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Ethanol affects the function of a neurotransmitter receptor protein without altering the membrane lipid phase

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Abstract

Using patch-clamp and fluorescence techniques we found that ethanol (10–200 mM) potentiated strychnine-sensitive glycine receptors without having detectable effects on lipid order parameters in mouse spinal cord neurons. Hepthanol (0.01–1 mM), in contrast, did not affect the glycine current, but it altered the core and surface of spinal neuron membranes as detected by changes in 1,6-diphenyl-1,3,5-hexatriene (DPH) and Laurdan fluorescence parameters. These findings support the idea that ethanol affects these membrane proteins without changing lipid fluidity. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Glycine receptor; Spinal neuron; Ethanol; Cl^- current; Laurdan; Anisotropy; Strychnine

1. Introduction

It is known that alcohols can affect several receptors and ion channels in neuronal membranes. For example, at concentrations detected during intoxication, ethanol inhibits *N*-methyl-D-aspartate (NMDA) (Lovinger et al., 1989) and potentiates chloride currents activated by gamma aminobutyric acid ($GABA_A$) and glycine in central mammalian neurons (Aguayo and Pancetti, 1994). The fundamental question of whether or not ethanol affects these receptors by direct interaction with the receptor protein or by disordering of the acyl chains of phospholipids in the neuronal membrane is still unresolved.

Recent work in our laboratory recording the electrophysiological activity of $GABA_A$ and glycine receptors in single brain neurons showed that ethanol can selectively affect these two receptors (Aguayo and Pancetti, 1994; Aguayo et al., 1996). Furthermore, the action of ethanol on $GABA_A$ and glycine receptors can be modified by altering the state of phosphorylation of these receptors (Aguayo and Pancetti, 1994; Aguayo et al., 1996; Wafford and Whiting, 1992; Weiner et al., 1994). We believe the above results support the conclusion that these effects of ethanol are mediated through specific changes in membrane proteins and not through changes in lipid domains. To study if the effects of ethanol on glycine receptors in central mammalian neurons are mediated by changes in the lipid microenvironment, we studied the effect of ethanol by using patch-clamp whole-cell recordings and two fluorescent probes that give an indication of membrane fluidity at different levels in the membrane (Parasassi and Gratton, 1995; Aguilar et al., 1996). The present study provides functional and physico–chemical evidence indicating that ethanol affects receptor function in spinal neurons without changing the membrane lipid order.

2. Materials and methods

2.1. Cultured neurons

A timed (13–14 days) pregnant mouse (C57BL/J6) was placed in a closed bucket containing paper tissue wetted with ether before cervical dislocation. The embryos were quickly decapitated and the spinal cords removed. Spinal neurons were plated at 150,000 cells/ml into 35-ml tissue culture dishes coated with poly-L-lysine (mol. wt. > 350 kDa, Sigma, St. Louis, MO, USA). The neuronal feeding medium consisted of 95% minimal essential

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medium (MEM, GIBCO), 5% heat-inactivated horse serum (GIBCO, Rockville, MD, USA) and a mixture of nutrient supplements (Aguayo and Pancetti, 1994). The neurons were treated for 1 day with a mixture of 5′-fluoro-2-deoxyuridine (15 mg/ml) and uridine (35 mg/ml) to stop the growth of background cells. The medium was changed every 3 days.

2.2. Recordings

The current recording was made using the patch-clamp technique (Hamill et al., 1981). The culture medium in the dish was changed to an external solution containing (in mM) 150 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4) and 10 glucose. The standard internal solution contained (in mM) 120 CsCl, 4.0 $MgCl_2$, 10 BAPTA, 10 HEPES and 2 ATP–Na_2 (pH 7.35). The cells were stabilized at 36°C for at least 30 min before the recordings were started. The whole-cell recordings were made by using an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA, USA). The holding potential was −60 mV. Electrodes were pulled from borosilicate capillary glass (WPI, Sarasota, FL, USA) in two stages on a vertical puller (Sutter Instruments, Novato, CA, USA). In order to rapidly apply increasing concentrations of glycine and ethanol, we used an array of tubes (200 μm in internal diameter) placed within 50 μm of the neuron. Recording of the generated junction potential after the normal extracellular solution was switched to one containing 1/4 NaCl (replaced with sucrose) revealed that full solution exchange occurred in less than 100 ms.

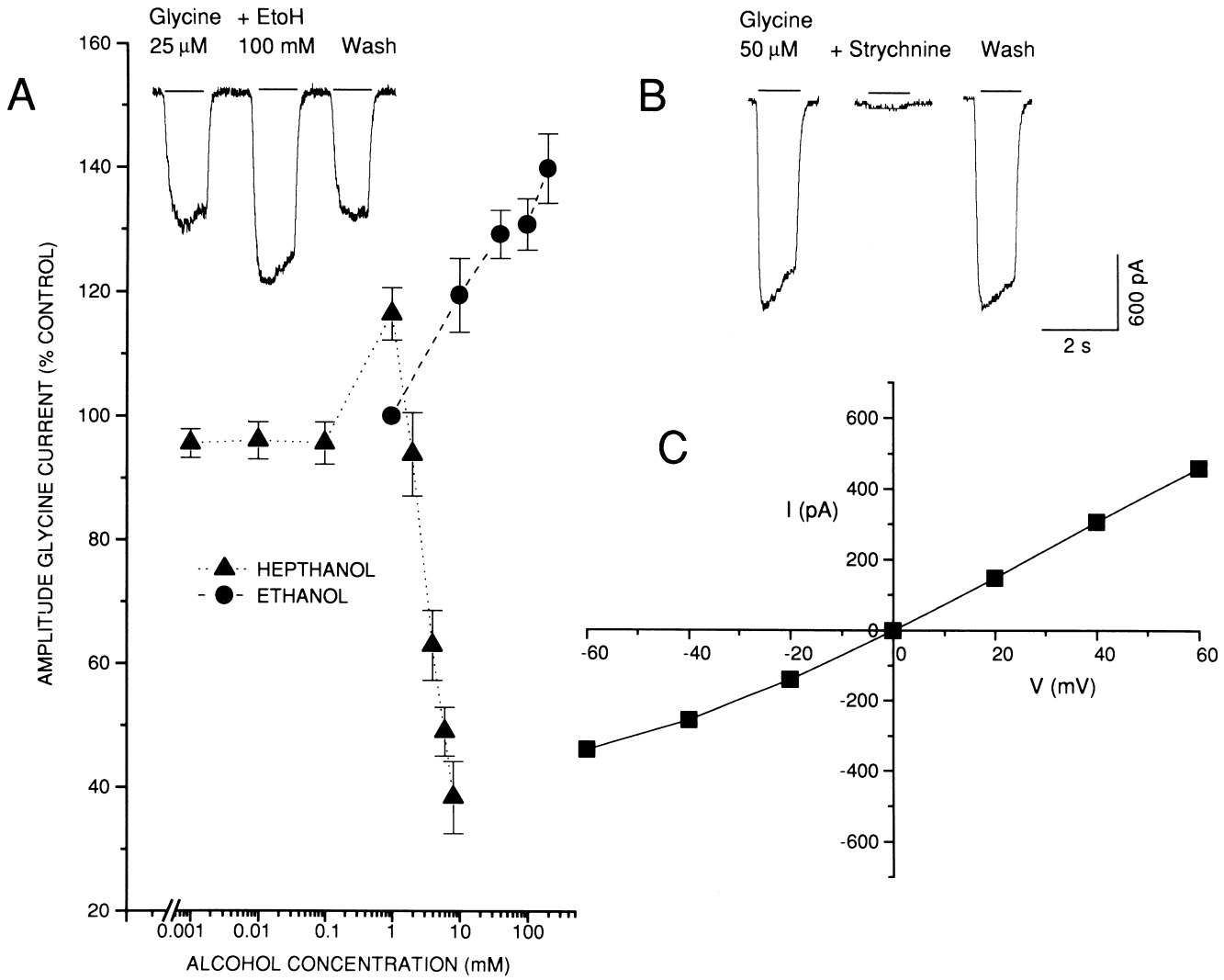

Fig. 1. Effect of ethanol and hepthanol on the Cl^- current. (A) The peak amplitude of the current was measured in the absence and presence of several concentrations of ethanol (closed circles) and hepthanol (closed triangles) at 36°C. Note that the effect of ethanol was concentration dependent. Data points represent the mean ± S.E.M. from 5 neurons. Current traces in inset show the reversible effect of 100 mM ethanol. (B) The current activated by glycine was reversibly blocked by 100 nM strychnine. (C) The data show a current–voltage relationship at voltages between −60 and +60 mV. The reversal potential of the glycine current was close to −3 mV.

2.3. Fluorescence spectroscopy

The anisotropy measurements were performed in a GREG 200 multifrequency phase-modulation spectrofluorometer (ISS, Champain, IL, USA) in the 'L' configuration, using Glan Thompson prism polarizers in both exciting and emitting beams, and was evaluated by:

$$r = \frac{(I_{\parallel}/I_{\perp}) - 1}{(I_{\parallel}/I_{\perp}) + 2} = \frac{(I_{VV}/I_{VH})1/G - 1}{(I_{VV}/I_{VH})1/G + 2}$$

where $G = I_{HV}/I_{HH}$, $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities when the emission beam polarizer is oriented parallel and perpendicular to the direction of the excitation beam polarizer (vertically oriented), respectively. I_{VV}, I_{VH}, I_{HH} and I_{HV} are the uncorrected fluorescence intensities of the four possible combinations of the excitation and emission polarizer orientations. The excitation light from a xenon arc lamp was set through the monochromator at a wavelength of 360 nm. The emission was measured through a WG-420 highpass filter (Schott Glaswerke, Mainz, Germany) which showed negligible fluorescence.

Laurdan's fluorescence spectrum shifts were quantified by using a general polarization (GP) parameter (Parasassi and Gratton, 1995) defined as;

$$GP = (I_g - I_{lc})/(I_g + I_{lc}),$$

where I_g and I_{lc} are the intensities observed at the wavelengths typical of maximum emission in the gel and in the liquid–crystalline phase, respectively. Fluorescent intensities (I_g and I_{lc}) were measured in a Fluorolog photon counting spectrofluorometer (Spex Industries, Metuchen, NJ., USA), using an excitation wavelength of 360 nm. The emission intensity at wavelengths of 440 and of 490 nm was used to calculate GP as follows:

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490}).$$

Both spectroflurometers were interfaced to a personal computer and appropriate software (ISS Scan 1.0) was used for data collection and analysis. Measurements were done with spinal neurons grown on glass plates placed vertically in a 1-cm path length square quartz cuvette with external solution at an angle of 45° with respect to the excitation beam. Concentrated solutions of fluorescent probes in dimethylsulfoxide (DMSO) were diluted in the culture medium to a final probe concentration of 1 μM for DPH and 10 μM for Laurdan, with an incubation period of 1 h at 37°C. DMSO concentration was always less than 0.1%. Prior to measurements, samples were repeatedly washed with external solution in order to remove unbound probe and culture medium which showed significant fluorescence. Background fluorescence was checked against blank unlabelled samples treated in the same way and was kept to a negligible level (< 5%). Sample temperature was

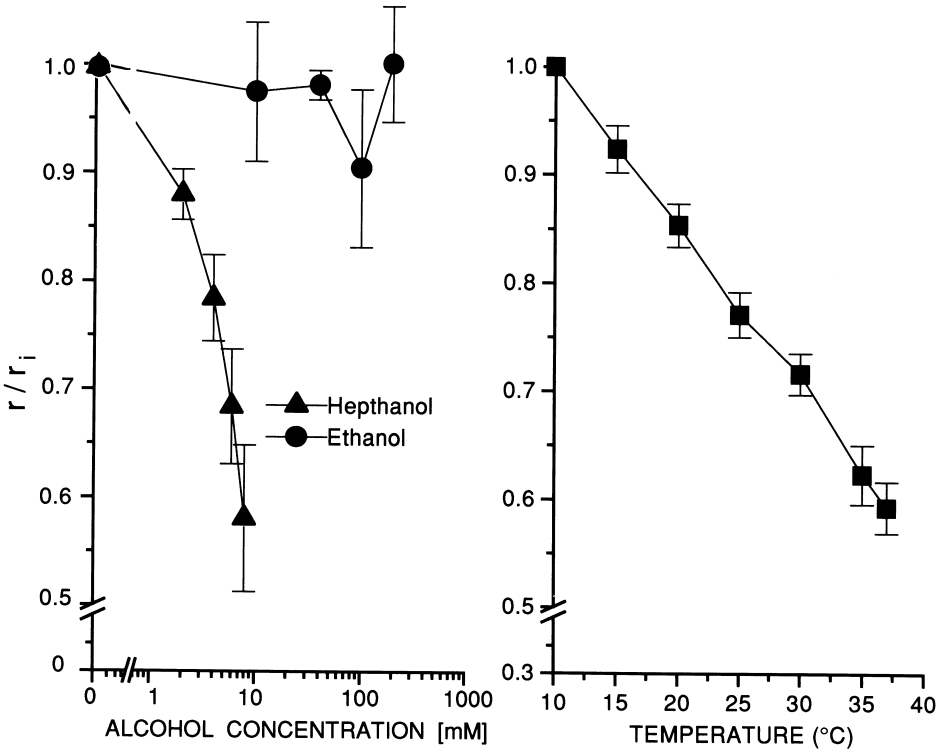

Fig. 2. Fluorescence anisotropy of DPH incorporated in mouse spinal neurons. Left panel, effect of ethanol (closed circles) and hepthanol (closed triangles). The given anisotropy values, at different concentrations, are relative to those obtained in the absence of the alcohol ($r_i = 0.16$). The temperature was 37°C. Right panel, effect of temperature on fluorescence anisotropy. The values are compared to those obtained at 10°C ($r_i = 0.29$). Data are means ± S.E.M. from 3 experiments.

controlled by an external bath circulator and the temperature was measured prior to and after each measurement with a digital thermometer.

1,6-diphenyl-1,3,5-hexatriene (DPH) and 2-dimethylamino-6-lauroylnaphthalene (Laurdan) were from Molecular Probes (Eugene, OR, USA). Ethanol (UVasol grade) was from Merck (Darmstadt, Germany) and *n*-hepthanol was from Aldrich (Milwaukee, WI, USA). Glycine, strychnine and DMSO were from Sigma.

3. Results

The data in Fig. 1 show that ethanol (closed circles) potentiated the Cl^- current at pharmacologically relevant concentrations between 10 and 200 mM (Rall, 1990). The Cl^- current activated by glycine had an EC_{50} of 25 μM (not shown) and reversed polarity at a potential of about -3 mV, which is close to the Cl^- reversal potential under the experimental conditions used (Fig. 1C). Because the glycine-activated current was inhibited by nanomolar concentrations of strychnine (Fig. 1B), we believe that these receptors contain the $\alpha 1$ subunit variant (Kuhse et al., 1990). Concentrations of hepthanol between 0.001 and 0.1 mM (Fig. 1A, closed triangles) had no effect on the amplitude of the glycine current. A small, but consistent increase in the current amplitude was found with 1 mM hepthanol (Fig. 1A). At concentrations of hepthanol between 2 and 10 mM, the amplitude of the current was strongly inhibited. Higher concentrations of hepthanol (> 10 mM) caused damage to the neurons and the gigaohm seal rapidly deteriorated.

Parallel experiments in the same set of spinal neurons showed that ethanol, at concentrations that potentiated the glycine current, had no effect on fluorescence polarization of DPH (Fig. 2, right panel, closed circles). In order to assure that the experimental set up was sufficiently sensitive to detect changes in lipid-packing order, we tested several concentrations of hepthanol and changed the temperature of the cells with the aim of increasing membrane lipid disorder. Unlike ethanol, hepthanol produced a concentration-dependent reduction of the fluorescence polarization of DPH (Fig. 2, right panel, closed triangles). Likewise, an increase in the temperature of the cells from 10 to 37°C had a clear effect on the lipid order parameter, indicating that an increase in membrane fluidity was achieved (Fig. 2, left panel). These data indicate that this experimental procedure is sensitive enough to detect changes in the membrane lipid order.

Very similar results were obtained with Laurdan, a probe that detects changes in molecular dynamics of the polar head regions of the lipid bilayer. Fig. 3A (right panel, closed circles), for instance, shows that ethanol (up to 400 mM) did not produce any change in the GP parameter. With hepthanol there was a 50% reduction in this parameter. Increasing the temperature of the neurons

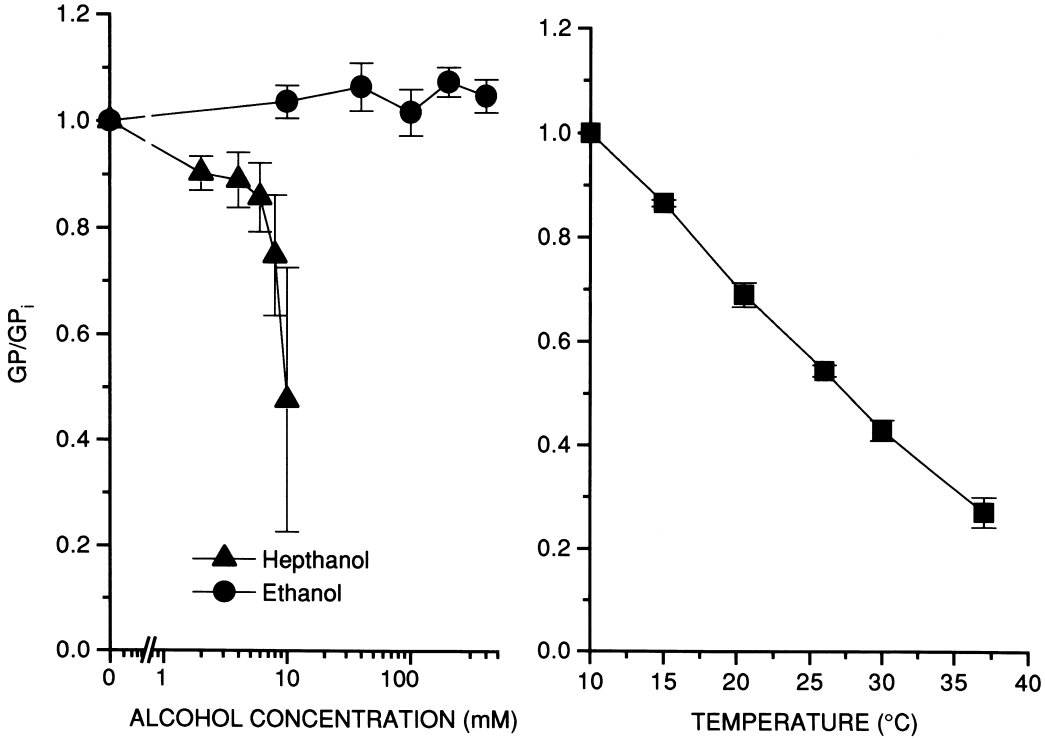

Fig. 3. GP of Laurdan incorporated in mouse spinal neurons. Left panel, effect of ethanol (closed circles) and hepthanol (closed triangles). The given values at different concentrations are relative to those in the absence of alcohol ($GP_i = 0.14$, 37°C). Right panel, effect of temperature. The values given are relative to those measured at 10°C ($GP_i = 0.46$). Data are means ± S.E.M. from 3 experiments.

also produced a decrease in this parameter (Fig. 3B, left panel, closed triangles).

4. Discussion

Glycine receptors are expressed in mammalian brain stem and spinal cord where they regulate neuronal excitability (Schmid et al., 1991; Bechade et al., 1994). Like $GABA_A$ receptors (Seighart, 1995), they are believed to have a pentameric nature and are composed of α and β subunit variants (Bechade et al., 1994; Grenningloh et al., 1990). These receptors are strongly inhibited by strychnine (Betz, 1991; Bechade et al., 1994) and potentiated by Zn^{2+} (Bloomental et al., 1994) and pharmacological concentrations of ethanol (Aguayo and Pancetti, 1994; Aguayo et al., 1996). In addition, a recent study showed that the α1 subunit, when expressed in *Xenopus* oocytes, is more sensitive than the α2 subunit to ethanol (Mascia et al., 1996). A single change in amino acid composition markedly reduces the sensitivity of the receptor to ethanol, suggesting that ethanol affects primarily the receptor protein (Mascia et al., 1996). We have recently suggested that the enhancement of glycine receptor-mediated inhibition in spinal neurons is associated with the depression of motor activity and respiratory function during intoxication with ethanol (Aguayo et al., 1996).

Two main theories may explain how ethanol affects the function of the nervous system (see Peoples et al., 1996): (1) direct binding to hydrophobic pockets in membrane proteins (i.e., ion channels, receptors, pumps), or (2) perturbations of membrane lipids with subsequent changes in membrane proteins (Sutherland et al., 1988). If ethanol affects membrane lipids, its site of action should be the polar region or the acyl chain of phospholipids. This study presents data indicating that the effect of ethanol on glycine receptors is not mediated by detectable changes in the physical properties of the lipid phase. Using fluorescent probes which are sensitive indicators of membrane lipid phase fluidity, we obtained experimental evidence that supports this hypothesis. Evaluation of DPH steady-state fluorescence an-isotropy provided information regarding the lipid order at the phospholipid acyl chain level. With its all-trans polyene distributed in the hydrophobic region of the membrane, the rotational motion of DPH is described as a hindered rotation due to the phospholipid acyl chain packing order. In this context, the steady-state fluorescence anisotropy is related primarily to the lipid order (Suwalsky et al., 1994). Laurdan, in contrast, is an amphipatic probe containing lauric acid anchored to the phospholipid acyl chains and its fluorescent moiety is at a shallow depth, at the hydrophobic–hydrophilic interface (Parasassi and Gratton, 1995). Thus, this probe provides information on the dynamic properties of membranes at this interface. Our results indicate that at concentrations that affect the glycine receptor, ethanol does not produce disordering of the lipid acyl chain and does not affect the dynamic properties of the membrane at the lipid polar head level.

As anticipated, we found that either hepthanol, an alcohol with higher membrane disordering potency than ethanol, or a raised temperature increased the fluidity of the neuronal membrane. Ethanol did not affect the fluorescence anisotropy of DPH at concentrations below 200 mM. This finding is in agreement with results obtained with similar concentrations of probes in mitochondrial and microsomal hepatic membranes (Sanchez-Amate et al., 1995). Thus, the main conclusion from our experiments is that ethanol does not affect the spinal glycine receptor by causing a generalized alteration of the fluidity of the membrane. This conclusion is in agreement with data concerning the activity of firefly luciferase and NMDA receptors (Franks and Lieb, 1995; Peoples and Weight, 1995).

In summary, our data indicates that although ethanol affects the glycine receptors in spinal neurons it does not change the macroscopic properties of the lipid phase.

Acknowledgements

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