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Ethanol Effects on Synaptic Glutamate Receptor Function and on Membrane Lipid Organization

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MICHAELIS, E. K., H. H. CHANG, S. ROY, J. A. McFAUL AND J. D. ZIMBRICK. *Ethanol effects on synaptic glutamate receptor function and on membrane lipid organization.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 1–6, 1983.—The enhancement of L-glutamic acid binding activity of brain synaptic membranes by low concentrations of ethanol (<50 mM) and the decrease in binding at high concentrations (>100 mM) was not due to a direct action by ethanol on the glutamate binding protein. Biphasic effects of ethanol on membrane protein complexes such as the glutamate binding sites might be the result of biphasic changes in membrane lipid organization. Low ethanol concentrations (0.1–4.0 mM) were shown to decrease fatty acid chain motion detected by the EPR probe 5-doxyl stearic acid, whereas high concentrations (>400 mM) increased lipid motion in egg phosphatidylcholine liposomes. The function of the L-glutamate receptor-ion channel complex in the presence of ethanol was also determined by measuring the changes in thiocyanate (SCN^-) influx brought about by L-glutamate or ethanol. A low concentration of ethanol (9.4 mM) diminished the L-glutamate-induced depolarization of synaptic membranes, while a high concentration (93.7 mM) increased the passive SCN^- influx and produced a transient overshoot in glutamate-stimulated SCN^- flux.

Ethanol Glutamate receptors Liposomes Membrane lipids Membrane structure EPR Synaptic membranes

INTERACTION of ethanol with neuronal membranes is known to affect depolarization-induced Na^+ and Ca^{2+} fluxes (e.g., [2, 9, 12, 30, 35]), to alter the activity of membrane-bound enzymes such as the ($Na^+ + K^+$) ATPase, adenylate cyclase, and 5′ nucleotidase (e.g., [11, 15, 18, 20, 22, 32]), and to change the interaction of neurotransmitters with their respective membrane receptor complexes (e.g., [15, 26, 28, 33]). The multiple effects of ethanol on the activity of membrane-associated ion channels, enzymes, and receptors are thought to be produced through a perturbation of the membrane lipid and protein organization which follows ethanol interaction with the membrane bilayer (e.g., [7, 8, 11, 13, 14, 16, 17, 19, 31]). The change in the physical state of the membrane lipid environment has most often been described as being one of increased phospholipid fatty-acyl chain motion caused by exposure of the membrane bilayer to increasing concentrations of ethanol [7, 8, 13, 17, 31].

In some studies the increase in fatty acid chain isomerizations brought about by ethanol has been shown to be a continuously increasing, monotonic function that is related to the ethanol concentration in the surrounding medium [7, 13, 17]. Yet, for a variety of membrane-associated enzymes, receptors, and divalent cation binding sites, the changes in activity produced by increasing ethanol concentrations are of a biphasic nature, i.e., they show enhanced activity at low ethanol concentrations, (<50 mM) and decreased activity at higher ethanol concentrations (>100 mM) [24–26, 28, 32]. These observations may indicate that the effect of ethanol on the activity of these membrane protein complexes is not due totally to the alcohol-induced perturbation of the organization of their lipid environment but may be in part the result of a direct action of ethanol on the protein conformation. Alternatively, ethanol may cause biphasic changes in lipid motion in a population of phospholipids that form the boundary region surrounding some of the membrane proteins.

We have previously shown that, in egg phosphatidylcholine (PC) liposomes which contained the electron paramagnetic resonance (EPR) probe 5-doxyl stearic acid, there was an indication of a biphasic effect of ethanol on fatty acid chain isomerizations [29]. This effect was seen most clearly when the more superficially located nitroxide group of 5-doxyl stearic acid was used, as compared to the more deeply located probes of 7- and 12-doxyl stearic acid [29]. Furthermore, the range of low ethanol concentrations that produced an apparent decrease in fatty acid chain motion included the ethanol concentrations which we have previously found to produce an increase in L-[^{3}H] glutamic acid and ^{45}Ca binding to synaptic membranes and in the activity of the Na^+-Ca^{2+} exchange transport in isolated-resealed synaptic membranes [24–26, 28].

We have explored in the present study the effects of ethanol on the activity of a purified membrane protein, the

purified L-glutamate binding protein, and compared these effects with ethanol's actions on L-[^{3}H] glutamate binding to intact synaptic membranes and on L-glutamate activation of the glutamate receptor-ion channel complex in these membranes. In addition, we have attempted to provide more detailed documentation of the decrease in fatty acid chain motion near the surface of PC liposome membranes produced by exposure to low ethanol concentrations.

METHOD

Synaptic Membrane Preparations and Glutamate Binding Protein Purification

Synaptic plasma membrane vesicles from adult, male Sprague Dawley rats were prepared and stored as previously described [4]. The glutamate binding protein was extracted and purified from freshly prepared batches of synaptic membranes according to the published procedure [23]. The purified protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing in the presence of Triton X-100 and was found to be homogeneous for the small M_r glycoprotein which was previously shown to be the glutamate binding protein [27].

L-[^{3}H] Glutamate Binding and [^{35}S]SCN- Uptake Measurements

Glutamate binding to the purified binding protein was measured by a Millipore filtration assay employing L-[^{3}H] glutamic acid (40–50 Ci/mmol, New England Nuclear Corporation) as was described elsewhere [23]. The uptake of [^{35}S] thiocyanate (30 Ci/mol, New England Nuclear Corporation) by synaptic plasma membrane vesicles was measured under conditions identical to those described in a previous paper [6].

EPR Spectroscopy of Egg PC Liposome Membranes

The preparation of egg PC liposomes, the incorporation of the 5-doxyl stearic acid EPR probe in the liposome structure, and the measurement of the EPR spectra were conducted according to the procedures reported previously [29]. The order parameter S from each spectrum was calculated according to the formula shown below which includes a correction for differences in solvent polarity:

$$S = \frac{T_{\parallel}' - T_{\perp}'}{T_{zz} - T_{xx}} \cdot \frac{a}{a'}$$

where $a = (1/3)(2T_{xx} - T_{zz})$ and

$$a' = (1/3)(T_{\parallel}' + 2T_{\perp}')$$

The values $2T_{\parallel}'$ and $2T_{\perp}'$ are the separations between the outer and inner hyperfine extrema of the 5-doxyl stearic acid spectra, and T_{zz} and T_{xx} were obtained from single crystal data [3].

RESULTS

The *in vitro* exposure of synaptic membranes to ethanol concentrations ranging between 2.5 and 25 mM produced an increase in L-[^{3}H] glutamic acid binding to the high affinity synaptic membrane binding sites [26,28]. Ethanol concentrations higher than 50 mM brought about a progressive decrease of L-[^{3}H] glutamate binding towards the baseline control level, and concentrations higher than 100 mM tended to produce a small degree of inhibition of the synaptic membrane L-[^{3}H] glutamate binding activity ([26,28] and unpublished observations). In order to determine whether these biphasic effects of ethanol on the activity of putative L-glutamate receptors in synaptic membranes are the result of a direct ethanol action on the glutamate binding protein, the effects of *in vitro* exposure of the purified glutamate binding protein to ethanol on the protein binding activity were determined (Fig. 1). As is shown in Fig. 1 ethanol at concentrations ranging between 2.5 and 25 mM caused a small to moderate degree of inhibition of L-[^{3}H] glutamate binding to the purified protein, while at concentrations of 50 and 100 mM, ethanol had no significant effect on the L-glutamate binding activity of the protein. Since the influence of ethanol on the binding activity of the purified protein was determined under conditions that were identical to those employed previously to measure the effects of ethanol on the membrane-attached glutamate binding sites, it would appear that the enhancement of L-[^{3}H] glutamate binding to the synaptic membranes at low ethanol concentrations cannot be ascribed to a direct effect of this alcohol on the glutamate binding protein.

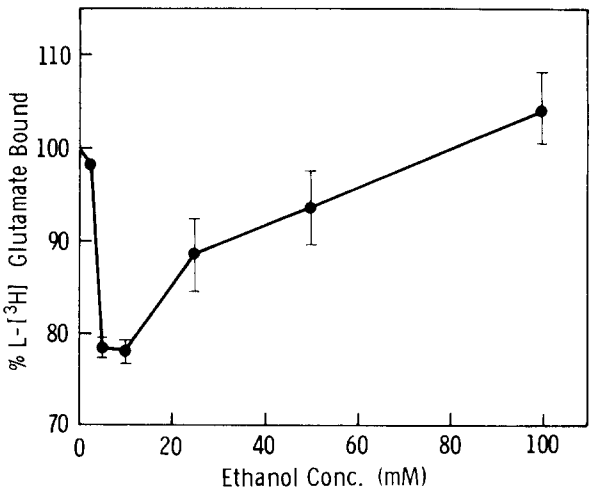

FIG. 1. Effects of increasing ethanol concentrations on L-[^{3}H] glutamate binding to the purified glutamate binding protein. All assays were conducted in triplicate at 24°C for 25 min, in the absence or presence of the ethanol concentrations shown, according to the procedures described in the Method section. Final protein concentration in each assay was 1 μg/ml and the concentration of L-[^{3}H] glutamic acid was 96 nM. The specific L-glutamate binding activity of the control samples in the absence of ethanol was 0.922±0.058 nmol/mg (mean±S.E.M.). Each value is the mean of three experiments.

The differences between the effects of ethanol on the synaptic membrane glutamate binding sites and those on the purified protein may be indicative of ethanol interactions with some synaptic membrane lipids which secondarily influence glutamate binding activity. Model lipid membranes, in particular egg PC liposomes, were used in the current attempts to detect the appearance of a biphasic ethanol-induced change in lipid organization within the bilayer. Since in our previous studies egg PC liposomes labeled with 5-doxyl stearic acid exhibited changes in EPR signals consistent with a small decrease in lipid motion following exposure to low ethanol concentrations (0.02–20 mM), the same liposome membrane system was used to examine in greater de-

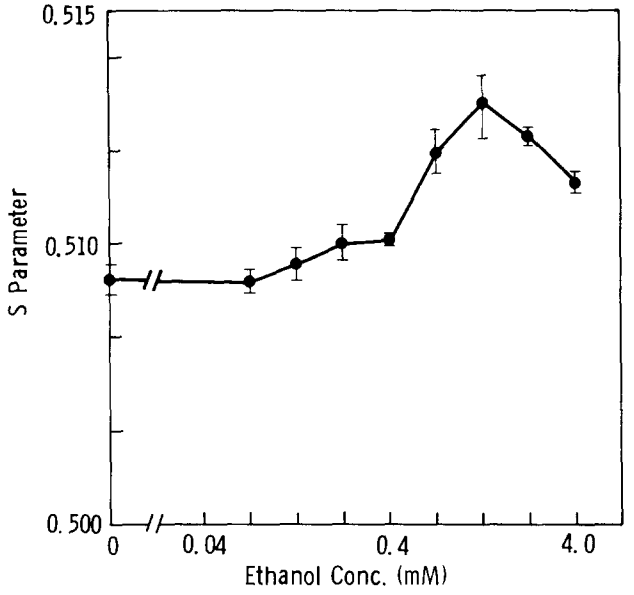

FIG. 2. Changes in egg PC order parameter in the presence of increasing ethanol concentrations. The egg PC liposomes were formed and labeled with 5-doxyl stearic acid as described in the Method section. The PC liposomes were formed in water and all measurements were obtained at 24°C following a 2 min preincubation with ethanol. Each data point is the mean (±S.E.M.) of 5 experiments.

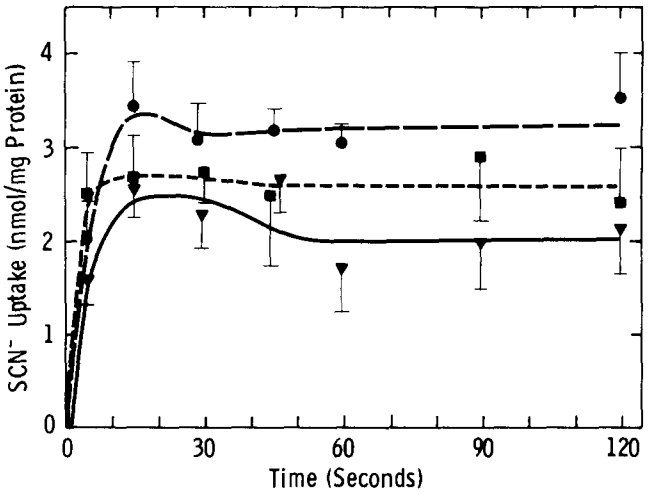

FIG. 3. Ethanol-induced changes in passive [^{35}S] SCN^- flux in synaptic plasma membrane vesicles. Synaptic membrane vesicles (40 μg protein) preloaded with sucrose buffer were diluted into 100 μg of external solution containing 50 mM Na_2SO_4/5 mM Tris-H_2SO_4/1 mM K ^{35}SCN/120 mM sucrose. Assays were conducted in duplicate at 24°C in the absence (▼) or in the presence of either 9.4 mM (■) or 93.7 mM (●) ethanol. All membrane aliquots were preincubated for 30 min at 4°C either with buffered sucrose or with sucrose containing ethanol. Each point is the mean (±S.E.M.) SCN^- uptake for 5 membrane preparations.

tail this phenomenon of ethanol-induced decreases in fatty acid chain isomerizations. The results of these studies are shown in Fig. 2. It is quite clear that in the egg PC liposomes, at 24°C, ethanol concentrations ranging between 0.14 and 1.62 mM produced a concentration-dependent increase in the lipid order parameter. Ethanol concentrations greater than 1.62 mM caused a decrease in the S parameter towards the baseline values (Fig. 2) and at 40 mM ethanol the S parameter was almost identical to the baseline values (data not shown). Ethanol concentrations greater than 400 mM produced a substantial decrease in the S parameter below the control values. The increases in the S parameter produced by ethanol concentrations between 0.4 and 1.62 mM were statistically significant at the 95% confidence level or higher. Statistical significance of differences was determined by Student's *t*-test for unpaired samples with a minimum of 15 *df* for any set of values tested. In addition, the slope of the line for the data points between 0.14 and 1.62 mM was significantly different from the zero slope as determined by the *t* statistic [10].

The net effect of ethanol on the activity of various membrane protein complexes may be due to a combination of the direct interaction of this alcohol with the proteins and of the changes in the organization of certain lipids within the plasma membrane. The glutamate binding protein is a hydrophobic, intrinsic membrane protein in the synaptic region of brain neurons [27] and its function could be influenced by both direct and lipid-mediated ethanol effects. However, the demonstration of ethanol-induced changes in glutamate binding activity described above does not necessarily indicate the ultimate effect of this alcohol on the activity of the L-glutamate receptor-ion channel complex in these membranes. The process of activation of these glutamate receptor-ion channel complexes in isolated-resealed synaptic plasma membranes has been studied in this laboratory by: (a) monitoring the L-glutamate initiated Na^+ flux across the vesicular membrane [4,5], and (b) determining the electrogenic nature, i.e., depolarizing effect, of this glutamate-stimulated ion flux by measuring the distribution of the lipophilic anion [^{35}S] thiocyanate into synaptic membrane vesicles [6]. The potential difference ($\Delta\psi$) in these membrane preparations can be calculated from the distribution of the lipophilic anion SCN^- according to the Nernst equation

$$\Delta\Psi = (RT/nF)\ \ln([SCN^-]_{in}/[SCN^-]_{out})$$

The latter method was used in the present studies to determine the effects of ethanol on passive ion fluxes in these membranes as well as on the glutamate-stimulated SCN^- accumulation.

The results shown in Fig. 3 demonstrate the effects of 9.4 and 93.7 mM ethanol on the passive SCN^- accumulation in the resealed synaptic plasma membrane vesicles. When these sucrose-loaded vesicles were incubated in a medium containing 50 mM Na_2SO_4, the SCN^- uptake reached a plateau level of approximately 2 nmol/mg protein. The plateau level of SCN^- accumulation under these incubation conditions was previously shown to be due to the greater membrane diffusion potential of Na^+ as compared to SO_4^{2-} [6]. Ethanol appeared to enhance the passive accumulation of SCN^- into synaptic membrane vesicles, especially when it was present at a concentration of 93.7 mM (Fig. 3). This might indicate a greater diffusion potential for Na^+ in the presence of increasing ethanol concentrations.

Exposure of the synaptic membranes to increasing concentrations of L-glutamate leads to increased SCN^- influx in these membranes which reaches a maximum at 1–10 μM L-glutamate [6]. When 5 μM L-glutamate was introduced

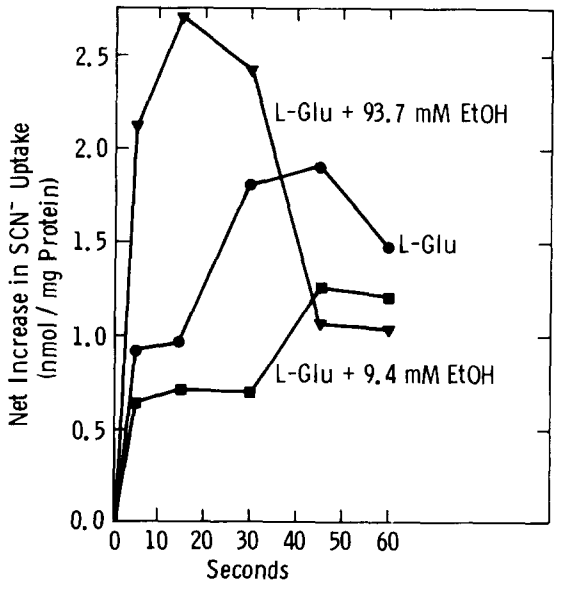

FIG. 4. Effects of ethanol on L-glutamate-stimulated SCN^- uptake in synaptic membranes. The net increase in SCN^- influx brought about by exposure of the membranes to 5 μM L-glutamic acid is shown. Both control and L-glutamate-exposed membrane vesicles were incubated in the Na_2SO_4 medium described in Fig. 3 in the absence of ethanol (●) or in the presence of either 9.4 mM (■) or 93.7 mM (▼) ethanol. Each point is the mean of duplicate determinations from 3 or 4 membrane preparations. The average standard error of the mean was 14%.

into the assay medium, there was a time-dependent net increase in SCN^- influx above that observed under passive diffusion conditions (Fig. 4). The L-glutamate-stimulated SCN^- accumulation was markedly inhibited by preincubation of the membranes in 9.4 mM ethanol (Fig. 4). On the other hand, 93.7 mM ethanol caused a large initial overshoot in glutamate-induced SCN^- uptake which rapidly returned to lower levels (Fig. 4). An intermediate ethanol concentration (56.3 mM) produced a lesser overshoot of L-glutamate-stimulated SCN^- influx than that observed in the presence of 93.7 mM ethanol (data not shown). Overall, a lower ethanol concentration (9.4 mM) appeared to produce an inhibition of both the initial and late phases of L-glutamate-stimulated SCN^- influx, whereas a higher ethanol concentration (93.7 mM) tended to enhance the initial phase of glutamate-induced SCN^- uptake.

DISCUSSION

The demonstration in the present study that the ethanol-induced increase in the L-glutamate binding activity of synaptic membranes was not due to a direct effect of ethanol on the glutamate binding protein was suggestive of a lipid-mediated action of ethanol on these membrane glutamate receptor sites. Partial support for this interpretation has previously been obtained by demonstrating the importance of a class of synaptic membrane lipids, the gangliosides, in the expression of ethanol-induced enhancement of the high affinity L-[^{3}H] glutamate binding to synaptic membranes at low ethanol concentrations (4 mM) [26]. The documentation in the present study of the biphasic response of lipid motion in egg PC liposomes to increasing ethanol concentrations represents further evidence that the biphasic actions of this alcohol on some membrane protein complexes may be produced in part through a lipid-mediated change. No direct link has been demonstrated between the biphasic effects of ethanol on L-[^{3}H] glutamate binding and those on egg PC liposome fatty acid-chain organization. It would be worthwhile, nevertheless, to pursue the idea that ethanol alters membrane organization in the direction of either greater or lesser acyl chain motion depending upon its concentration and upon the lipid composition of a given membrane.

It has previously been suggested that ethanol may alter the dielectric constant in the environment of transmitter receptors and that such a change may cause an alteration in the rate of the decay of synaptic currents [1]. If ethanol did produce a change in the interaction between a group of charged membrane lipids, such as the phospholipids or gangliosides, and the glutamate receptor protein complex, then it might affect not only the receptor's binding activity but also the ion conducting properties of this receptor complex. The results obtained in our studies of glutamate-induced depolarization in synaptic membranes determined by the distribution of the lipophilic anion SCN^-, were indicative of an inhibition of glutamate-stimulated Na^+ diffusion by low ethanol concentrations (9.4 mM). Ethanol at this low concentration did not inhibit the passive synaptic membrane permeability to Na^+. On the contrary, both lower and higher ethanol concentrations have been found to cause a concentration-dependent increase in passive SCN^- influx (Fig. 3 and unpublished observations). Thus, the inhibition of glutamate-induced SCN^- flux cannot be attributed to a non-specific effect of ethanol on the passive ion permeability of synaptic membranes.

Higher concentrations of ethanol that would normally be considered within the general anesthetic range tended to produce a large initial overshoot of glutamate-induced ion fluxes which rapidly returned to lower levels. This overshoot may be due in part to the increase in passive membrane permeability brought about by ethanol at high concentrations, although the background increase in SCN^- influx with either 9.4 or 93.7 mM ethanol was subtracted from the values obtained for glutamate-enhanced SCN^- uptake determined in the presence of each ethanol concentration. On the basis of these results it was apparent that the response to ethanol of the glutamate receptor-ion channel complex did not follow the same pattern as the binding of L-[^{3}H] glutamic acid. Whereas glutamate binding to synaptic membrane sites was increased by low ethanol concentrations [26,28], the glutamate-induced ion flux was decreased in the presence of low alcohol concentrations. Concentrations of ethanol around 100 mM did not produce significant changes in L-[^{3}H] glutamate binding to synaptic membranes yet, similar ethanol concentrations led to a transient enhancement of receptor-coupled ion fluxes.

The effects of ethanol on brain synaptic membrane glutamate receptors may be comparable to the effects of volatile and local anesthetics on cholinergic receptors. These agents have been shown *in vitro* to increase the rate of transition of the acetylcholine receptors in *Torpedo* electroplax membranes and in brain neuronal membranes to a high affinity binding but desensitized state [21,34]. If ethanol produces similar changes in synaptic membrane glutamate receptors, then the increase in binding activity brought about by exposure of the membranes to low ethanol concentrations may be related to a desensitized state of the receptor-ion channel complex. Alternatively, ethanol may alter the kinetics of

glutamate receptor-ion channel activation or the decay of ion currents in the synaptic region. This latter action of ethanol has been described previously for glutamate-induced excitatory postsynaptic potentials in invertebrate neuromuscular junctions [1]. The kinetic characterization of ion fluxes needed to distinguish between these two mechanisms would require measurements of ion flux changes within the range of milliseconds, which we have not yet achieved in our studies. Such characterization will nevertheless be needed in order to achieve a better understanding at the molecular level of ethanol's actions on the glutamate excitatory synaptic activity in the mammalian central nervous system.

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