MOLECULAR PHARMACOLOGY, 17, 52–55

Adaptation to Ethanol-Induced Fluidization of Brain Lipid Bilayers: Cross-Tolerance and Reversibility

DAVID A. JOHNSON,[1] NANCY M. LEE,[2] ROGER COOKE AND HORACE LOH[3]

Langley Porter Neuropsychiatric Institute and Departments of Psychiatry, Biochemistry/Biophysics and Pharmacology, University of California, San Francisco, San Francisco, California 94143

Received May 27, 1979; Accepted August 28, 1979

SUMMARY

JOHNSON, D. A., N. M. LEE, R. COOKE AND H. H. LOH. Adaptation to ethanol-induced fluidization of brain lipid bilayers: Cross-tolerance and reversibility. *Mol. Pharmacol.* 17: 52–55 (1980).

Apparent tolerance to the ability of ethanol to increase membrane lipid thermal motions or membrane fluidity of artificial membranes formed from the lipid extracts of tolerant mice synaptosomal membranes has recently been observed by us. We attempted to determine whether this apparent tolerance was correlated with tolerance to the anesthetic actions of ethanol. We, consequently, measured the ability of ethanol to "fluidize" artificial membranes prepared from the membrane lipid extracts from pentobarbital, from morphine, and from pre- and postwithdrawal ethanol-tolerant mice. Membrane fluidity was assessed by measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene incorporated into the membranes. We observed that ethanol showed cross-tolerance with pentobarbital but not with morphine. Also, after withdrawal the effects of chronic ethanol treatment were reversible. The data suggest 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.

INTRODUCTION

Chin and Goldstein observed that chronic *in vivo* exposure to ethanol attenuates ethanol-induced fluidization of intact synaptosomal membranes (1). Membrane lipid but not protein compositional changes were later shown by us to be responsible for this phenomenon, since ethanol was less able to fluidize bilayers prepared from lipid extracts of membranes from ethanol-tolerant mice (2, 3). Because these observations correlate with the development of tolerance to the anesthetic actions of ethanol, they appear to suggest that changes in membrane lipid composition are directly involved in the expression of cellular tolerance to ethanol and they lend support to the hypothesis that the primary mechanism of action of ethanol, and other anesthetics, is to fluidize membrane lipids and, in turn, perturb membrane protein functions (4, 5). However, before these observations can support any theory of tolerance or of anesthesia, one question must be answered. Namely, is the attenuated ability of ethanol to fluidize tolerant membranes related to the anesthetic actions of ethanol or is it just a nonspecific drug effect?

To attempt to answer this question, we determined whether a correlation exists between the attenuated fluidizing effects of ethanol following chronic ethanol exposure and the development of tolerance to the anesthetic actions of ethanol. Since anesthetics show at least partial cross-tolerance among themselves and not with other classes of drugs (6–9) and since tolerance to anesthetics is eventually lost after cessation of exposure (10), we measured the ability of ethanol to fluidize bilayers prepared from the lipid extracts of synaptosomal membranes from pentobarbital, from morphine and from pre- and post withdrawal ethanol-tolerant mice. If ethanol tolerance is related to the attenuated ability of ethanol to fluidize tolerant membranes, we hypothesized that it should be possible to observe at least partial cross-tolerance with pentobarbital but not with morphine and reversal of the attenuation of ethanol's fluidizing effects after withdrawal.

METHODS

In separate experiments, male ICR mice (25–30 g) obtained from Simonsen (Gilroy, Calif.) were rendered tolerant to pentobarbital or morphine by 72-hr pellet implantation following methods previously described (11,

This investigation was supported in part by USPHS Grant DA01696.

[1] Present address: Division of Pharmacology, Department of Medicine, University of California at San Diego, La Jolla, Calif. 92093.

[2] Recipient of Career Development Award 5-K02-DA-00020.

[3] Recipient of Career Research Scientist Development Award K2-DA-70554.

0026-895X/80/010052-04$02.00/0

12). Control groups received placebo pellets. Mice were rendered tolerant to ethanol with twice daily intraperitoneal injections of 4.5 g/kg of ethanol (20%, v/v, in isotonic saline) at 9 AM and 4 PM for 7 days. Control groups received saline via the same route and schedule, and the food intake was restricted to that of the ethanol-treated group as previously described (3). On the eighth day, half the mice from each group were given an additional injection of either ethanol or saline two hours prior to decapitation. Only those animals whose sleep times decreased by 40% were included. The remaining ethanol-treated and control mice were placed on *ad lib* food and water for 12 additional days without ethanol treatment before decapitation.

The crude synaptosomal membranes were isolated as previously described (3). Lipids were extracted following the method of Bligh and Dyer (13) and lipid bilayers were prepared from these extracts in Krebs–Ringer–bicarbonate buffer, pH 7.4, containing 1 μM 1,6-diphenyl-1,3,5-hexatriene (DPH)[4] (Aldrich, Milwaukee, Wis.) following the method of Bangham *et al.* (14).

Steady state fluorescence depolarization of DPH incorporated into lipid bilayers was measured at 37° as previously described (3) with one small difference. In addition to the incident beam grading monochromometer, two filters (Corning 7-60 and Turner 110-810) were placed in the path of the excitation beam. These filters reduced the apparent fluorescence polarization produced by incident beam light scattering and enhanced drug-induced changes in fluorescence depolarization. The fluorometer measured $I_\parallel$ and $I_\perp$ simultaneously, so it was possible to calculate the fluorescence polarization, P, electronically on line from the formula:

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp},$$

where $I_\parallel$ is the fluorescence intensity parallel to and $I_\perp$ is the intensity perpendicular to the plane of polarization of the incident beam.

RESULTS

Pentobarbital has been repeatedly shown to display cross-tolerance with ethanol (6–8). This effect was also evident in the properties of the bilayers. Ethanol (0.7 M) was less able to depolarize the fluorescence of DPH incorporated into the bilayers prepared from the lipid extracts of synaptosomal membranes from pentobarbital-tolerant mice (t (10) = 2.02, $p < 0.05$, one-tailed) (Table 1). (The use of the "one-tailed" criterion, we believe, is justified here because the same results were observed in a replication. Moreover, we have the same adaptive effect in intact synaptosomal membranes in two separate experiments, data not presented.) While it is difficult to quantitate due to the inherent experiment-to-experiment variability of this technique, the magnitude of the attenuation of the fluidizing effects of ethanol was less for the "pentobarbital-tolerant" than for the "ethanol-tolerant" bilayers based on the lower level of statistical significance and the larger sample size required to detect tolerance

[4] The abbreviations used are: DPH, 1,6-diphenyl-1,3,5-hexatriene; P, fluorescence polarization.

TABLE 1

Differences in lipid bilayer fluidity and in ethanol-induced changes in lipid bilayer between chronic drug-treated and control mice as measured by fluorescence polarization of DPH incorporated into bilayers prepared from the lipid extracts of their synaptosomal membranes

Each value represents mean (±SEM) differences (drug-treated minus control group) in fluorescence polarization in the absence of ethanol (ΔP) and in the change in fluorescence polarization $\Delta(\Delta P)$ produced by the addition of 0.7 M ethanol. Positive ΔP's represent more rigid membranes compared to controls; negative $\Delta(\Delta P)$'s represent greater resistance to the fluidizing effects of ethanol (0.7 M). Measurements were performed in duplicate for each bilayer preparation. In separate experiments mice were rendered tolerant to pentobarbital or morphine by 72-hr pellet implantation. Control groups for these experiments received placebo pellets. Mice were rendered tolerant to ethanol with twice daily injections of ethanol for seven days. Ethanol-tolerant mice were decapitated on the eighth day and recovered ethanol-tolerant mice were decapitated 12 days later. Control groups for these experiments received saline injections. For the control groups the average (±SEM) fluorescence polarization in the absence of ethanol was 0.226 (±0.004) and the change in fluorescence polarization produced by the addition of 0.7 M ethanol was −0.0204 (±0.0013).

	N[a]	ΔP	$\Delta(\Delta P)$
Pentobarbital tolerant	6	+0.002 (±0.0001)*	−0.0019 (±0.0005)*
Morphine tolerant	6	+0.001 (±0.0028)	−0.001 (±0.0006)
Recovered ethanol tolerant	4	−0.003 (±0.0013)	−0.002 (±0.0014)
Ethanol tolerant	4	+0.009 (±0.0017)**	−0.0025 (±0.0005)**

[a] Number per group.
* $p < 0.05$, one-tailed.
** $p < 0.01$, two-tailed.

(N = 6 compared to 4). Moreover, the "pentobarbital-tolerant" bilayers were slightly less fluid than controls, since the fluorescence polarization of DPH in the absence of drug was higher (Table 1, t (10) = 1.89, $p < 0.05$, one-tailed).

Opiates and ethanol do not show cross-tolerance and are generally thought to act via different mechanisms (9). Similarly, no cross-tolerance was observed in "morphine-tolerant" bilayers. The results of this experiment are shown in Table 1. No differences were observed in either the fluorescence polarization DPH in the absence of ethanol (t (10) = 0.07) or in the change in fluorescence polarization of DPH produced by the addition of 0.7 M ethanol (t (10) = 0.43).

Finally, like anesthetic tolerance development, the attenuation of ethanol's fluidizing effects following long-term exposure to ethanol are reversible. The results of this experiment are also shown in Table 1. Twelve days after the withdrawal of ethanol, no differences were observed in either the fluorescence polarization of DPH in the absence of ethanol (t (5) = 1.66) or in the changes in fluorescence polarization of DPH produced by the addition of 0.7 M ethanol (t (5) = 0.223). This is in contrast to clear differences observed immediately after ethanol treatment in the fluorescence polarization in the absence of ethanol (t (6) = 3.75, $p < 0.01$, two-tailed) and the ethanol-induced changes in fluorescence polarization of DPH (t (6) = 4.89, $p < 0.01$, two-tailed) between ethanol treated and control groups (Table 1).

DISCUSSION

These data provide evidence that the change in brain membrane lipid composition responsible for the attenuation of ethanol-induced fluidization of "tolerant" membranes is related to tolerance to the anesthetic actions of ethanol. This conclusion is based on the correlation between the *in vivo* activity of ethanol on mice tolerant to pentobarbital, morphine and ethanol (pre- and postwithdrawal) and fluidizing properties of ethanol on bilayers prepared from lipid extracts of synaptosomal membranes of these mice. Just as ethanol has been shown to display cross-tolerance with pentobarbital and not with morphine, we observed cross-tolerance between the membrane fluidizing properties of ethanol and pentobarbital but not with morphine. Similarly, just as ethanol tolerance is reversed 12 days after cessation of exposure (10), we found that 12 days after ethanol withdrawal it was no longer possible to measure differences in fluidizability between bilayers prepared from lipid extracts of synaptosomal membranes from ethanol-tolerant and control mice.

Besides the correlation between the *in vivo* activity and fluidizing properties of ethanol following the development of tolerance to pentobarbital, morphine and ethanol, the relative magnitude of the intrinsic fluidity (in the absence of any drug) of these bilayers is also correlated with ethanol tolerance. However, differences in intrinsic fluidity is a poor indicator of tolerance because it is not observed in ethanol-tolerant C-57BL mice, although clear differences are seen in the ethanol-induced fluidizability.[5]

A variety of observations have previously shown a correlation between the membrane fluidizing and pharmacological activities of anesthetics and, consequently, have been used to support the lipid membrane fluidization theory of anesthesia. These observations include the following: (1) anesthetics fluidize membranes at pharmacologically relevant concentrations (15, 16); (2) the anesthetic potencies of a variety of agents correlate with their membrane fluidizing properties (17); and (3) high pressure reverses both anesthesia and anesthetic-induced membrane fluidization (4). The data presented in this paper also support the fluidization theory insofar as it shows that the attentuation of ethanol's fluidizing properties following chronic exposure to ethanol is related to the expression of tolerance to the anesthetic properties of ethanol.

However, while the expression of tolerance to ethanol's fluidizing properties appears to be related to *in vivo* anesthetic tolerance, and is thus consistent with the fluidization theory of anesthesia, there are at least two observations that pose problems for this theory. First, Richards *et al.* (18) showed that there was no correlation between the nerve blocking and membrane fluidizing properties of butanol and dodecanol. Both butanol and dodecanol block axonal conduction; however, butanol increases while dodecanol decreases the fluidity of lipid bilayers. Second, the magnitude of the change in fluidity of synaptosomal membranes produced by intoxicating levels of ethanol is smaller than the change in fluidity of these same membranes produced by the normal daily fluctuations of body temperature (which, of course, do not produce anesthesia). A one degree change of temperature produces a 0.0027–0.0032 decrease in the fluorescence polarization of DPH incorporated into rat synaptosomal membranes, while the addition of 22 mM ethanol (the legal intoxicating blood level in California) produces less than a 0.0008 decrease in the fluorescence polarization of DPH incorporated into these same membranes based on a linear interpolation of the dose–response curve.[6] Thus, increases in fluidity per se do not produce anesthesia and, consequently, modification of the fluidity theory of anesthesia may be in order.

It should be emphasized that, while problems exist for the membrane fluidization theory of anesthesia, these problems do not detract either from the importance of our basic observation that a membrane lipid compositional change occurs with the development of tolerance to ethanol or from the importance of the study of drug-induced membrane fluidization. Ethanol-induced membrane fluidization need not have functional significance for this lipid compositional change to be a primary event in the expression of tolerance to ethanol. A change in membrane lipid composition could alter the functional activity of membrane proteins independent of an alteration of fluidity. The study of ethanol-induced membrane fluidization provides a simple biophysical index of tolerance and may well play a pivotal role in determining what membrane compositional changes have functional consequences for cellular tolerance.

ACKNOWLEDGMENT

We would like to acknowledge the editorial and typing assistance of Kaye Welch.

REFERENCES

1. Chin, J. H. and D. B. Goldstein. Drug tolerance in biomembranes: A spin-label study of the effects of ethanol. *Science* **196:** 684–685 (1977).
2. Johnson, D. A., N. M. Lee and R. Cooke. Adaptation to ethanol-induced fluidization of brain lipid bilayers. *Drug. Alc. Depend.* **4:** 197–202 (1979).
3. Johnson, D. A., N. M. Lee, R. Cooke and H. H. Loh. Ethanol-induced fluidization of brain lipid bilayers: Required presence of cholesterol in membranes for the expression of tolerance. *Mol. Pharmacol.* **15:** 739–746 (1979).
4. Trudell, J. R., D. G. Payon, J. H. Chin and E. N. Cohen. The antagonistic effect of an inhalation anesthetic and high pressure on phase diagram of mixed dipalmitoyl-dimyristoyl phosphatidylcholine bilayers. *Proc. Nat. Acad. Sci. USA* **72:** 210–213 (1975).
5. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24:** 583–655 (1972).
6. Fraser, H. F., A. Wikler, H. Isbell and H. K. Johnson. Partial equivalence of chronic alcohol and barbiturate intoxication. *Quart. J. Stud. Alc.* **18:** 541–551 (1957).
7. Wahlström, G. Cross tolerance between hexobarbital and ethanol in rats induced by forced drinking of intoxicating amounts of ethanol, in *Biological Aspects of Alcohol Consumption* (O. Forsander and K. Eriksson, eds.). The Finnish Foundation for Alcohol Studies, 241–249 (1972).
8. Frankel, D., J. M. Khanna, A. E. LeBlanc and H. Kalant. Effect of p-chlorophenylalanine on development of cross tolerance between pentobarbital and ethanol. *Can. J. Physiol. Pharmacol.* **55:** 954–957 (1977).
9. Kalant, H. Comparative aspects of tolerance to, and dependence on, alcohol, barbiturates and opiates, in *Alcohol Intoxication and Withdrawal*, Vol. IIIb (M. M. Gross, ed.). Plenum, New York, 169–186 (1977).
10. LeBlanc, A. E., H. Kalant, R. J. Gibbins and N. D. Berman. Acquisition and loss of tolerance to ethanol by the rat. *J. Pharmacol. Exp. Ther.* **168:** 244–250 (1969).
11. Ho, I. K., I. Yamamoto and H. H. Loh. A model for the rapid development of dispositional and functional tolerance to barbiturates. *Eur. J. Pharmacol.* **30:** 164–171 (1975).

[5] Johnson *et al.*, unpublished observations.

[6] Hitzemann and Johnson, unpublished observations.

12. Way, E. L., H. H. Loh and F.-H. Shen. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J. Pharmacol. Exp. Ther.* **167**: 1–8 (1969).
13. Bligh, E. G. and W. J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Biophysiol.* **37**: 911–917 (1959).
14. Bangham, A. D., J. DeGier and G. D. Greville. Osmotic properties and water permeability of phospholipid liquid crystals. *Chem. Phys. Lipids* **1**: 225–246 (1967).
15. Chin, J. H. and D. B. Goldstein. Effects of low concentrations of ethanol on the fluidity of spin-labeled erythrocyte and brain membranes. *Mol. Pharmacol.* **13**: 435–441 (1977).
16. Trudell, J. R., W. L. Hubbell and E. N. Cohen. The effect of two inhalation anesthetics on the order of spin-labeled phospholipid vesicles. *Biochim. Biophys. Acta* **291**: 321–327 (1973).
17. Jain, M. K., N. Y.-M. Wu and L. V. Wray. Drug-induced phase change in bilayer as possible mode of action of membrane expanding drugs. *Nature* **255**: 494–495 (1975).
18. Richards, C. D., K. Martin, S. Gregory, C. A. Keightley, T. R. Hesketh, G. A. Smith, G. B. Warren and J. C. Metcalfe. Degenerate perturbations of protein structure as the mechanism of anaesthetic action. *Nature* **276**: 775–779 (1978).

Send reprint requests to: Dr. David A. Johnson, Division of Pharmacology, Department of Medicine, University of California at San Diego, La Jolla, Calif. 92093.