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Ethanol-Induced Fluidization of Brain Lipid Bilayers: Required Presence of Cholesterol in Membranes for the Expression of Tolerance

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SUMMARY

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The effects of ethanol on fluidity of reconstituted membranes formed from lipid extracts of tolerant and control mice synaptosomal membranes were assessed by fluorescence polarization techniques. Ethanol was less able to fluidize reconstituted membranes of ethanol-tolerant mice than controls. Acute *in vivo* administration of ethanol did not alter ethanol-induced fluidization of the reconstituted membranes. Since increased membrane cholesterol has been suggested to account for tolerance, the cholesterol and trace nonpolar lipids from the lipid extracts were removed and then cholesterol was added back so as to equalize its level in all groups. Following removal of cholesterol, it was not possible to detect any differences between the tolerant and control groups. However, when the cholesterol was added back so that all groups had a cholesterol/phospholipid ratio of 0.54, i.e., the control group level, it was possible to measure differences between tolerant and control lipid extracts. These results suggest that changes in the lipid composition of membranes can account in part for tissue adaptation to ethanol-induced membrane fluidization. Moreover, while the presence of cholesterol in the membranes appears to be required for the expression of tolerance, the small changes in the cholesterol content of membranes observed following development of tolerance probably are not responsible for the attenuation of ethanol-induced membrane fluidization.

INTRODUCTION

Ethanol, like other anesthetics, expands and increases the thermal motion, or what is commonly termed the "membrane fluidity," of both biological and artificial membranes (1, 2). While the functional significance of ethanol-induced membrane fluidization has not been demonstrated, the recent observations by Chin and Goldstein (3) that fluidization occurs at pharmacologically relevant concentrations and that tolerance develops (4) add much support to the suggestion that fluidization is the primary mechanism of action of ethanol in anesthesia.

Hill and Bangham recently proposed that the adaptation mechanism to long-term exposure to anesthetics involves changes in the lipid composition of the membranes (5). Subsequently, Littleton and John (6) and Chin *et al.* (7) demonstrated that the level of cholesterol and saturated fatty acids both increase slightly in synaptosomal membranes of tolerant mice. Although the

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degree of fatty acid saturation and the amount of cholesterol in membranes have been associated with greater order of the bulk hydrocarbon regions of membranes (8, 9), it is unclear whether these small changes are sufficient to explain tolerance. Consequently, we attempted to determine whether alterations occur in the membrane lipids per se which could account for the expression of tolerance and whether cholesterol plays any role in tolerance to ethanol-induced membrane fluidization. To this end, the lipids from crude brain synaptosomal membranes of ethanol-tolerant and control mice were extracted, and the ability of ethanol to fluidize bilayers formed from both groups was then measured using the fluorescence depolarization of DPH[1] incorporated into the bilayers as a molecular probe of fluidity (10, 11).

METHODS

Male ICR mice weighing 25–30 g were obtained from Simonsen, Gilroy, CA., and rendered tolerant to ethanol with twice daily intraperitoneal injections of 20% ethanol in saline (0.9%) (4.5 g/kg) at 9 a.m. and 4 p.m. for seven days. Control groups received saline via the same route and schedule. The animals were housed six per cage; weighed food pellets (Purina Mouse Chow) were placed in the cage at 4 p.m. every day. The leftover pellets were weighed the next morning to determine the amount consumed. Since ethanol-treated mice eat less, the control group was always limited to the amount consumed by the ethanol group. The calorie intake for each mouse averaged 15–21 kcal/day. The average calories contributed by the ethanol injections were approximately 1.5 kcal/day, so that the ethanol injections provided less than 10% additional calories. Body weights were measured each morning. Both groups lost weight during the first three days of injections (average weight loss, 1 g/day); however, by the end of the 7 days the average weights were at the initial weight or higher. Those mice who lost 2 g/day were excluded. Each mouse's sleeping was assessed individually on the first, second, sixth and seventh day of injection. On the first day sleeping time averaged 60–90 min and the average sleep times decreased 40–50% for different shipments of mice. Only those animals whose sleeping time decreased by at least 40% were included. (This injection schedule is admittedly unusual, but it allows for the study of large numbers of animals at a minimal cost.)

The brains were homogenized in 19 vol of suspended buffer (0.32 M sucrose and 5 mM HEPES, pH 7.8) with a teflon homogenizer and subsequently centrifuged at 1000 $\times g$, 10 min. The supernatants were centrifuged at 17,000 $\times g$ for 10 min and pellets were then washed once with the original suspending buffer. The final pellets were stored at −20° until the lipids were extracted following the method of Bligh and Dyer (12). The lipid extracts were dissolved in chloroform-methanol (2:1 by vol) and the concentration of lipid phosphorus was determined (13). The extracts were stored at −20°.

Lipid bilayers were prepared following the method of Bangham *et al.* (14). Briefly, the lipid extracts, dissolved in chloroform-methanol, were rotary evaporated on the walls of 25 ml pear-shaped flasks, then placed under high vacuum for one hour. Bilayers were then formed by adding glass beads and Krebs-Ringer-bicarbonate buffer, pH 7.4, which also contained 1 μM DPH, and gently swirling the beads around the flask in a 50° water bath until the lipid was dispersed.

Steady state fluorescence depolarization of DPH as a function of time was measured with a fluorometer, a schematic of which is shown in Fig. 1. Photomultiplier tubes were placed to the right and left of the sample cell; one emission beam was polarized parallel and one perpendicular to the plane of the 360 nm polarized excitation beam. Corning 3-74 cutoff filters were placed in the path of the emission beams. The signals from the photomultiplier tubes were fed into Pacific Instrument amplifiers and the outputs of these were fed into an Ithaco Ratiometer. The ratiometer was operated in the polarization mode, $P = (I_{\parallel} - I_{\perp}/I_{\parallel} +$

[1] The abbreviations used are: DPH, 1,6-diphenylhexatriene; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

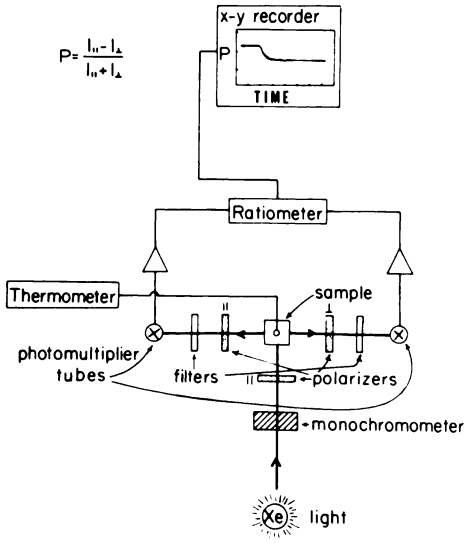

FIG. 1. *Schematic diagram of fluorometer*

$I_\perp$) where $I_\parallel$ is the fluorescence intensity parallel to and $I_\perp$ is the intensity perpendicular to the plane of polarization of the excitation beam. Prior to running the samples, the outputs of the photomultiplier amplifiers were equalized to correct for the difference in the detection efficiency of the two emission beams using a horizontally polarized excitation beam. Temperature was regulated by circulating water through the sample cell holder, and the sample was continuously mixed magnetically.

After preparation of the lipid bilayers, and prior to the polarization measurements, the lipid bilayers were incubated at 45° for at least 90 min to allow the DPH to equilibrate throughout the sample. Polarization measurements were performed by adding a 2 ml aliquot of the samples to a 1 × 1 cm quartz cuvette. After the sample reached 37° in the fluorometer, the polarization was noted prior to the injection of 80 μl of the various ethanol solutions. After the polarization had reached a new steady state (ca. 2 min), the polarization was again noted.

Analysis of phospholipids was performed by spotting the lipid extracts (200 nmol lipid phosphorus) on silica gel G thin-layer chromatography plates and developing with chloroform-ethanol-water (70:30:4 by vol). Lipids visualized with iodine vapor were scraped and lipid phosphorus determined.

Cholesterol was qualitatively detected by this same procedure except that visualization was performed by spraying with 0.6% potassium dichromate in 50% sulfuric acid and heating at 170° for 20 min. Cholesterol was quantitated by the method of Zlatkis *et al.* (15).

Cholesterol and trace non-polar lipids were separated from the polar lipids via silicic acid chromatography. Silicic acid (15 g, Sigma's SIL-LC grade) equilibrated with chloroform was placed in glass columns (2.5 cm dia). The lipid extracts (25 μmole lipid phosphorus) were loaded on the columns, followed by elution with 10 column volumes of chloroform and then 10 column volumes of methanol. The methanol eluates were rotary evaporated to near dryness, dissolved in chloroform-methanol (2:1 by vol), and stored at −20°. Aliquots (200 nmole lipid phosphorus) of the methanol eluates were analyzed qualitatively for cholesterol as described above. No cholesterol could be detected in any of the methanol eluates.

RESULTS

Reconstituted membranes formed from the lipid extracts of crude synaptosomal membranes of ethanol-tolerant mice were less fluidizable by ethanol in a dose-related manner than those formed from controls. This is indicated by the decreased ability of ethanol to depolarize the fluorescence of DPH incorporated into these reconstituted membranes ($F = 12.51$, $df = 1,4$, $p < 0.025$) (Fig. 2). The intrinsic fluidity of the reconstituted membranes of ethanol-tolerant mice brain lipid extracts was also slightly lower than the controls. The mean (± SEM) absolute fluorescence polarization of the DPH incorporated into the reconstituted membranes was 0.233 (± 0.001) for the tolerant and 0.227 (± 0.001) for the controls ($t = 3.89$, $df = 4$, $p < 0.02$).

To show that the results were due to the long-term exposure to ethanol, mice were injected once with either 4.5 g/kg of 20% ethanol in saline or saline 2 hr prior to decapitation. No significant differences were observed between the membranes pre-

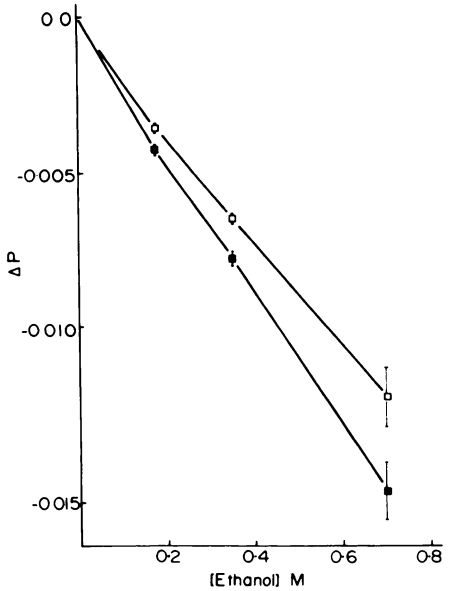

FIG. 2. *Mean (± SEM) changes in fluorescence polarization (ΔP) of DPH incorporated into bilayers composed of lipid extracts from tolerant* (□—□) *and control* (■—■) *mice synaptosomal membranes in Krebs-Ringer-bicarbonate buffer, pH 7.4, at 37° on the addition of ethanol*

Measurements were performed in duplicate for each sample. Decrease in polarization reflects an increase in membrane fluidity. Each point represents three membrane preparations.

pared from the lipid extract of the acute ethanol or control groups. The mean (± SEM) depolarization of DPH produced by the *in vitro* addition of 0.7 M ethanol from acute-ethanol treated and control mice were −0.0157 ± 0.0007 and −0.0148 ± 0.0006, respectively (n = 3 per group).

To determine whether membrane cholesterol is required for the expression of tolerance, the cholesterol was removed and the fluidizing effects of ethanol examined. The results of this experiment are shown in Fig. 3. Following removal of the cholesterol and nonpolar lipids from the extracts, no differences were detected in ethanol-induced fluidization of bilayers composed of lipids from the two groups. Also, it was no longer possible to detect differences between the intrinsic fluidity of the reconstituted membranes from tolerant and control groups.

To show that removal of cholesterol was specifically responsible for the elimination of the differential efficacy of ethanol to fluidize the bilayers and not a change in the composition of polar lipids, an analysis of the phospholipids before and after silicic acid chromatography was performed. We observed that the ratios of phosphatidylcholine: phosphatidylethanolamine: sphingomyelin: phosphatidylserine + phosphatidylinositol were virtually identical before and after silicic acid chromatography (1: 0.988: 0.141: 0.386 and 1: 0.994: 0.148, 0.390, respectively). (The amounts of phosphatidylserine and phosphatidylinositol were combined because they could not be separated with our TLC system.)

The ratio of cholesterol to total phospholipid was also determined in all the lipid extracts prior to silicic acid chromatography. The lipid extracts from tolerant mice had a slightly higher mean (± SEM) cho-

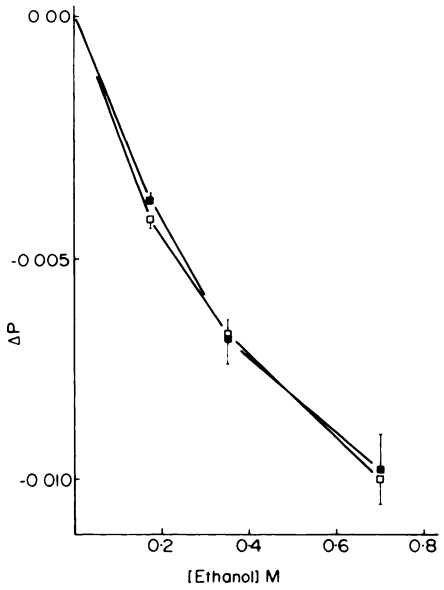

FIG. 3. *Mean (± SEM) changes on the addition of ethanol in fluorescence polarization (ΔP) of DPH incorporated into bilayers composed of lipid extracts from tolerant* (□—□) *and control* (■—■) *mice synaptosomal membranes after cholesterol was removed; in Krebs-Ringer-bicarbonate buffer, pH 7.4, at 37°*

Decrease in polarization reflects an increase in membrane fluidity. Each point represents the average of duplicate measurements made on each of three membrane preparations.

lesterol/phospholipid molar ratio (n = 3 for each group) although it was nonsignificant (0.58 ± .02 compared to 0.54 ± .03). Since we wanted to determine whether a small increase in the cholesterol could account for the expression of tolerance, we first added back cholesterol to the lipid extracts to equalize the amount of cholesterol relative to phospholipid (0.54) so that extracts from tolerant mice had the same amount of cholesterol as initially measured in the control group. The results of this experiment are shown in Fig. 4. Equalization of the amount of cholesterol relative to phospholipid restored the differential efficacy of ethanol to fluidize the bilayers composed of lipids from ethanol-tolerant and control synaptosomal membranes ($F = 13.34$, df = $1, 4, p < 0.01$). Interestingly, it was no longer possible to measure differences in the intrinsic fluidity between the two groups of membranes.

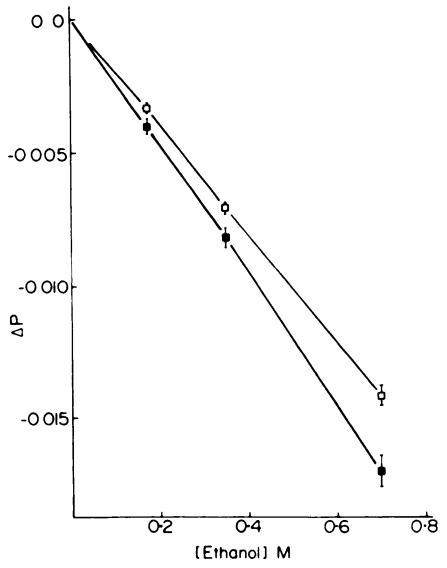

FIG. 4. *Mean (± SEM) changes on the addition of ethanol in fluorescence polarization (ΔP) of DPH incorporated into bilayers composed of lipid extracts from tolerant (□—□) and control (■—■) mice synaptosomal membranes after the removal then readdition of equal amounts of cholesterol (cholesterol/phospholipid ratio = 0.54 in all samples); in Krebs-Ringer-bicarbonate buffer, pH 7.4, at 37°*

Decrease in polarization reflects an increase in membrane fluidity. Each point represents the average of duplicate measurements made on each of three membrane preparations.

To evaluate what effect alterations of bilayer cholesterol had on ethanol-induced fluidization, we added various amounts of cholesterol back into control lipid extracts from which the cholesterol and other nonpolar lipids had been removed and then determined the intrinsic fluidity and the effect of the addition of 0.7 M ethanol on bilayer fluidity. As expected from previous reports, the addition of cholesterol increased the intrinsic polarization in almost a linear manner, as shown in Fig. 5A. Contrary to the suggestion of Chin *et al.* (7) that higher concentrations of membrane cholesterol would attenuate ethanol-induced membrane fluidization, the addition of cholesterol in our system enhanced the effect of ethanol to fluidize the bilayers up to about a 0.25 cholesterol/phospholipid ratio and then plateaued (Fig. 5B). Thus, in the range of cholesterol/phospholipid ratios we observed for tolerant and control lipid extracts, differences in the level of cholesterol could not account for the observed tolerance to ethanol-induced membrane fluidization.

DISCUSSION

The experiments presented here were designed to show that tolerance to the fluidizing effects of ethanol on synaptosomal membranes results, at least in part, from alterations in the membrane lipid composition and to ascertain what role, if any, cholesterol plays in the expression of tolerance. To this end, we extracted the lipids from synaptosomal membranes of tolerant mice and measured the effect of ethanol on membrane fluidity. As would be expected if tolerance occurred because of modifications of the lipid composition, bilayers formed from tolerant mice had a slightly lower intrinsic fluidity and were less fluidizable by ethanol in a dose dependent manner. The decreased fluidizability of the lipids from tolerant mice resulted from a long-term exposure to ethanol (or at least more than a single injection), since acute administration of ethanol did not alter ethanol's ability to fluidize lipid bilayers.

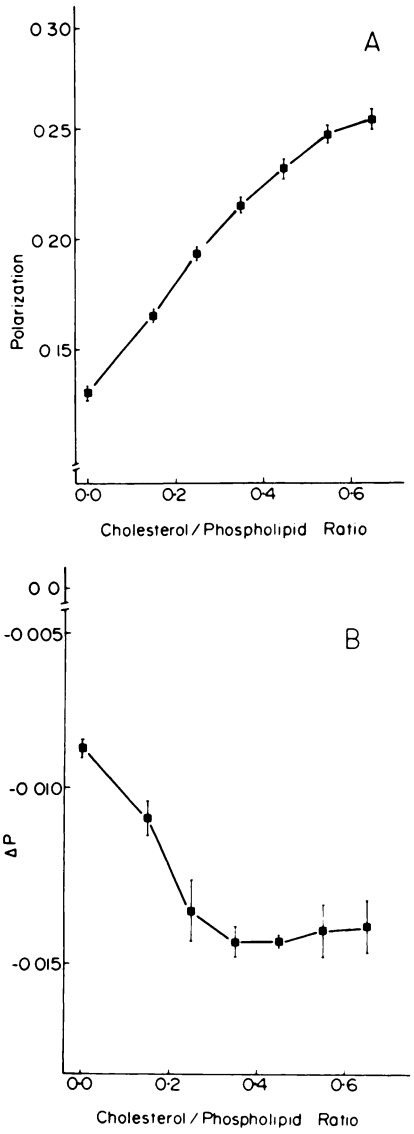

FIG. 5. *(A) Mean (± SEM) polarization of DPH incorporated into bilayers of lipid extracts (100 μM with respect to lipid phosphorus) of mice synaptosomal membranes that have had the cholesterol removed and various amounts of cholesterol added back prior to the formation of the bilayers in Krebs-Ringer-bicarbonate buffer, pH 7.4, at 37° (in triplicate)*

(B) Mean (± SEM) changes in fluorescence polarization (ΔP) of DPH incorporated into the above lipid bilayers on the addition of 0.7 M ethanol.

Our results suggest that the presence of cholesterol in the membrane is required for the expression of tolerance but the small changes in the cholesterol content of membranes seen following development of tolerance probably are not responsible for the observed decreased ethanol-induced membrane fluidizability. This conclusion is supported by the following observations: Although removal of cholesterol from the lipid extracts eliminates the differential efficacy of ethanol to fluidize membranes, it was still possible to measure differences in fluidizability if the amounts of cholesterol relative to phospholipid were equalized in all groups. Finally, alterations of the cholesterol content of lipid bilayers, comparable to those found in tolerant mice brains, had little or no detectable effect on ethanol-induced membrane fluidization.

Our results, therefore, suggest that it is still unclear which changes in membrane lipid composition are responsible for tolerance to ethanol-induced membrane fluidization. Changes in the level of membrane cholesterol do not appear responsible for tolerance, because it was still possible to observe differences in ethanol-induced membrane fluidization when the amount of cholesterol was equalized in both groups. Other non-polar lipids are probably not involved because it was also possible to measure differences after the non-polar lipids were removed with the cholesterol and the cholesterol content equalized. Moreover, the fact that our lipid extraction procedure does not extract gangliosides (sialic acid/phospholipid ratio < 0.005) suggests that at least changes in ganglioside composition cannot account for all membrane tolerance. Finally, decreased membrane fatty acid unsaturation may account for tolerance to ethanol-induced membrane fluidization; however, verification of this awaits additional evidence, because the time course of the development of tolerance to ethanol does not correlate well with the decrease in membrane fatty acid unsaturation. Littleton and John (6) reported little or no differences in membrane fatty acid unsaturation between 2 and 10 hr of continuous exposure to ethanol, while Grieve and Littleton (16), using the same strain of mice and presumably the type of exposure system, reported that there was a clear difference in the level of cellular tolerance be-

tween 2 and 5 hr of continuous exposure to ethanol. If membrane fatty acid saturation is related to tolerance development, one would expect differences in the fatty acid unsaturation between 2 and 10 hr of exposure.

Clearly, our approach to reconstitute lipid membranes and to assess ethanol's ability to fluidize these membranes rests on many assumptions, perhaps the weakest of which is that the homogeneous dispersion of lipids we used represents the actual distribution in intact membranes. While the distribution of lipids along the surface of synaptosomal membranes has not been established, asymmetrical distribution of lipids across synaptosomal membranes has been demonstrated (17). It is unclear whether our results would still be valid if it was possible to distribute lipids in artificial membranes as they are in intact membranes. However, considering the large number of biochemical changes associated with the development of tolerance (6, 7, 18–22), methods are needed to assess the significance of these changes to the expression of tolerance. The basic consistency of our results with the *in vitro* data (4) tends to support the validity of our approach and, indeed, offers a much needed method to test the potential significance of particular changes in membrane composition following the development of tolerance to ethanol.

Based on the widespread belief that cell membranes require a certain intrinsic fluidity for normal functioning and that perturbation of this optimal fluidity can cause adaptive changes in the lipid composition so as to return the fluidity to the optimal values, Hill and Bangham (5) predicted that tolerance to anesthetics involves a decrease in the intrinsic membrane fluidity. Unfortunately, the data thus far show that there is little relation between the intrinsic fluidity and the ability of anesthetics to fluidize membranes. Chin and Goldstein (4) observed decreased ethanol-induced fluidizability of synaptic plasma membranes from ethanol-tolerant mice, not changes in the intrinsic membrane fluidity. Moreover, although we report here that bilayers formed from lipid extracts of tolerant mice had slightly lower intrinsic fluidity, there was no correlation between intrinsic fluidity and ethanol-induced fluidizability of the bilayers when the cholesterol content and, consequently, the intrinsic fluidity was varied. When the cholesterol content was equalized in artificial membranes formed from tolerant and control brain extracts, it was possible to measure differences in the drug-induced membrane fluidization, but it was no longer possible to measure differences in intrinsic fluidity. Furthermore, when cholesterol was removed from lipid brain extracts and various amounts of cholesterol added back, the intrinsic fluidity of the bilayers decreased almost linearly with added cholesterol; however, the ability of ethanol to fluidize these bilayers surprisingly increased up to a cholesterol/phospholipid ratio of about 0.25 after which it plateaued. These results suggest that it may be more important to focus on the drug-induced fluidizability than the intrinsic fluidity in an analysis of the effects of anesthetics on biomembranes.

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