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ADAPTATION OF BRAIN LIPID BILAYERS TO ETHANOL-INDUCED FLUIDIZATION

SPECIES AND STRAIN GENERALITY*

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Abstract—Membrane lipid compositional changes have recently been shown by us to be at least partially responsible for the apparent tolerance to the membrane-fluidizing effects of ethanol [D. A. Johnson, N. M. Lee, R. Cooke and H. H. Loh, *Molec. Pharmac.* **15**, 739 (1979)]. Because not all effects of ethanol are generalizable to all strains and species, we attempted to determine whether these lipid compositional changes are related to the anesthetic actions of ethanol. Consequently, the effects of ethanol on the fluidity of reconstituted membranes formed from lipid extracts of synaptic membranes from tolerant C57BL/6J mice and Sprague–Dawley rats were assessed by using the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, incorporated into the membranes, as a relative index of fluidity. We observed apparent tolerance to the fluidizing effects of ethanol in both the C57BL/6J mice and the Sprague–Dawley rats. Acute *in vivo* ethanol treatment did not alter ethanol-induced fluidity of the reconstituted membranes. The results further support the suggestion that the change in brain membrane lipid composition, responsible for the apparent tolerance to the membrane-fluidizing effects of ethanol, is related to tolerance to the anesthetic actions of ethanol.

Ethanol has been shown to produce tolerance and physical dependence upon repeated administration. Although the mechanisms responsible for these changes are unknown, the initial site of action of ethanol is generally believed to be on the nerve membrane [1]. Chin and Goldstein [2] observed that chronic *in vivo* exposure to ethanol attenuated ethanol-induced fluidization of intact synaptosomal membranes. Membrane lipid, but not protein, compositional changes were later shown by us to be at least partially responsible for this phenomenon, since ethanol was less able to fluidize bilayers prepared from lipid extracts of membranes from ethanol-tolerant ICR mice [3]. We have also shown [4] that these changes in lipid composition are reversed after removal of drug and are observed in extracts from pentobarbital- but not morphine-tolerant mice. These results suggest that changes in lipid composition can account for tolerance to the membrane-fluidizing effect of ethanol, and that these lipid compositional changes are related to the anesthetic actions of ethanol. In order to establish further that such changes are indeed related to the anesthetic action of ethanol, we have studied, in the work described below, ethanol-induced fluidizability of lipid bilayers formed from crude synaptosomal lipid extracts isolated from C57BL/6J mice and from Sprague–Dawley rats.

MATERIALS AND METHODS

C57BL/6J mice (Simonsen Laboratories, Gilroy, CA) were chronically treated with ethanol, using essentially the same procedure described by Johnson *et al.* [3]. The degree of tolerance was also measured as described. The average sleep time was decreased from 97 ± 2.5 min to 53. 6 ± 2.1 min (N = 29).

Sprague–Dawley rats (Simonsen Laboratories) with a mean (± S.E.) body weight of 377 ± 5.5 g were intubated intragastrically while under light CO_2 anesthesia three times daily for 3 days, using a maximally tolerable dosage regimen, as previously described [5, 6]. Briefly, just prior to intubation the degree of intoxication was determined by judging the righting ability of the animals. The dose was adjusted according to the degree of intoxication with possible doses of 6.0, 4.0, 3.0, 2.0 and 0.0 g of ethanol/kg. The intervals between doses were not exactly 8 hr, so the doses were prorated accordingly. The ethanol solution was 20%, w/v, in Slender (Carnation Co.). Additional Slender was administered so that the total volume of each intubation was 14 ml. In order to maintain body weight, a fourth daily 14 ml intubation of Slender was given at irregular times at least 2 hr removed from an ethanol intubation. Control animals were intubated with a 35%, w/v, sucrose solution in Slender which was isocaloric to the ethanol solution. Each control animal received an amount of sucrose diet proportional to the mean

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amount of ethanol diet given to the experimental group. Total volume of the sucrose intubation was also made up to 14 ml with additional Slender, and a fourth daily intubation of Slender was given. Food was removed from the cage at the start of intubation; water was available *ad lib*.

Rats from which lipid bilayers were prepared were killed 1 hr after their first missed dose, a time at which blood alcohol levels begin to decline but at which withdrawal has not yet commenced. Dependence was assessed in another group by measuring seizure susceptibility 9 hr after the first missed dose. Each animal was injected i.p. with 50 mg/kg of pentylenetetrazol (Metrazol) in a volume of 4 ml/kg and placed in an individual observation chamber. Three variables were determined: time to onset (latency) of clonic seizure, duration of seizure and intensity of seizure, scored as 0 for no seizure, 1 for clonic seizure, 2 for tonic seizure, and 3 for death following seizure. If no seizure occurred, a latency of 180 sec was assigned. Maximum seizure duration recorded was 10 min.

Tolerance to ethanol was also produced by this dosage regimen as evidenced by attenuation of ethanol-induced analgesia following similar treatment [5]. Furthermore, less severe treatment (3.0 g/kg twice daily for 3 days) produced tolerance with regard to both ethanol-induced analgesia and sleep time (unpublished observations, 1979).

Lipid bilayers were prepared and extracted, and steady state fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), incorporated into these bilayers, was measured as described previously [3].

RESULTS AND DISCUSSION

In order for the mechanism of ethanol tolerance development to be initiated at the membrane level by changing membrane physical properties, the changes must be common to all animals, since the acute and chronic effects of ethanol can differ greatly among various strains and species of rodents. Acute sensitivity differences have been found in inbred mouse strains in that sleeping time (duration of ethanol-induced narcosis) is quite short for C57B2 mice but long for the BALB strain [6]. Lower doses produce qualitative as well as quantitative differences; locomotor activity was greatly reduced in C57BL mice treated with 0–2.25 g/kg of ethanol but was enhanced in BALB mice [7]. Furthermore, the rate, and possibly the degree, of tolerance development is related to initial differences in sensitivity in rats and mice. A rat line which is less sensitive to acute ethanol (LA) developed more tolerance than a more sensitive (MA) line [8], although with mice the less acutely sensitive DBA strain developed less tolerance than the more sensitive C57BL strain [9].

Since chronic administration of ethanol produces tolerance and physical dependence in all animals, we reason that if the ethanol-induced attenuation of membrane fluidizability is indeed related to an ethanol tolerance mechanism, we should observe a similar change in the ability of ethanol to fluidize lipid bilayers formed from other strains of mice rendered tolerant to ethanol as well as for other species such as rats.

Table 1. Ethanol-induced fluidizability of lipid bilayers prepared from crude synaptosomal membranes of tolerant C57BL/6J mice*

	P†	ΔP
Saline control (N = 3)	0.2262 ± 0.0033	−0.0267 ± 0.0012
Ethanol tolerant (N = 4)	0.2284 ± 0.0069	−0.0224 ± 0.0010‡

* Mean (± S.E.) fluorescence polarization of DPH incorporated into bilayers prepared from lipid extracts of C57BL/6J mice synaptic membranes before the addition of ethanol (P) and mean (± S.E.) changes in fluorescence polarization of DPH in these same bilayers produced by the addition of 0.7 M ethanol (ΔP). In comparison to controls, higher P values indicate a more 'rigid' membrane and smaller ΔP values indicate an attenuated ability of ethanol to fluidize membranes.

† P = fluorescence polarization.

‡ $P = < 0.05$, $t = 2.64$, $d.f. = 5$.

After C57BL/6J mice were rendered tolerant to ethanol using the same schedule used with the ICR mice (see Materials and Methods), the crude synaptosomal fractions were isolated and washed, and lipid was extracted. Using the fluorescence polarization (P) of DPH as a relative index of membrane fluidity, significant differences in the change in DPH polarization induced by the addition of 0.7 M ethanol were observed between the saline-control and chronic-ethanol groups (Table 1). Thus, it indicates that the differences in ethanol-induced fluidizability that we observed in ICR mice [3], using DPH fluorescence depolarization, were also observed in C57BL mice ($t = 2.64$, $d.f. = 5$).

In our previous study using ICR mice, we reported significant changes in absolute fluidity of the lipid bilayers [10]. However, in the present study the absolute fluorescence polarization of DPH in lipid bilayers composed from the lipid extracts of synaptosomal membranes of C57BL/6J tolerant animals was not different from that of the control group (Table 1). Chin and Goldstein [2] also reported no differences in the intrinsic fluidity in ethanol-tolerant DBA/2J mice even though they observed decreased ethanol-induced fluidizability. The intrinsic fluidity change observed in ICR mice after chronic exposure to ethanol was also not evident in the rat and, therefore, may be limited to ICR mice.

In order to assess further whether or not other species rendered tolerant to ethanol would also exhibit changes with regard to the ability of ethanol to fluidize lipid bilayers, we extended our work to Sprague–Dawley rats treated with ethanol by intubation three times daily for 3 days (see Materials and Methods). With this treatment schedule the animals were both tolerant to and physically dependent on ethanol. Table 2 reveals that after chronic ethanol treatment these rats had significant differences in seizure latencies, duration and intensity compared to the sucrose control group. Lipids were extracted and bilayers formed from the crude synaptosomal membranes from these animals and the

Table 2. Seizure measures of ethanol withdrawal in rats

	Seizure latency (sec)	Seizure duration (min)	Seizure intensity
Sucrose control	117.5 ± 19.7	1.83 ± 1.10	1.13 ± 0.04
Ethanol dependent*	60.9 ± 13.5†	7.52 ± 1.10‡	2.27 ± 0.30§

* Dependence was produced by three daily maximally tolerable doses of ethanol over 3 days. Withdrawal was assessed 9 hr after the first missed dose by i.p. injection of 50 mg/kg of Metrazol. Maximum latency to clonic seizure recorded was 180 sec and maximum duration was 10 min; intensity was scored as 0 for no seizure, 1 for clonic seizure, 2 for tonic and 3 for death following seizure. Values are means ± S.E.M.

† $P < 0.05$, $t = 2.33$, $d.f. = 17$.
‡ $P < 0.01$, $t = 3.57$, $d.f. = 17$.
§ $P < 0.05$, $t = 2.23$, $d.f. = 17$.

ability of ethanol to fluidize these bilayers was measured. Table 3 shows that, although the fluidity of the bilayer before the addition of ethanol was again unchanged by chronic ethanol treatment, the relative ethanol fluidizability was altered. When animals were treated with ethanol acutely, there was no change in ethanol fluidization in membrane lipid bilayers (data not shown). These results indicate that the attenuation of the fluidizing effects of ethanol following long-term exposure to ethanol occurs in both mice and rats.

Our failure to find species and strain generality in the intrinsic membrane fluidity following chronic ethanol administration further reinforces our previous observation that there is little relation between the intrinsic membrane fluidity and the ability of anesthetics to fluidize membranes [3]. We previously reported that there was no correlation between intrinsic fluidity and ethanol-induced fluidization of bilayers when the cholesterol content and, consequently, the intrinsic fluidity were varied. The addition of various amounts of cholesterol into bilayers composed of brain polar lipids decreased the intrinsic fluidity almost linearly with each addition of cholesterol; however, the ability of ethanol to fluidize these bilayers increased up to a cholesterol/phospholipid ratio of about 0.25, after which it plateaued [3].

These observations pose difficulties for the theory that chronic exposure to anesthetics, which perturbs some optimal fluidity, causes adaptive changes in lipid composition in order to return the fluidity to this optimal value [11]. Which modifications of this theory are necessary is unclear. What is clear is that certain brain membrane lipid compositional changes occur following long-term exposure to ethanol, which cause an apparent tolerance to the ethanol-induced fluidizability of brain membranes. These changes correlate with the anesthetic actions of ethanol insofar as they show cross-tolerance with pentobarbital and not with morphine, are reversible, and are generalizable to at least three strains of mice and one strain of rats. Discovery of the lipid composition change responsible for this alteration of ethanol-induced fluidizability should lead to an understanding of the molecular basis of tolerance to anesthetics. Greater emphasis should, therefore, be placed on anesthetic-induced fluidizability of neural membranes rather than absolute fluidity.

Chronic ethanol treatment alters at least one biophysical property of brain membranes, ethanol-induced fluidizability. Any change occurring at the membrane level would most likely be transferred to the next step. Therefore, a membrane alteration induced by ethanol may be the cause for a change of membrane function, such as some biochemical

Table 3. Effect of ethanol on lipid bilayers prepared from crude synaptosomal membranes of tolerant-dependent rats*

	P†	ΔP
Sucrose control (N = 5)	0.2456 ± 0.0017	−0.0181 ± 0.0003
Ethanol tolerant and dependent (N = 4)	0.2478 ± 0.0029	−0.0168 ± 0.0004‡

* Mean (± S.E.) fluorescence polarization of DPH incorporated into bilayers prepared from lipid extracts of Sprague–Dawley rat synaptic membranes before the addition of ethanol (P) and mean (± S.E.) changes in fluorescence polarization of DPH in these same bilayers produced by the addition of 0.7 M ethanol (ΔP). In comparison to controls, higher P values indicate a more 'rigid' membrane and smaller P values indicate an attenuated ability of ethanol to fluidize membranes.

† P = fluorescence polarization.
‡ $P < 0.05$, $t = 2.69$, $d.f. = 7$.

event at the membrane level. The final outcome of the drug action is the change in behavior of the animal. The relative change in ethanol fluidizability after chronic ethanol treatment may be small, yet a small change in the state or composition of lipids may cause a magnified alteration in some membrane protein functions and even greater changes in the behavior of the animal.

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