various depths within the bilayer with an orientation parallel to the plane of the membrane. Typical spectra obtained from these EPR probes are shown in Fig. 2. The parameters which were measured and plotted in subsequent Figures are indicated on the spectra. The spectral parameter $2T_\perp$ was used to measure membrane lipid fluidity in the case of the doxyl stearate probes, and the low field line width at one-half maximum amplitude was used in the case of the cholestane spin label. An increase in $2T_\perp$ is indicative of increasing membrane fluidity [7]. However, $2T_\perp$ was difficult to measure in the spectra of the cholestane probe since the lines were not sharp and they varied in shape. The low field line width of the cholestane probe spectrum was a more stable parameter which correlated very well with the fluidity of lipids within the membrane as will be described below (e.g., Fig. 5).

Egg lecithin liposomes labeled with 5-, 7-, and 12-doxyl stearic acid probes were prepared in a solvent system consisting of H_2O only. The vesicles were then suspended in various ethanol-H_2O mixtures and the EPR spectra for each sample were recorded. Ethanol at concentrations of 0.2 mM to 200 mM had minimal or no effect on membrane fluidity in the deeper areas of the bilayer as judged by the lack of

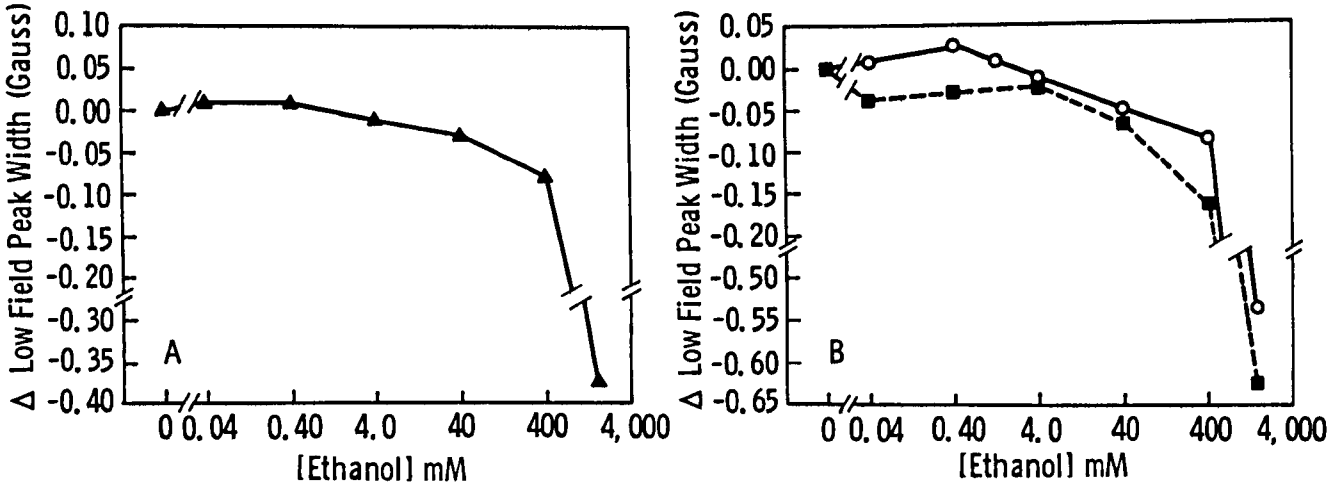

FIG. 4. Ethanol effects on cholestane-labeled membranes. The changes in the low field peak width of the ethanol-exposed liposomes as compared to the control (no ethanol) vesicles are shown. Cholestane-labeled egg lecithin (A) and bovine brain phospholipid vesicles (B) were used in these studies. In (B) measurements were taken in the absence (○) and in the presence of 50 mM NaCl (■) in the incubation medium. Each point is the mean of 6 determinations. The mean low field peak width in Gauss for the control liposomes was: egg lecithin 3.90 G, bovine brain lipids 4.42 G, bovine brain lipids +NaCl 4.48 G.

changes in the $2T_{\perp}$ parameter of 7- and 12-doxyl stearate EPR spectra (Fig. 3). On the other hand, the $2T_{\perp}$ parameter of the EPR spectrum of 5-doxyl stearic acid was consistently decreased by approximately 0.5 Gauss even with the lowest concentration of ethanol tested (0.2 mM) (Fig 3). This observation might be suggestive of an interaction of ethanol with the surface of the lipid bilayer which has caused an increase in rigidity (decrease in $2T_{\perp}$) of the more superficially positioned 5-doxyl stearic acid probe.

Higher concentrations of ethanol (0.2–2.0 M) caused an increase in the $2T_{\perp}$ parameter of the 7- and 12-doxyl stearate probes, while the same measure in the 5-doxyl stearic acid probe spectra was brought back to baseline levels by the introduction of these ethanol concentrations into the incubation medium. These changes in $2T_{\perp}$ may be indicative of ethanol's penetration deep into the bilayer and of the consequent fluidization of the membrane when this agent is present at high concentrations.

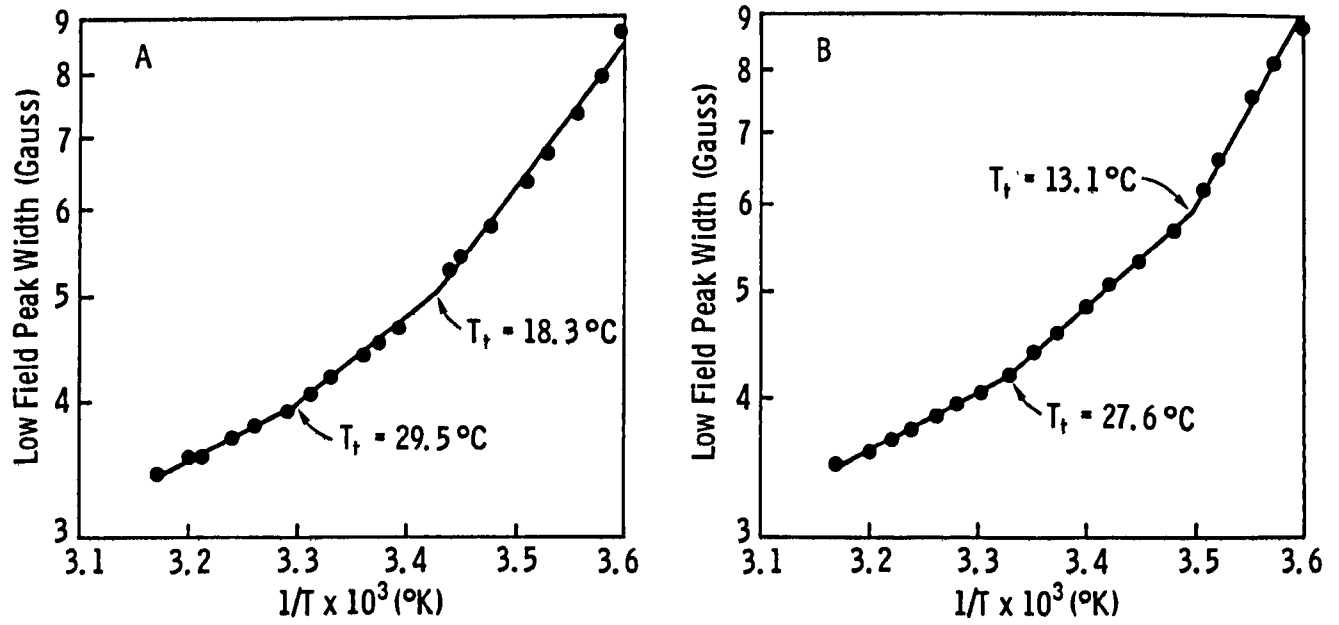

FIG. 5. Arrhenius-type plots of the cholestane low field peak width from labeled bovine brain phospholipid vesicles. Electron paramagnetic resonance spectra were recorded from two different preparations of spin-labeled liposomes: control (A) and ethanol-exposed (B). The ethanol concentration was 4 mM.

Ethanol's biphasic effects on lipid organization near the surface of the liposome membranes were also demonstrated by the use of the cholestane probe. Egg lecithin and Type III bovine brain phospholipid vesicles were labeled with the cholestane probe and their EPR spectra determined at various concentrations of ethanol. A small increase in the low field line width parameter of the EPR spectra was observed upon exposure of these vesicles to low concentrations of ethanol (below 4 mM ethanol) (Figs. 4A and 4B). Concentrations of this alcohol in the range of 40 mM to 2 M brought about a marked decrease in the width of the low field peak. The inclusion of 50 mM NaCl in the solvent in which the bovine brain phospholipids were suspended completely eliminated the apparent biphasic effects of ethanol on the line width parameter of the cholestane spectra (Fig. 4B). In the presence of 50 mM NaCl there was a somewhat greater fluidizing effect (decrease in the peak width) of ethanol at all concentrations tested as compared to its effects on lipid vesicles suspended in H_2O.

The fact that an increase in the low field peak width was a reflection of greater ordering of the lipid structure in the membrane bilayer was substantiated by the results of the studies shown in Figs. 5A and 5B. In these Arrhenius-type plots it can be seen that this spectral parameter exhibited marked increases with decreasing ambient temperature of the vesicle suspension, reaching a value of 8.8 Gauss at 5°C. There were two T_t's observed for the vesicles which were suspended in H_2O: the first T_t was 18.3°C and the second was 29.5°C (Fig. 5A). The presence of 4 mM ethanol in the suspension medium led to a marked shift in the lower T_t from 18.3°C to 13.1°C, while it produced a smaller shift in the higher T_t (from 29.5 to 27.6°C). It should be pointed out that 4 mM ethanol produced very small changes in the low field peak width of the cholestane spectra at room temperature (Fig. 4B), and yet, this concentration of ethanol caused an earlier transition from an ordered to a fluid state of the membrane lipids in response to increases in ambient temperatures (Fig. 5B).

DISCUSSION

The possibility that ethanol influences the glutamate receptor recognition function in brain synaptic membranes through a perturbation of this protein's lipid environment was substantiated by the observations described in this study. The presence of 50 mM ethanol in the incubation medium brought about a marked decrease in T_t of the glutamate binding activity of these receptors in isolated synaptic plasma membranes. These findings correlate quite well with our previous observations of ethanol's interference with the interaction of glutamate receptors in synaptic membranes with a specific class of membrane lipids, the gangliosides [10]. The apparent promotion by ethanol of a more rapid transition from ordered to fluid phase in the microenvironment of the receptor protein may be the process through which even low concentrations of ethanol (2.5–50 mM) bring about disruption of the normal modulation of receptor activity by surrounding membrane lipids such as the gangliosides. However, the effects of ethanol on lipid molecular organization may be more complex than the mere disordering or fluidization which has been described before [3, 4, 14].

Exposure of liposomes to small concentrations of ethanol (0.04–2 mM) led to an apparent increase in lipid ordering near the surface of the bilayer as judged by the decrease in the $2T_\perp$ spectral parameter of 5-doxyl stearic acid and by the increase in the low field line width of the cholestane probe. It is of interest that this apparent ordering effect of ethanol near the surface of the membrane is eliminated by the addition of salt solutions such as NaCl (5–50 mM). Both monovalent and divalent cations are known to cause greater ordering of lipids in membranes [1]. We have also observed a large increase in the low field line width of the cholestane EPR spectrum following the addition of millimolar concentrations of Ca^{2+} into the medium of bovine brain phospholipid vesicles (unpublished observations). Thus, it is possible that the small increase in membrane rigidity near the membrane surface which is brough about by ethanol may be masked by the effects of the cations found in physiological fluids.

Ethanol concentrations greater than 4 mM produced an increase in motion of the cholestane probe, whereas increases in lipid mobility in the environment of the 5-doxyl stearic acid probe were not detected until ethanol concentrations greater than 200 mM were achieved. Since both the location of these EPR probes in lipid membranes, as well as the molecular motion detected by them are different, it would be difficult to state categorically that one or the other probe was detecting changes in membrane organization representative of those brought about by ethanol in the microenvironment of the glutamate receptors in synaptic membranes.

All EPR probes used in this study have shown a definite fluidization of lipid membranes at high concentrations of ethanol. It was interesting that even though 4 mM ethanol caused only a slight change in the EPR spectrum of the cholestane probe, it nevertheless brought about a rather marked lowering in the phase transition temperatures of these lipid membranes (Fig. 5B). On the basis of these observations and of the ethanol effects on the transition temperature of the glutamate binding activity of synaptic membranes, it would seem reasonable to suggest that subtle effects of this alcohol on membrane organization may be revealed best through studies of ethanol's influence on the temperature-induced phase transitions in membrane lipids.

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