MOLECULAR PHARMACOLOGY, 27:256–262

Ethanol-Induced Alterations in Rat Synaptosomal Plasma Membrane Phospholipids

Relationship to Changes in the Phospholipid Methyltransferases

JANET D. MAGRUDER, MADELINE WAID-JONES, AND RONALD C. REITZ

Department of Biochemistry, School of Medicine, University of Nevada, Reno, Nevada 89557

Received June 14, 1984; Accepted November 12, 1984

SUMMARY

The effects of ethanol ingestion on the lipids of the synaptic plasma membrane (SPM) have been measured and correlated with the time frame for the development of physical dependence. Alterations were observed in three of the phospholipid fractions: phosphatidylcholine (PC) increased, and the phosphatidylethanolamine (PE) and phosphatidylserine (PS) plus phosphatidylinositol (PI) fractions decreased. These alterations occurred after the animals showed signs of dependence. Because PC can be synthesized from PE by the methyltransferase pathway, synaptosomal methyl group incorporation was measured. Rats were fed ethanol for 6 days before an increase was observed in methyl incorporation, a shorter length of time than was necessary to demonstrate physical dependence or phospholipid alterations (10 to 14 days). After ethanol withdrawal, 7 days of control diet feeding were required for methyl group incorporation to return to control values. *In vitro* ethanol (10–250 mM) additions to the methyltransferase incubations resulted in a slight increase in methyl incorporation. These data suggest that synaptic membrane lipid alterations may be related to ethanol dependence and that changes in the PC/PE ratio may be the result of an increase in the incorporation of methyl groups into synaptosomal phospholipids.

INTRODUCTION

The manner in which ethanol induces biochemical alterations resulting in physical dependence continues to be an area of intensive investigation. It is known that ethanol enters the hydrophobic region of biomembranes causing fluidity changes and alterations in membrane function (1). Evidence also indicates dependency may be due to membrane adaptation to the constant presence of ethanol (1). The effects of ethanol may relate to alterations in membrane lipids; however, reports from other laboratories concerning ethanol-induced lipid changes in SPM[1] have been controversial. Ethanol-induced increases in cholesterol levels in brain (2) and SPM (3) have been reported; however, other groups (4, 5) have found no change in brain cholesterol levels. Some laboratories have reported changes in phospholipid composition (4, 6) or phospholipid turnover (7). In contrast, other laboratories have not found changes in the phospholipids (3, 5). There are reports of ethanol-induced alterations in synaptic membrane fatty acids (5, 6, 8–10) while another report showed no change in fatty acid composition (3). These membrane changes are of relatively small magnitude, and the fact that there seems to be no consensus regarding the ethanol-induced alterations may be due to differences in experimental design. However, the continued investigation of small changes in membrane structure may eventually lead to insight as to the biological effects of ethanol.

Because PC and PE are the major phospholipids in the SPM, alterations in their metabolism as a result of ethanol ingestion might be very important. One of the pathways for membrane PC synthesis is the SAM-mediated methylation of PE by phosphatidylethanolamine-*N*-methyltransferase (EC 2.1.1.17). Crews *et al.* (11) have identified and characterized two methyltransferases that convert PE to PC in rat brain synaptosomes. The first enzyme, methyltransferase I, methylates PE to form MMPE. The second enzyme, methyltransferase II, adds two more methyl groups to form PC. That ethanol ingestion may affect these enzymes has been suggested by

Support for this research was provided by National Institutes of Health Grant AA 05426, from the University of Nevada, Reno Foundation, and from the University of Nevada, Reno Research Advisory Board.

[1] The abbreviations used are: SPM, synaptic plasma membranes; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; MMPE, phosphatidyl-*N*-monomethylethanolamine; DMPE, phosphatidyl-*N*,*N*-dimethylethanolamine; SAM, *S*-adenosyl-L-methionine; EtOH, ethanol.

0026-895X/85/020256-07$02.00/0

Littleton's group (12) who found that chronic ethanol inhalation resulted in an increase in methyl group incorporation into rat brain synaptosomes. Further, Fallon *et al.* (13) have observed a similar increase in the methylation pathway in liver.

In view of the diversity of animal models used, as well as differences in method and length of time of ethanol administration, it is difficult to determine how these synaptic membrane changes may relate to physical dependence. However, we believe it would be useful to determine if alterations in synaptosomal phospholipid composition and metabolism occur in a time-related manner, and to establish whether this pattern of alterations would coincide with the onset of ethanol dependence. The treatment time required for rats to become physically dependent on ethanol was determined by conducting withdrawal studies using animals that had been fed a nutritionally complete, ethanol-containing diet for 3, 6, 10, or 14 days. The rats became physically dependent within 10 to 14 days after the feeding regimen began, and subsequently the same 3-, 6-, 10-, and 14-day time frame was used to examine the phospholipid composition of the SPM. There was no change in the total phospholipid content; however, when the various phospholipids were separated and quantitated individually, an increase was seen in the PC fraction marked by a decrease in the PE and PS + PI fractions. We also report that an increase in the ability of synaptosomes to incorporate methyl groups into PC preceded the changes in membrane composition.

MATERIALS AND METHODS

Animals and diet. Male Long-Evans rats (Simonsen Laboratories, CA) (140–160 g) were weight-paired, housed in individual cages, and then pair-fed a liquid diet (Dyets, Bethlehem, PA). This was a low fat (4.6% of calories), fiber-containing, suspended diet similar in composition to that previously used (14). One animal from each pair was fed a diet in which ethanol provided 35% of the calories. In some cases, one control animal was paired with two ethanol-fed animals. Maltose dextrin replaced ethanol calories in the control diet. All the animals were fed the control liquid diet for at least 2 days prior to pairing and receiving ethanol. Rats fed the control diet for 14 days showed a weight gain of about 4.2 g/day while consuming an average of 63.6 kcal/day. Ethanol-fed animals (ingesting 13–16 g of ethanol/kg of body weight/day and consuming the same number of calories/day as the control) averaged a daily weight gain of about 3.7 g. It was observed that the difference in weight gain occurred during the first few days of ethanol feeding with the ethanol-treated gaining an average of 1.4 g/day during the first 3 days. After the initial 3 days, the rate of weight gain for the control and ethanol-fed groups was similar.

Withdrawal studies. Withdrawal studies were conducted using a procedure modified from that reported by Numan and Gilroy (15) and by Majchrowicz (16). Four groups of rats (6 rats/group) were fed the ethanol-containing liquid diet for 3, 6, 10, or 14 days. The study was programmed so that all the ethanol-treated groups were withdrawn at the same time. In addition, a group of six control rats were weight-paired with the rats receiving ethanol for 14 days and pair-fed the liquid control diet. These animals served as controls for all four groups of ethanol-fed animals. At 10 p.m. the night before withdrawal, blood was taken from the tail vein and ethanol levels were determined (17). The animals were withdrawn from ethanol at 7 a.m. and given rat chow and water. Withdrawal signs were monitored until absent in all the animals (15 hr). The observations and scorings of symptoms were done as a blind study. The rats (including the treated animals and the controls) were coded and their individual cages were scrambled randomly while the observers were absent. For observation, five cages at a time (containing one rat per cage) were removed from the feeding room and taken to an observation room where each animal was monitored for 5–10 min. Each animal was examined every 2 hr by two independent observers who looked for teeth chatter, tail stiffening, body rigidity, tremors, hyperexcitability, and audiogenic seizures (induced by jangling keys). The observers rated each withdrawal sign from absent to 4+ depending on severity. At the end of the study these data were combined into a single numerical score for each rat. Based on these scores, animals were rated from 0 (no signs) to 4 (severe signs of withdrawal). Only rats undergoing audiogenic seizures rated a score of 4. The final scores from the two observers were then pooled, the scores were averaged for each animal, and the identity of the rats was decoded. The data for each group were pooled and averaged, and the various groups were compared.

Synaptosome and SPM isolation. Rats were fed ethanol for 3, 6, 10, and 14 days and killed by decapitation while still intoxicated. For the experiments with withdrawn animals, the rats were fed ethanol for 14 days before being withdrawn to a rat chow and water diet. Alternatively, the animals were fed the control liquid diet for the withdrawal period. The two methods yielded indistinguishable results. In some cases, blood was collected at the time of decapitation for ethanol analysis (17). The brains were removed immediately after killing and placed on a watch glass on ice where the brain stem and all visible white matter was removed by aspiration. The remaining gray matter from the cerebrum and cerebellum was quickly homogenized in ice-cold Tris (50 mM, pH 7.4)-buffered 0.32 M sucrose containing 1 mM $MgCl_2$ and 1 mM EDTA. For some experiments, tissues from 2–12 animals were pooled. Synaptosomes and SPM were obtained using a differential and gradient centrifugation procedure (18) and were immediately subjected to lipid extraction (SPM) or a methyl group incorporation assay (synaptosomes) as described below. Cytochrome oxidase (EC 1.9.3.1) activity was determined (19) as a measure of mitochondrial contamination. There was a 3× reduction of cytochrome oxidase activity during the final SPM enrichment procedure, which included separation of the lysed synaptosomes by density gradient centrifugation. Protein analysis was by the method of Lowry *et al.* (20). All reagents were analytical grade.

Phospholipid analysis. The SPM were subjected to lipid extraction (21), and the phospholipids and appropriate standards were separated by TLC on Silica Gel G using a chloroform:methanol:acetic acid:water (100:55:16:6) solvent system. The individual phospholipids were located with 2′,7′-dichlorofluorescein and eluted from the TLC scrapings (22). Total and individual phospholipids were quantitated using the Bartlett phosphate assay (23). All assays were done in duplicate or triplicate.

Methyl group incorporation. Synaptosomal methyl group incorporation was determined using a method modified from that reported by Crews *et al.* (11). Following isolation, synaptosomes were washed and resuspended in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MgCl_2$. The methylation of phospholipids was measured by following the incorporation of the [^{3}H]methyl group from *S*-adenosyl-[*methyl*-^{3}H]methionine. Synaptosomes were incubated in either 25 mM Tris-glycylglycine buffer, pH 7.5, 1 mM $MgCl_2$, 2 μM SAM, or in 25 mM Tris-glycylglycine buffer, pH 10.5, 1 mM $MgCl_2$, 200 μM SAM. The SAM (Boehringer Mannheim) was purified prior to use on a Dowex 1-HCO^-_3 column (24) and the concentration was determined spectrophotometrically (E_m = 15,400 at 260 nm) in water (24). One μCi of [^{3}H] SAM (80 Ci/mmol, New England Nuclear) was added to each incubation. In some cases, ethanol was added to the incubations in the concentrations described in the text. Volumes were adjusted to result in total assay volumes of 0.4 ml. Reaction buffers were prepared on ice immediately before use. The reactions were started by adding 0.4 mg of protein and placing the mixture in a 37° shaking water bath. After 30 min, reactions were stopped by adding 1.0 ml of 40% sodium trichloroacetate, pH 1.0. The samples were placed on ice for 25–30 min and then centrifuged at 2000 rpm in a Sorvall GLC-1 type M rotor for

20 min. The precipitated membranes were washed twice with 20% trichloroacetate, pH 1.0, and subjected to lipid extraction (21). The resulting chloroform phase was transferred to vials and dried for scintillation counting. All incubations and extractions were done in triplicate. Methyl group incorporation increased linearly with protein in the range of protein concentration tested (0.2–0.8 mg/assay). The rate of incorporation was linear with time for 5 min and then gradually declined. Due to the low activity of the synaptosomal methyltransferases (11), a 5-min incubation did not allow sufficient incorporation of label for reproducible measurements; thus, incubations were for 30 min, and results are reported as total incorporation/mg/30 min.

For identification of the reaction products, incubations were done as described above except that 2–5 μCi of [^{3}H]SAM were added to each incubation mixture. The chloroform layers from the lipid extraction steps were applied to Silica Gel G plates. Standards, including PC, PE, LPC, (Serdary Research Laboratories), MMPE, and DMPE (Larodan Fine Chemicals, Sweden), were applied to each chromatogram. Separation was by TLC in a chloroform:1-propranol:propionic acid:water (2:3:2:1) solvent system (11). The developed chromatogram was masked with paper, except for the standards which were visualized by spraying their lane with 2′,7′-dichlorofluorescein. Fractions corresponding to the position of each phospholipid standard were scraped from the plates, and the amount of tritium label present was determined directly by scintillation counting in a thixotropic gel [0.4% 2,5-diphenyloxazole (Sigma), 4.0% fuming silica (Sigma) in toluene] (25).

Statistical analysis. The data in Table 1 were subjected to simple linear regression analysis which yielded the line: (score or y) = 0.67 + 0.12 (time or x). To determine if the scores were significantly different, 95% confidence intervals were determined around the mean score for each timepoint and then compared. Confidence limits for data in Table 3 were based on Student's t test for two means. In Table 4, the combined data for the controls were analyzed by linear regression. The slope calculated for the control data was essentially zero showing there was no change with time. Therefore, the composite control data were treated as a single group and subsequently compared to each of the ethanol-treated and withdrawn groups using Student's t test for two means. Data from the *in vitro* ethanol incubation studies were initially evaluated using Student's t test for two means to determine if there were differences in incorporation between the control-fed and ethanol-fed groups at any of the concentrations of exogenous ethanol tested. Within each group, paired t tests were used to compare the methyl incorporation at each concentration of exogenous ethanol used to the incorporation when added ethanol was absent (Table 5). The data then were expressed as a percentage of the value for the incorporation when ethanol was omitted. These normalized data were subjected to a two-way analysis of variance to determine if *in vitro* ethanol resulted in selective differences in methyl incorporation when control-fed and ethanol-fed animals were compared.

RESULTS

Withdrawal studies. To determine the time frame for onset of ethanol dependence, withdrawal studies were conducted after ethanol ingestion for 3, 6, 10, and 14 days. The animals were rated in a blind study according to severity of withdrawal (Table 1). The mean scores for each group increased with time of ethanol feeding and by day 10, the average rating was greater than 2.0 (moderate withdrawal symptoms) and was statistically significantly elevated ($p < 0.05$) when compared to the control mean score. These data suggest that dependence had occurred in most of the individuals by this time. By day 14, the score for the ethanol-fed animals was also statistically significantly different ($p < 0.05$) compared to the score for the 3-day ethanol-treated animals. The mean blood ethanol concentration for each group ranged between 99 and 176 mg/100 ml at 10 p.m. (the night before killing) and showed a slight increase with length of time on the diet until day 10 (Table 2). Mean blood ethanol concentrations were lower (41–63 mg/100 ml) in the morning (at the time of killing) (Table 2) and were not different for any of the four time groups.

TABLE 1

Withdrawal scores after varying lengths of time of ethanol feeding

Withdrawal symptoms were monitored in rats fed ethanol for 3, 6, 10, and 14 days and in a control group fed only liquid control diet for 14 days. There were six rats in each group. The rats were scored in a blind study in which the symptoms were rated as: 0 = symptoms absent; 1 = mild; 2 = moderate; 3 = strong; and 4 = severe.

Days of treatment	Mean score (± SD)	Range
Control (No EtOH)	0.7 ± 0.8	0.0–2.0
3-day EtOH	0.9 ± 0.8	0.0–2.5
6-day EtOH	1.4 ± 0.9	0.5–2.5
10-day EtOH	2.1 ± 1.2[a]	0.5–4.0
14-day EtOH	2.2 ± 1.0[a,b]	1.0–4.0

[a] $p < 0.05$ compared to control.
[b] $p < 0.05$ compared to 3-day EtOH.

TABLE 2

Blood ethanol levels

The blood ethanol concentrations were determined as described in Materials and Methods. These data represent two separate experiments. The blood drawn at 10 p.m. was from the animals used for the withdrawal studies (see Table 1). The blood noted as 8 a.m. (at killing) represents that taken from the animals used for the biochemical studies reported in the subsequent tables. Numbers in parentheses represent n for each group, and values are means ± SD.

Days of ethanol treatment	10 p.m. (before withdrawal)	8 a.m. (at killing)
	mg/100 ml	
3	99 ± 44 (6)	62 ± 52 (49)
6	123 ± 47 (6)	42 ± 41 (49)
10	176 ± 41 (6)	41 ± 29 (48)
14	117 ± 41 (6)	63 ± 41 (47)

Phospholipid analysis. To determine if structural changes occurred in the SPM at times correlating with onset of dependence, phospholipid analysis was done on SPM fractions from rats fed ethanol for 3, 6, 10, and 14 days. No change was found in the total phospholipid content of these membranes. The lipid phosphorus/protein ratio for all control timepoints combined was 0.63 ± 0.17 nmol of phosphate/mg of protein and was 0.60 ± 0.17 nmol of phosphate/mg of protein for the ethanol-treated animals (excluding withdrawn individuals). However, when the phospholipids were separated by TLC and individually quantitated, significant changes were observed (Table 3). By day 10, a slight but not statistically significant increase was observed in the PC fraction and in the PC/PE ratio. A significant decrease was found in the fraction representing PS + PI. By day 14, the increases in PC and PC/PE ratio were statistically significant as was the decrease in PE. In addition, a significant decrease was still present in the PS + PI fraction.

Methyl group incorporation. Crews *et al.* (11) have shown that rat synaptosomal methyltransferases I and II have different pH optimums (7.5 and 10.5, respectively). In addition, methyltransferase I has a low appar-

TABLE 3

Effects of ethanol on synaptic plasma membrane phospholipids

Phospholipids were separated by TLC on Silica Gel G using a chloroform:methanol:acetic acid:water (100:55:16:6) solvent system. The individual phospholipids were eluted from the TLC scrapings and the phosphate was quantitated (23). Each value was calculated as (nanomoles of phosphate/fraction) ÷ (total nanomoles of phosphate for all fractions combined) × 100 and is the mean ± SD. The number of groups analyzed is shown below each set of data. Brains from 2–12 animals were pooled for each group

TLC fraction	Days of treatment			
	3	6	10	14
	nmol %			
Control				
Solvent front	2.2 ± 0.6	2.7 ± 2.4	3.6 ± 2.4	3.2 ± 1.5
PE	38.1 ± 0.6	39.0 ± 1.3	38.3 ± 1.7	38.3 ± 2.1
PS & PI	9.4 ± 0.7	10.1 ± 2.3	8.0 ± 1.8	9.0 ± 0.6
PC	43.8 ± 3.1	42.4 ± 2.7	43.6 ± 2.7	42.9 ± 2.3
Sphingomyelin	5.4 ± 0.6	4.8 ± 0.5	5.5 ± 2.4	5.5 ± 0.4
LPC	0.7 ± 0.8	0.7 ± 1.2	0.7 ± 0.6	0.6 ± 0.2
Origin	0.4 ± 0.5	0.3 ± 0.6	0.3 ± 0.2	0.5 ± 0.5
	(4 groups)	(4 groups)	(11 groups)	(6 groups)
PC/PE ratio	1.15 ± 0.10	1.10 ± 0.11	1.15 ± 0.08	1.12 ± 0.04
Ethanol				
Solvent front	3.6 ± 1.1	2.9 ± 2.4	4.0 ± 2.1	3.7 ± 1.3
PE	37.3 ± 1.2	38.4 ± 1.1	37.8 ± 4.5	34.2 ± 3.3[a]
PS & PI	9.1 ± 0.5	9.3 ± 1.8	6.3 ± 0.5[b]	6.1 ± 0.6[c,d]
PC	43.5 ± 3.5	43.8 ± 2.7	45.8 ± 3.1	48.4 ± 3.0[d,e]
Sphingomyelin	5.0 ± 0.8	4.8 ± 0.4	5.1 ± 0.8	5.9 ± 0.9
LPC	0.9 ± 1.1	0.5 ± 0.6	0.7 ± 0.5	0.9 ± 0.7
Origin	0.6 ± 0.9	0.3 ± 0.3	0.3 ± 0.5	0.8 ± 0.9
	(4 groups)	(4 groups)	(11 groups)	(6 groups)
PC/PE ratio	1.17 ± 0.13	1.15 ± 0.09	1.24 ± 0.25	1.42 ± 0.12[c]

[a] Compared to control, $p < 0.05$.
[b] Compared to 6-day EtOH, $p < 0.01$.
[c] Compared to control, $p < 0.001$.
[d] Compared to 6-day EtOH, $p < 0.05$.
[e] Compared to control, $p < 0.01$.

ent K_m for SAM and requires Mg^{2+} (11), whereas, methyltransferase II has a high apparent K_m for SAM and does not require Mg^{2+} (11). When incorporation of methyl groups from [^{3}H]SAM into synaptosomal lipids was measured under optimum conditions for methyltransferase I (pH 7.5, 2 μM SAM, 1 mM $MgCl_2$) (11), no difference was observed in incorporation into synaptosomes from the ethanol-fed animals as compared to the controls for any of the timepoints tested. The methyl group incorporation into the control synaptosomes from all the timepoints combined was 1.34 ± 0.38 pmol/mg/30 min (n = 15), while the incorporation into synaptosomes from ethanol-fed animals from all timepoints (excluding withdrawn animals) was 1.31 ± 0.25 pmol/mg/30 min (n = 20). When synaptosomes were incubated with [^{3}H]SAM at optimum conditions for methyltransferase II (pH 10.5, 200 μM SAM, and with 1 mM $MgCl_2$ to allow *in situ* substrate formation by methyltransferase I) (11), it was found that ethanol treatment resulted in increased incorporation of label into the synaptosomal lipids (Table 4). Synaptosomal methyl group incorporation was not different between any of the control groups

TABLE 4

Effects of ethanol treatment on the incorporation of methyl groups into synaptosomal phospholipids

Incorporation of methyl groups from [^{3}H]SAM was done as described in Materials and Methods. Values are in picomoles/mg of protein/30 min ± SD. The per cent change represents the difference between the composite control figure and the specific ethanol-treated group and is calculated as % = (incorporation into the ethanol-treated) ÷ (incorporation into the composite control) × 100. n, number of individual animals, is in parentheses. All assays were done in triplicate. Compared to the composite figure, [a] $p < 0.02$, [b] $p < 0.01$, and [c] $p < 0.001$.

Length of treatment	Control	EtOH	%
	pmol/mg/30 min	*pmol/mg/30 min*	
3 days	33.1 ± 4.9 (4)	33.4 ± 9.0 (8)	98
6 days	36.7 (2)	41.5 ± 10.6[a] (4)	122
10 days	35.0 ± 3.8 (3)	40.3 ± 2.7[b] (5)	119
14 days	33.9 ± 2.4 (7)	47.2 ± 6.8[c] (10)	139
24-hr withdrawn	32.8 (2)	48.3 ± 4.4[c] (3)	142
3-day withdrawn	34.3 ± 3.5 (3)	44.1 ± 3.9[c] (6)	130
7-day withdrawn	33.4 (2)	35.2 ± 6.0 (4)	104
Composite for all controls	34.0 ± 3.6 (23)		

at any of the timepoints and averaged 34.0 ± 3.6 pmol/mg/30 min (n = 23) for all timepoints combined. Because none of the control timepoints were different, each of the ethanol-fed groups was compared to the composite figure for all controls. After 3 days of ethanol feeding, there was no difference in the values for methyl incorporation as compared to the combined controls. However, in the 6-day ethanol-fed animals, incorporation was 122% ($p < 0.02$) of the control value. Incorporation remained significantly elevated in the 10-day ethanol-fed group (119%, $p < 0.01$), and by 14 days, incorporation had increased to 139% ($p < 0.001$) of the value for the controls. Incorporation remained elevated (142%, $p < 0.001$) in the 24-hr withdrawn animals. At 3 days following withdrawal, incorporation had decreased but was still significantly elevated (130%, $p < 0.001$). Methyl incorporation returned to control levels 7 days after the animals had been withdrawn from ethanol.

To determine the identity of the products that resulted from the increase in synaptosomal methyl group incorporation in the 14-day ethanol-treated animals, incubations with [^{3}H]SAM were done as described above. Following extraction, the phospholipids were subjected to TLC using a solvent system that separated PE, MMPE, DMPE, PC, and LPC (11). The radiolabel present in each of these fractions and in the solvent front and origin fractions was measured and calculated as a percentage of total radiolabel present. The portion of the radiolabel that was incorporated into the TLC fraction corresponding in R_F value with the PC standard increased from 16% in the control to 19% in the ethanol-treated animals. There was also an increase observed in the methylation of the LPC fraction (from 10 to 12%). There is very little LPC normally found in synaptic plasma membranes (Table 3), and we believe that the substantial amount of radiolabel present in the LPC fraction is due to phospholipase A_2 (EC 3.1.1.4) cleavage of PC (26) during the 30-min incubation at 37°. Ethanol treatment resulted in

an increase in label found in the MMPE fraction (from 6 to 8%), but the percentage of total label did not change in the DMPE fraction (10% for both groups). There was a large reduction (from 27 to 17%) in the label found at the origin. A large portion of the total radiolabel (25% for the control and 28% for the ethanol-treated animals) chromatographed ahead of the phospholipids. Zatz *et al.* (27) have presented evidence that radiolabeled fatty acid methyl esters are formed after rat lung tissue is incubated with [^{3}H]SAM. Thus, it is possible that the radiolabel found in the solvent front fraction is due to [^{3}H]SAM methylation of endogenous free fatty acids as well as free fatty acids released by phospholipase A_2 cleavage during incubation. We interpret these data to show that the observed ethanol-induced increase in synaptosomal lipid methyl group incorporation included a specific increase in the PC fraction.

To measure the effect of *in vitro* ethanol on methyl incorporation, 10–250 mM (46–1152 mg/100 ml) ethanol was added to the incubations (Table 5). Synaptosomes used in the study were obtained from animals treated for 14 days with either the ethanol-containing or the control liquid diet. As expected from the data in Table 4, methyl group incorporation was statistically significantly elevated in the ethanol-treated group at all the concentrations of exogenously added ethanol tested (statistical data not shown; see Materials and Methods for procedures used). Methyl group incorporation in the presence of increasing amounts of added ethanol was compared to incorporation without exogenous ethanol for both the control-fed and ethanol-fed groups. The addition of 100 mM ethanol produced a statistically significant increase in methyl group incorporation in the control-fed group, and 50, 100, and 250 mM ethanol resulted in significant increases in the ethanol-fed group (Table 5). Methyl group incorporation then was expressed as a percentage of the value for the incorporation when exogenous ethanol was omitted. These normalized data were subjected to a two-way analysis of variance which showed there were no selective effects of ethanol. We conclude from these data that exogenous ethanol increases methyl group incorporation but that there are no differences when control-fed and ethanol-fed animals are compared.

TABLE 5

Effects of in vitro additions of ethanol on methyl group incorporation

Synaptosomes from animals treated for 14 days with either ethanol-containing (n = 7) or control (n = 10) diet were used to measure the effects of exogenous EtOH (10–25 mM) on the incorporation of methyl groups from [^{3}H]SAM. The incubations were done as described in Materials and Methods. Methyl group incorporation is expressed both as picomoles/mg/30 min and as a percentage (incorporation with exogenous ethanol present + ethanol absent × 100). All assays were done in triplicate.

EtOH concentration	Control-fed	%	Ethanol-fed	%
mM	*pmol/mg/30 min*		*pmol/mg/30 min*	
No added EtOH	35.3 ± 3.0	100	49.5 ± 7.3	100
10	37.7 ± 3.6	107	48.5 ± 4.4	98
25	34.7 ± 8.1	98	53.5 ± 13.3	108
50	40.4 ± 9.7	114	67.5 ± 9.9[a]	136
100	45.6 ± 9.6[b]	129	63.6 ± 9.6[a]	128
250	39.9 ± 7.2	111	60.1 ± 10.3[a]	121

[a] $p < 0.02$ compared to no EtOH for ethanol-fed.
[b] $p < 0.01$ compared to no EtOH for control-fed.

DISCUSSION

Time course studies have shown that an ethanol-induced increase in the PC/PE ratio occurred in rat SPM at a time corresponding to that established for the onset of physical dependence. The individual SPM phospholipids were quantitated after 3, 6, 10, and 14 days of ethanol treatment. By 14 days, there was a significant increase in the PC fraction as well as a decrease in the PE fraction. In addition, an increase in the incorporation of methyl groups into synaptosomal lipids was found to precede the changes in membrane composition. Separation and identification of the radiolabeled phospholipids showed that the increase in methyl group incorporation was predominantly an increase in methyl incorporation into the PC fraction. Synaptosomal methyl group incorporation was found to remain statistically elevated for 3 days after removing ethanol from the diet and to return to control levels after 7 days. Fallon *et al.* (13) have observed an increase in phospholipid methylation in liver, and Nhamburo *et al.* (12) recently presented data which showed that ethanol administered by inhalation resulted in an increase in methyl group incorporation into rat synaptosomal phospholipids. Thus, our data showing a time-related increase in PC and decrease in PE coupled with an increase in the incorporation of methyl groups into the synaptosomal phospholipids are consistent with an ethanol-induced increase in the methylation pathway for PC synthesis from PE.

Rat brain synaptosomal methyltransferase activity is very low (11) when compared, for an example, to rat liver methyltransferase activity (13), and incubations done at pH 7.5 and with 2 μM SAM (optimum conditions for methyltransferase I) (11) resulted in such low levels of incorporation that small changes were difficult to detect. At pH 10.5 and with 200 μM SAM (optimum conditions for methyltransferase II) (11), the incorporation of methyl groups to form PC must depend on endogenous MMPE originally present in the membrane or produced by the methyltransferase I during the course of the incubation. Because our experiments measured synaptosomal lipid methyl group incorporation without the addition of exogenous phospholipid substrate, a limiting amount of endogenous MMPE substrate may partially explain our observation that methyl group incorporation was not totally linear with time. Additional characterization of the apparent ethanol-induced increase in methyltransferase activity is needed before an understanding of the observed increase in methyl group incorporation into the synaptosomal phospholipids can be reached.

The withdrawal and *in vitro* ethanol addition studies done in our laboratory produced results that differed from those previously reported. Nhamburo *et al.* (12) showed synaptosomal methyl group incorporation to return to control levels within 6–8 hr after withdrawing their rats from ethanol treatment by inhalation, whereas incorporation remained statistically elevated in our animals at 3 days following withdrawal from an ethanol-

containing liquid diet. In addition, we found *in vitro* additions of 10–250 mM ethanol to be stimulatory to synaptosomal methyl group incorporation while Nhamburo *et al.* (12) reported inhibitory effects at concentrations of 10–100 mM. There were many experimental differences between our work and that reported by Nhamburo *et al.* (12), which may help to explain the dissimilarities in the results. Animals in our study ingested ethanol for 14 days prior to the *in vitro* ethanol addition studies or to the withdrawal studies. Nhamburo *et al.* (12) treated their animals by ethanol inhalation for 5–10 days and did not report nutritional information (i.e., weight gain or loss, calories consumed). The route and length of time of ethanol administration may affect experimental findings, especially if the nutritional state of the animal is altered. We have noted an initial lag in utilization of ethanol calories as compared to the maltose-dextran calories of the control diet (see Materials and Methods: *Animals and diet*). Thus, it is possible that the biological effects of ethanol administered by inhalation may be somewhat different from those of dietary ethanol, especially when the caloric content of the dietary ethanol is considered. There are other differences in experimental design which should be mentioned including the fact that our rats had blood ethanol levels (Table 2) that were lower than those noted for the inhalation-treated rats (12). In addition, there were differences in synaptosomal preparation and in incubation pH (12). The effects of ethanol on SPM methyltransferase activity may depend on a variety of experimental conditions, and care must be taken in comparing results from different laboratories. In any case, ethanol treatment, both inhalation and ingestion, resulted in similar findings with respect to synaptosomal methyl group incorporation: it was increased.

In addition to changes in synaptic membrane PC and PE, we found a decrease in the SPM PS + PI fraction. Other studies done in this laboratory (28) have linked ethanol treatment to a reduction in the PS + PI fraction from rat spinal cord. A possible explanation for these observations is provided by work done by Virtanen (29) who used *in vivo* labeling experiments with [^{14}C]serine to show that ethanol treatment resulted in a decrease in serine incorporation into rat synaptosomal phospholipids. Harris *et al.* (30) have recently shown that chronic ethanol ingestion resulted in a decrease in both membrane fluidity and ethanol sensitivity in mouse synaptic plasma membranes, but their analysis of the membrane lipids found only a small decrease in the unsaturated acyl groups of PS. Changes in the total fatty acid composition of SPM from ethanol-treated rats have not been noted in our laboratory; however, an increase in octadecenoate (18:1) in PC marked by an increase in docosahexenoate (22:6, $n-3$) in PE has been observed after 14 days of ethanol ingestion.[2] Others (8, 9) have observed more rapid alterations in the fatty acid composition of synaptosomal membranes from animals treated by inhalation. Harris *et al.* (30) have suggested that ethanol treatment may alter the lipid arrangement or the lipid-protein interactions of the membrane. Earlier work from our laboratory (31) has linked changes in mitochondrial membrane phospholipids to alterations in mitochondrial respiration and energy-producing ability. The fact that synaptosomal methyltransferase I is located on the cytosolic side of the membrane with methyltransferase II on the opposite side (32) suggests that phospholipids may flip-flop from inside to outside during methylation. Evidence has been presented that shows the β-adrenergic receptor is regulated in part by the methylation of small amounts of membrane phospholipid (33). These observations give weight to the theory that small ethanol-induced changes in membrane phospholipid structure may cause biologically significant changes in membrane function, and our data suggest that these changes could contribute to the development of a physical dependence on ethanol.

[2] J. D. Magruder and R. C. Reitz, unpublished observations.

ACKNOWLEDGMENTS

The authors thank Dr. Donald Book and Dr. Clifton Blincoe for their help with the statistical analyses.

REFERENCES

1. Hill, M. W., and A. D. Bangham. General depressant drug dependency: a biophysical hypothesis. *Adv. Exp. Med. Biol.* **59**:1–9 (1975).
2. Chin, J. H., L. M. Parsons, and D. B. Goldstein. Increased cholesterol content of erythrocyte and brain membranes in ethanol-tolerant mice. *Biochim. Biophys. Acta* **513**:358–363 (1978).
3. Smith, T. K., and M. J. Gerhart. Alterations in brain lipid composition of mice made physically dependent to ethanol. *Life Sci.* **31**:1419–1425 (1982).
4. Rawat, A. K. Lipid metabolism in brain from mice chronically-fed ethanol. *Res. Commun. Chem. Pathol. Pharmacol.* **8**:461–469 (1974).
5. Alling, C., S. Liljequist, and J. Engel. The effects of chronic ethanol administration on lipids and fatty acids in subcellular fractions of rat brain. *Med. Biol.* **60**:149–154 (1982).
6. Sun, G. Y., and A. Y. Sun. Chronic ethanol administration induced an increase in phosphatidylserine in guinea pig synaptic membranes. *Biochem. Biophys. Res. Commun.* **113**:262–268 (1983).
7. Lee, N. M., H. J. Friedman, and H. H. Loh. Effects of acute and chronic ethanol treatment on rat brain phospholipid turnover. *Biochem. Pharmacol.* **29**:2815–2818 (1980).
8. Littleton, J. M., and G. John. Synaptosomal membrane lipids of mice during continuous exposure to ethanol. *J. Pharm. Pharmacol.* **29**:579–580 (1977).
9. Littleton, J. M., G. R. John, and S. J. Grieve. Alterations in phospholipid composition in ethanol tolerance and dependence. *Alcohol. Clin. Exp. Res.* **3**:50–56 (1979).
10. Sun, G. Y., and A. Y. Sun. Effects of chronic ethanol administration on phospholipid acyl groups of synaptic plasma membrane fraction isolated from guinea pig brain. *Chem. Pathol. Pharmacol.* **24**:405–408 (1979).
11. Crews, F. T., F. Hirata, and J. Axelrod. Identification and properties of methyltransferases that synthesize phosphatidylcholine in rat brain synaptosomes. *J. Neurochem.* **34**:1491–1498 (1980).
12. Nhamburo, P. T., G. R. John, and J. Littleton. Alterations in phospholipid methylation in rat brain synaptosomal membranes produced by ethanol *in vivo* and *in vitro*. *Biochem. Pharmacol.* **31**:3936–3938 (1982).
13. Fallon, H. J., P. M. Gertman, and E. L. Kemp. The effects of ethanol ingestion and choline deficiency on hepatic lecithin biosynthesis in the rat. *Biochim. Biophys. Acta* **187**:94–104 (1969).
14. Thompson, J. A., and R. C. Reitz. Effects of ethanol ingestion and dietary fat levels on mitochondrial lipids in male and female rats. *Lipids* **13**:540–550 (1978).
15. Numan, R., and A. M. Gilroy. Induction of physical dependence upon ethanol in rats using intravenous infusion. *Pharmacol. Biochem. Behav.* **9**:279–282 (1978).
16. Majchrowicz, E. Induction of physical dependence upon ethanol and the associated behavioral changes in rats. *Psychopharmacologia (Berl.)* **43**:245–254 (1975).
17. Sigma Chemical Company. *Ethyl Alcohol in Blood, Serum, Plasma, Urine or Other Fluids at 340 mμ, Sigma Technical Bulletin No. 331-UV.* Sigma, St. Louis, MO (1972).
18. Sun, G. Y., and A. Y. Sun. Phospholipids and acyl groups of synaptosomes and myelin membranes isolated from the cerebral cortex of squirrel monkey (*Saimiri sciureus*). *Biochim. Biophys. Acta* **280**:306–315 (1972).
19. Duncan, H. M., and B. Mackler. Electron transport system of yeast. III. Preparation and properties of cytochrome oxidase. *J. Biol. Chem.* **241**:1694–1697 (1966).
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein

measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
21. Bligh, E. G., and W. J. Dyer. A rapid method of total lipid extraction. *Can. J. Biochem.* **37**:911–917 (1959).
22. Arvidson, G. A. D. Reversed-phase partition thin-layer chromatography of rat liver lecithins to yield eight simple phosphatidycholines. *J. Lipid Res.* **8**:155–158 (1967).
23. Bartlett, G. R. Phosphorous assay in column chromatography. *J. Biol. Chem.* **234**:469–471 (1959).
24. Shapiro, S. K., and D. J. Ehniger. Method for the analysis and preparation of adenosylmethionine and adenosylhomocysteine. *Anal. Biochem.* **15**:323–333 (1966).
25. Blomquist, G. J., and L. J. Jackson. Incorporation of labelled dietary n-alkanes into cuticular lipids of the grasshopper *Melanoplus sanguinipes*. *J. Insect. Physiol.* **19**:1639–1647 (1973).
26. Cooper, M. F., and G. R. Webster. The differentiation of phospholipase A_1 and A_2 in rat and human nervous tissue. *J. Neurochem.* **17**:1543–1554 (1970).
27. Zatz, M., P. A. Dudley, Y. Kloog, and S. P. Markey. Nonpolar lipid methylation: biosynthesis of fatty acid methyl esters by rat lung membranes using *S*-adenosylmethionine. *J. Biol. Chem.* **256**:10028–10032 (1981).
28. Starich, G. H., and R. C. Reitz. Effects of chronic ethanol digestion on the fatty acids and phospholipids of spinal cord. *Toxicol. Lett.* **4**:1–5 (1979).
29. Virtanen, P. Labeling of rat brain synaptosomal phosphatidylserine in the after state of acute alcoholism intoxication and in the withdrawal state. *Adv. Exp. Med. Biol.* **85A**:193–202 (1977).
30. Harris, R. A., D. M. Baxter, M. A. Mitchell, and R. J. Hitzemann. Physical properties and lipid composition of brain membranes from ethanol tolerant-dependent mice. *Mol. Pharmacol.* **25**:401–409 (1984).
31. Schilling, R. J., and R. C. Reitz. A mechanism for ethanol-induced damage to liver mitochondrial structure and function. *Biochim. Biophys. Acta* **603**:266–277 (1980).
32. Crews, F. T., F. Hirata, and J. Axelrod. Phospholipid methyltransferase asymmetry in synaptosomal membranes. *Neurochem. Res.* **5**:983–991 (1980).
33. Strittmatter, W. J., F. Hirata, and J. Axelrod. Regulation of the β-adrenergic receptor by methylation of membrane phospholipids. *Adv. Cyclic Nucleotide Res.* **14**:83–91 (1981).

Send reprint requests to: Ronald C. Reitz, Department of Biochemistry, School of Medicine, University of Nevada, Reno, NV 89557.