Chronic Ethanol Increases Liver Plasma Membrane Fluidity†

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ABSTRACT: Purified plasma membrane fractions of cultured well-differentiated Reuber H35 hepatoma cells were studied after growth in the presence or absence of ethanol. Growth of cells in the presence of ethanol significantly increased plasma membrane 5′-nucleotidase activity but did not influence sodium–potassium adenosinetriphosphatase activity. Fluorescence polarization of lipophilic probes was used to study membrane lipid structure. Steady-state polarization of diphenylhexatriene (DPH), a probe of the hydrophobic core, was significantly lower in plasma membranes from cells grown in 80 mM ethanol for 3 weeks, compared to controls. Decreased polarization of DPH in plasma membranes was observed after 3-weeks growth of cells in as little as 1 mM ethanol. A 1-h exposure to 80 mM ethanol had no effect. Altered DPH polarization was due to a decrease in the order parameter of the probe. The rotational correlation time of the probe was virtually unchanged. Chronic ethanol treatment of cells did not alter the polarization of the membrane surface probe trimethylammoniodiphenylhexatriene. Plasma membranes from cells grown in 80 mM ethanol had decreased contents of both phospholipid and unesterified cholesterol, but the cholesterol to phospholipid ratio was unchanged. The percentages of sphingomyelin and phosphatidylserine in plasma membrane phospholipids were significantly decreased after ethanol treatment, while the phosphatidylcholine/sphingomyelin ratio was increased by 42%. Vesicles prepared from total plasma membrane lipids of ethanol-treated cells, as well as vesicles prepared from polar lipids alone, showed the same alterations in DPH polarization as did plasma membranes. The importance of ethanol metabolism in the observed plasma membrane changes was demonstrated in two ways. First, growth of the fibroblast cell line CHO-K1, which lacks alcohol dehydrogenase activity, in the presence of 80 mM ethanol had no effect on DPH fluorescence polarization in purified plasma membrane fractions. Second, the ethanol-mediated change in H35 plasma membrane DPH polarization was completely prevented by simultaneous administration of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase activity. In summary, the H35 hepatoma cell plasma membrane becomes disordered after chronic ethanol administration. This structural change may be due in part to changes in plasma membrane polar lipid composition and appears to be dependent on ethanol oxidation.

Alteration of membrane lipid structure can influence the activities of membrane-associated enzymes and transport proteins (Stubbs & Smith, 1984). Thus, the ability of ethanol to directly increase the fluidity[1] of membranes in vitro has been invoked to explain many of its pharmacologic effects. Other effects of ethanol, such as the development of tolerance and dependence, occur only after chronic ethanol administration, and so are presumed to arise after some cellular response to its membrane-disordering properties (Harris & Hitzemann, 1981).

Ethanol is a known hepatotoxin. Acutely, moderate consumption leads to reversible deposition of neutral lipids in the hepatocyte (Baraona & Lieber, 1979). Chronic alcohol abuse is a major risk factor in the development of liver cirrhosis in humans, although only a minority of chronic alcoholics develop this disease (Burnett & Sorrell, 1981). The underlying mechanisms of ethanol-induced hepatocellular necrosis are not well understood.

A number of investigators have studied the effects of chronic ethanol consumption of the structure and composition of hepatic membrane lipids. In general, these studies have emphasized mitochondrial (Cunnigham et al., 1982; Hosein et al., 1980; Waring et al., 1982) or microsomal (Cunningham et al., 1982) membranes. Few studies have focused on the liver plasma membrane, despite the importance of this membrane in nutrient and ion transport and in maintenance of cellular integrity.

Studies of the effects of chronic ethanol in animal models are complicated. Ethanol may influence nutrient absorption, and unless carefully administered, ethanol-containing diets may be poorly tolerated. Pair feeding can alleviate some of these problems, but switching animals from chow to liquid diets can alter plasma membrane lipid composition, even in the absence of ethanol.[2] Furthermore, the liver contains different cell types which may respond differently to ethanol.

We chose to study the effects of chronic ethanol on the plasma membrane of well-differentiated Reuber H35 hepatoma cells, a homogeneous population of liver-derived cells in culture. These cells maintain a number of hepatocyte-specific functions, including the ability to oxidize ethanol (Bertolotti & Weiss, 1972). We previously showed that these cells, like hepatocytes in vivo, had increased contents of triacylglycerol and cholesteryl ester after short- or long-term exposure to ethanol (Polokoff et al., 1983). This effect did not occur in cultured Chinese hamster ovary cells treated similarly. In the present study, cells were grown for up to 3 weeks in the presence or absence of ethanol, and the lipid composition and

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[1] The term "fluidity" in this report refers to the relative motional freedom of membrane constituents, as sensed by fluorescent probe molecules. It encompasses both rotational diffusion, a function of microviscosity, and hindered anisotropic rotations, a function of lipid order. Except where stated explicitly in the text, no attempt is made to distinguish these types of motion when reference is made to fluidity.

[2] T. Zysset, M. A. Polokoff, and F. R. Simon, unpublished observations.

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fluidity of purified plasma membrane fractions were determined. We compared the effects of ethanol in these liver-derived cells with effects in cultured Chinese hamster ovary cells.

Experimental Procedures

Cell Lines and Culture. Well-differentiated Reuber H35 rat hepatoma cells, clone ACl-1, and the Chinese hamster ovary cell line CHO-K1 were grown in monolayer culture essentially as described previously (Polokoff et al., 1983), except that Ham's F12 medium was used throughout. For growth of H35-ACl-1, this medium was supplemented with 4% (v/v) each of calf serum and fetal calf serum. Growth medium for CHO-K1 was supplemented with 5% (v/v) fetal calf serum.

Reagent-grade 95% ethanol was redistilled prior to addition to growth media. To prevent evaporation, growth media were preequilibrated with a 95% air/5% CO_2 atmosphere, then ethanol was added, and flasks were immediately capped tightly. Control flasks were treated identically. Media and ethanol were replenished every 3 days. At the highest level of ethanol used in these studies, 80 mM, little change in the concentration of ethanol in the media could be detected after 3 days of incubation with cells. However, at an initial concentration of 10 mM, a decrease in the amount of ethanol remaining in the growth medium was evident by the second day. At an initial concentration of 1 mM, the lowest level used in our studies, ethanol was undetectable after the second day of incubation.

As reported previously (Polokoff et al., 1983), viability of H35-ACl-1 was unaffected by growth in the presence of up to 80 mM ethanol. The population doubling time of these cells (24 h) was not altered by the presence of up to 10 mM ethanol but was increased to 34 h in 80 mM ethanol. In contrast, 80 mM ethanol had no measurable effect on the viability or doubling time (14 h) of CHO-K1 cells. The addition of 0.2 mM 4-methylpyrazole did not affect the growth rate or viability of H35-ACl-1.

Plasma Membrane Purification. Fifteen nearly confluent flasks (75 cm^2 growth area) of cells were pooled for a membrane preparation. All subsequent steps were performed at 4 °C. The cell monolayers were washed twice with isotonic phosphate-buffered saline. Cells were hypotonically lysed by incubation for 20 min with 1 mM sodium bicarbonate. The lysate (homogenate) was centrifuged at 27000*g* for 15 min. The pellet, containing the total cellular membranes (total membranes), was resuspended in 46% (w/v) sucrose, adjusted to 44% sucrose, and overlayed with 1 mL of 41% sucrose and 5 mL of 38% sucrose. The gradient was centrifuged for 2.5 h at 90000*g* in an SW 41 Ti rotor. The opalescent material at the top of the gradient was collected, diluted 20-fold with 1 mM sodium bicarbonate, and centrifuged at 48000*g* for 30 min. The pellet (plasma membrane) was resuspended in 1 mM sodium bicarbonate and frozen at −20 °C under argon for up to 2 days before analysis. In a typical preparation, 500–1000 μg of plasma membrane protein was recovered.

Enzyme Assays. Sodium–potassium adenosinetriphosphatase (Zysset et al., 1983) and 5′-nucleotidase (Song & Bodansky, 1967) were employed as plasma membrane marker enzyme activities. Mitochondrial contamination of plasma membrane preparations was assessed by measurement of cytochrome *c* oxidase activity (Straus, 1956) and microsomal contamination by NADPH–cytochrome *c* reductase activity (Baron & Tephly, 1969). All assays were performed at 37 °C.

Fluorescence Polarization. (*A*) *Steady-State Measurements.* An HH-1 T-format polarization spectrofluorometer (BHL Associates, Burlingame, CA) with fixed excitation and emission polarization filters was used to measure fluorescence intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the polarization plane of the exciting light (Harris et al., 1984). Polarization $[(I_\parallel - I_\perp)(I_\parallel + I_\perp)]$ and fluorescence intensity $(I_\parallel + 2I_\perp)$ were calculated by an on-line microprocessor. For all probes, the excitation wavelength was 362 nm. Excitation filters (03FCG001) were obtained from Melles Griot, Irvine, CA, and emission filters (KV389) from Schott Optical, Duryea, PA. Membrane samples were diluted to a final concentration of 50 μg of protein/mL in phosphate-buffered saline for analysis. Fluorescent probes were incorporated into membrane samples or lipid vesicles at 35 °C for 10 min with frequent vortexing. Probes (Molecular Probes, Inc., Junction City, OR) were used at a ratio of 1 mol of probe to 500 mol of phospholipid. The temperature dependence of fluorescence polarization was studied by placing the sample in a thermostated compartment at the highest temperature tested and then reducing the temperature by 0.5 °C/min using a refrigerating water bath. The data were analyzed in the form of Arrhenius plots of anisotropy parameter vs. temperature (Brasitus et al., 1980). Steady-state fluorescence polarization (P)[3] was converted to steady-state anisotropy (r_s) by the equation $r_s = 2P/(3 - P)$. The anisotropy parameter, $[(r_0/r_s) - 1]^{-1}$, was calculated by using 0.362 as the maximal limiting anisotropy of DPH (Shinitzky & Barenholz, 1974).

(*B*) *Fluorescence Lifetime Measurements.* The fluorescence lifetimes (τ_F) of DPH in plasma membranes from control and ethanol-treated cells were obtained by using a Photochemical Research Associates Model 3000 nanosecond lifetime fluorometer, housed at the Solar Energy Research Institute, Golden, CO. The method of single-photon counting has been described in detail previously (Gudgin et al., 1981). The fluorescence decay data were fitted to a monoexponential curve by the instrument to generate the lifetimes.

(*C*) *Calculation of the Order Parameter and Rotational Correlation Time of DPH.* The order parameter (S) and the rotational correlation time (τ_c) were calculated form the steady-state fluorescence anisotropy and fluorescence lifetime data (Heyn, 1979; Van Blitterswijk et al., 1981). The structural component of anisotropy, r_∞, is equal to $^4/_3 r_s - 0.1$. The order parameter (S) can then be calculated from $S = (r_\infty/r_0)^{0.5}$. The kinetic component of anisotropy, r_f, is equal to $r_s - r_\infty$. The rotational correlation time is then equal to $\tau_F r_f/(r_0 - r_s)$.

Membrane Lipid Extraction and Analysis. (*A*) *Compositional Studies.* Membrane lipids were extracted by the Bligh–Dyer method (Bligh & Dyer, 1959). The lipid extract was washed once with the aqueous phase of a blank extraction. Methods for quantitation of total phospholipid composition, unesterified cholesterol composition, phospholipid polar head-group species composition, and lipid acyl chain composition have been described previously (Polokoff et al., 1983). Gas–liquid chromatogram peak areas were analyzed by using an automatic integrator. Identification of individual peaks was made by coretention with standard compounds.

(*B*) *Membrane Lipid Vesicle Preparation.* Total lipid fractions were prepared either by the Bligh–Dyer method or by a nonaqueous extraction procedure (Harris et al., 1984).

[3] Abbreviations: DPH, diphenylhexatriene; TMA-DPH, trimethylammoniodiphenylhexatriene; P, steady-state fluorescence polarization; r_s, steady-state fluorescence anisotropy; r_0, maximum limiting fluorescence anisotropy; NS, not significant; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; ns, nanosecond.

Table I: Purification of H35 Plasma Membranes

fraction	protein (% of total)	marker enzyme enrichment[a] 5′-nucleotidase	(Na,K)-ATPase	NADPH–cyt *c* reductase	cyt *c* oxidase
homogenate	100.0	1.0 (100)[b]	1.0 (100)	1.0 (100)	1.0 (100)
total membranes	52.1	1.5 (81)	1.5 (81)	1.4 (78)	1.4 (79)
plasma membrane	2.5	12.2 (27)	10.4 (27)	1.5 (4)	2.2 (6)

[a] Enrichment is defined as specific activity/specific activity of the homogenate. [b] The numbers in parentheses indicate the percent of the total homogenate activity recovered at each step.

Polar lipids were separated from neutral lipids by silicic acid chromatography of the lipids extracted by the latter procedure. Neutral lipids were eluted from the column with chloroform, and then polar lipids were eluted separately with methanol. Vesicles were prepared from total lipids or polar lipids by first drying the extracts to a thin film under argon, resuspending in phosphate-buffered saline, and purging again with argon, and finally placing the samples in a bath-type sonicator (Model B-200, 125-W power; Branson Equipment Co., Shelton, CT) for 1 min at 35 °C. Vesicles formed under these conditions are mostly large multilamellar structures. Vesicles were used for fluorescence polarization studies immediately after preparation.

Statistical Analysis. Comparisons of the disordering effects of in vitro ethanol on plasma membrane (Figure 1) were made by using an analysis of variance program for repeated measures on a microcomputer. A computer program was used to analyze Arrhenius plots for potential break points (Molitoris & Jones, 1984). In this program, an F test is used to determine whether the data are better fitted to a single line or to two lines connected at a break point. Other comparisons between treatment groups were made by a two-tailed Student's *t* test for unpaired samples. Statistical significance was defined as $p < 0.05$. All data are expressed as mean ± standard deviation.

Results

Reuber H35-ACl-1 Hepatoma Cells: Plasma Membrane Purification from Control and Ethanol-Treated Cells. The purification and yield of plasma membranes prepared from control ACl-1 cells were assessed by marker enzyme analysis. A typical preparation is shown in Table I. Two activities located in the plasma membrane, 5′-nucleotidase and (Na,-K)-ATPase, were enriched 10–12-fold over the total cellular homogenate and recovered with an overall yield of greater than 25%. The mitochondrial activity cytochrome *c* oxidase and the microsomal activity NADPH–cytochrome *c* reductase were enriched only about 2-fold over the homogenate specific activities and were recovered with overall yields of 6% and 4%, respectively. Growth of cells in media containing 80 mM ethanol for up to 3 weeks had no significant effect on the enrichment or overall recovery of these marker enzymes in plasma membrane fractions.

Activities of Plasma Membrane Enzymes. The specific activity of 5′-nucleotidase was significantly increased from 196 ± 56 to 259 ± 68 μmol h^{-1} (mg of protein)$^{-1}$ in purified plasma membranes of H35-ACl-1 following 3 weeks of growth in 80 mM ethanol ($n = 15$; $p < 0.005$). A similar increase was seen in homogenates. Mixing control and ethanol-treated fractions gave the average of the two specific activities, indicating that no inhibitors or activators of 5′-nucleotidase were present in the preparations. In contrast to these results, the specific activity of (Na,K)-ATPase was unaffected by growth in ethanol in plasma membrane fractions (control = 6.9 ± 1.2 μmol h^{-1} mg^{-1}; ethanol = 6.3 ± 0.5 μmol h^{-1} mg^{-1}; $n = 5$; NS).

Fluorescence Polarization in Membrane Fractions. The fluidity of membrane fractions prepared from cells grown in the presence or absence of 80 mM ethanol for 3 weeks was compared by using steady-state fluorescence polarization. Diphenylhexatriene (DPH) was used to probe the hydrophobic core of the membrane. In plasma membrane fractions, a significant decrease was observed in the ethanol-treated group. In 15 separate experiments, DPH polarization in control plasma membranes was 0.205 ± 0.006, while in plasma membranes from cells grown in ethanol the value was 0.182 ± 0.006 ($p < 0.001$). No significant change occurred in DPH polarization of total membrane fractions (control = 0.170 ± 0.007; ethanol-treated fraction = 0.172 ± 0.008; $n = 3$; NS). Trimethylammoniodiphenylhexatriene (TMA-DPH) probed the region close to the membrane surface. No significant differences between the two groups were seen in TMA-DPH fluorescence polarization of either plasma membranes (control = 0.287 ± 0.010; ethanol treated = 0.290 ± 0.009; $n = 8$; NS) or total membranes (control = 0.259 ± 0.002; ethanol treated = 0.261 ± 0.003; $n = 3$; NS).

The disordering effects of ethanol in vitro were compared in plasma membranes of cells grown in the presence or absence of 80 mM ethanol (Figure 1). As expected, addition of ethanol to membranes produced a dose-dependent decrease in the fluorescence polarization of DPH. Plasma membranes from cells grown in ethanol were more resistant to disordering than

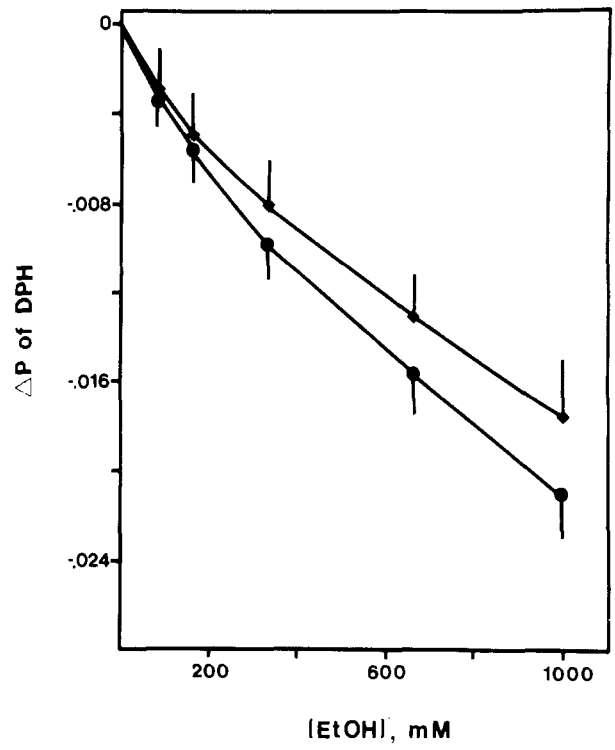

FIGURE 1: Effect of in vitro ethanol addition on DPH fluorescence polarization of H35-ACl-1 plasma membrane fractions at 35 °C. (●) Control plasma membranes ($n = 15$); (♦) plasma membranes from cells grown 3 weeks in the presence of 80 mM ethanol ($n = 15$). An analysis of variance for repeated measures indicated that trends for the two groups were significantly different ($p < 0.001$).

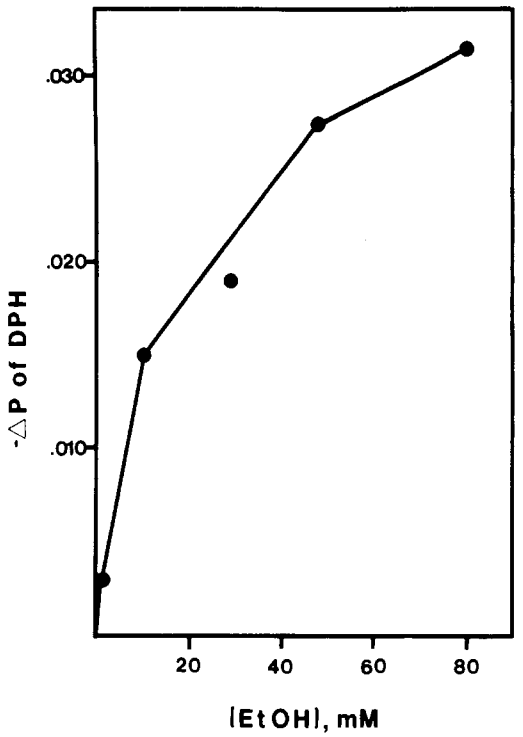

FIGURE 2: Effects of growth of H35-ACl-1 cells in ethanol on plasma membrane DPH fluorescence polarization measured at 35 °C. Cells were grown for 3 weeks in the presence of 0, 1, 10, 24, 48, or 80 mM ethanol. Each value represents a single determination. The absolute polarization value in the absence of ethanol was 0.210.

control plasma membranes ($p < 0.001$). Note that the in vitro effects of 80 mM ethanol were small and did not account for the difference in base-line DPH polarization between the two treatment groups. In fact, addition of about 1 M ethanol in vitro was necessary to alter DPH polarization of control plasma membranes to the same degree as did growth in 80 mM ethanol.

The decrease in plasma membrane DPH fluorescence polarization after exposure of H35-ACl-1 cells to ethanol was dependent on dose and time. A measurable effect was seen after 3 weeks of growth in the presence of as little as 1 mM ethanol (Figure 2). At 80 mM ethanol, no change in DPH polarization occurred after a 1-h exposure, while 3 days of exposure produced a significant change (data not shown).

The temperature dependence of plasma membrane DPH polarization was measured, and the results were expressed in the form of Arrhenius plots (Figure 3). Computer analysis (Molitoris & Jones, 1984) of the data for both control and ethanol-treated cells revealed no break points in the range 14–39 °C. The change in DPH polarization produced by chronic ethanol was observed throughout this range of temperatures.

The DPH fluorescence lifetimes (τ_F) were determined by single-photon counting for one representative plasma membrane sample each of control ($\tau_F = 9.64 \pm 0.06$ ns) and ethanol-treated ($\tau_F = 9.24 \pm 0.07$ ns) cells. These data, along with the mean steady-state polarization data, were used to calculate the order parameters and the rotational correlation times of DPH. The order parameter of DPH in plasma membranes was decreased from 0.52 to 0.45 after chronic ethanol treatment, while rotational correlation times were essentially identical for the two groups (2.28 and 2.25 ns, respectively).

Composition and Fluidity of Plasma Membrane Lipids. The lipid compositions of plasma membranes from control and

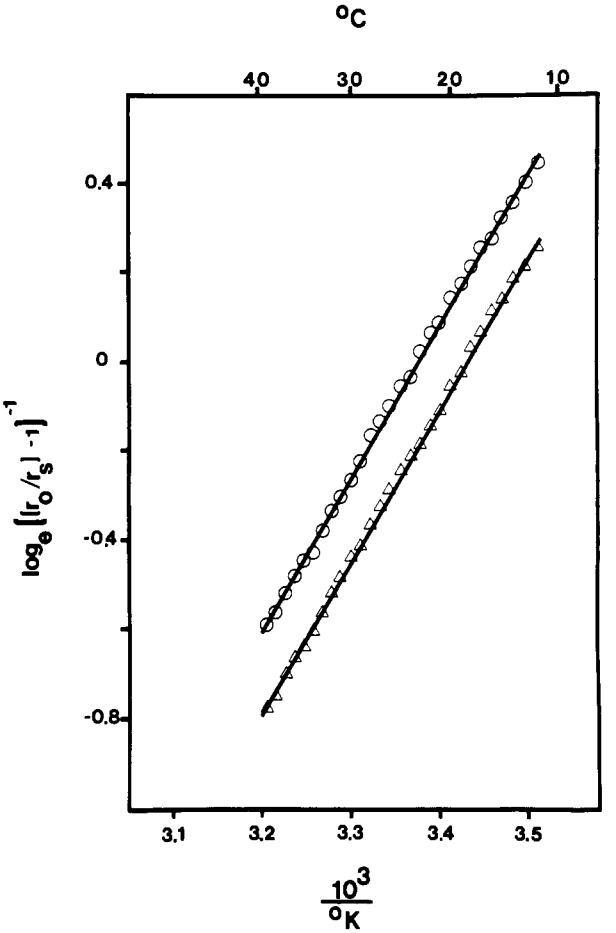

FIGURE 3: Effect of temperature on DPH anisotropy parameter in H35-ACl-1 plasma membranes. Polarization values were converted to the anisotropy parameter, $[(r_0/r_s) - 1]^{-1}$, as described in the text. (○) Control plasma membrane; (△) plasma membrane fraction from cells grown for 3 weeks in the presence of 80 mM ethanol.

Table II: Phospholipid and Cholesterol Contents of H35 Plasma Membranes[a]

growth medium	phospholipid (nmol/mg of protein)	cholesterol (nmol/mg of protein)	cholesterol/phospholipid (mol/mol)
control	1100 ± 218	380 ± 76	0.36 ± 0.11
+80 mM ethanol	876 ± 170[b]	309 ± 77	0.36 ± 0.07

[a] Cells were grown for 3 weeks as indicated. Data are the means and standard deviations of 10 experiments. [b] Significantly different from control ($p < 0.02$).

ethanol-treated cells were compared. After 3 weeks of growth in 80 mM ethanol, the plasma membrane content of total phospholipid relative to protein was decreased significantly by 20% (Table II). The unesterified cholesterol content relative to protein was decreased an average of 20%, although statistical significance was not achieved ($p < 0.1$). As a result, the molar ratio of cholesterol to phospholipid in the plasma membrane was unaltered by ethanol treatment.

Phospholipid polar head-group species composition was determined after separation by two-dimensional thin-layer chromatography (Figure 4). Sphingomyelin and phosphatidylserine were reduced significantly by 23% and 34%, respectively, after ethanol treatment. The ratio of sphingomyelin to phosphatidylcholine was increased significantly by 42% following ethanol treatment.

The fatty acid composition of total plasma membrane polar lipids was determined after removal of neutral lipids by silicic acid chromatography. Following ethanol treatment of cells,

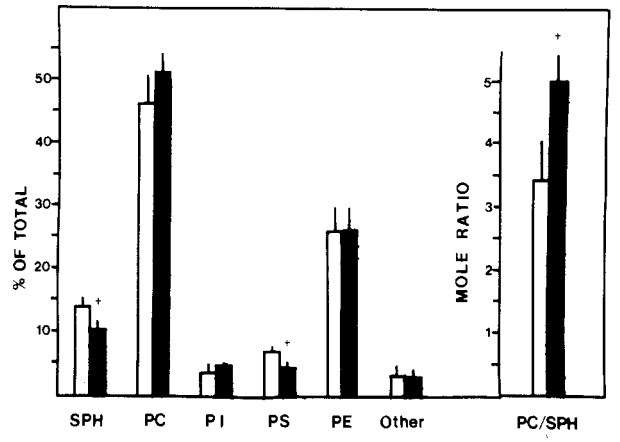

FIGURE 4: Phospholipid composition of H35-ACl-1 plasma membranes. Light bars are data from control plasma membranes (n = 5). Dark bars are data from plasma membranes of cells grown for 3 weeks in the presence of 80 mM ethanol (n = 5). The symbol "+" indicates that data are significantly different from control data (p < 0.01).

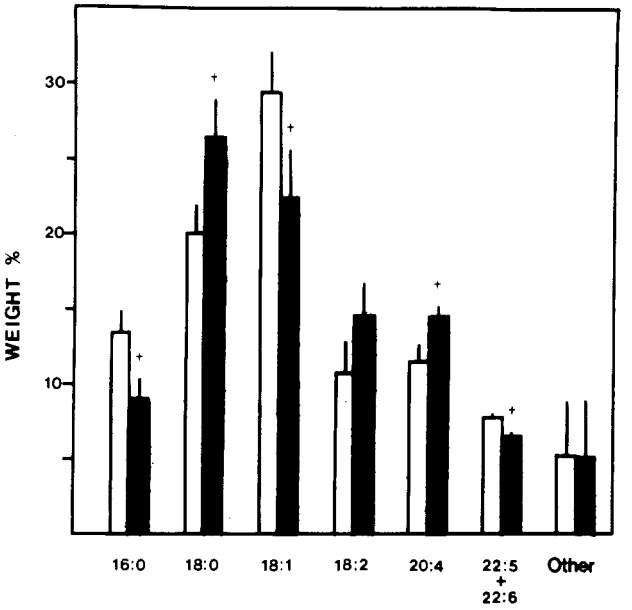

FIGURE 5: Acyl chain composition of total polar lipids from H35-ACl-1 plasma membranes. Light bars represent controls (n = 3). Dark bars represent cells grown for 3 weeks in 80 mM ethanol (n = 3). The symbol "+" indicates that data are significantly different from control data (p < 0.05).

the contents of 16:0, 18:1, and 22:5 + 22:6 were decreased, while 18:0, 18:2, and 20:4 were increased (Figure 5). However, the ratio of saturated to unsaturated fatty acids was not significantly affected.

To determine whether the change in the polarization of DPH observed in plasma membranes of ethanol-treated cells was due to alterations in membrane lipid composition, vesicles prepared by sonication of protein-free lipid extracts were probed with DPH. With the nonaqueous extraction protocol, vesicles prepared from plasma membranes of cells grown in ethanol displayed decreased polarization compared to control vesicles (0.160 ± 0.009 vs. 0.179 ± 0.007; n = 8; p < 0.01). After removal of neutral lipids by silicic acid chromatography, vesicles of the total polar lipids from the ethanol group also showed decreased DPH polarization (0.115 ± 0.008 vs. 0.127 ± 0.008; n = 4; p < 0.01). Virtually identical results were obtained using vesicles prepared from Bligh–Dyer-extracted plasma membranes.

Effects of 4-Methylpyrazole. To investigate the potential role of alcohol dehydrogenase mediated ethanol oxidation in the alteration of H35-ACl-1 plasma membrane structure, cells grown for 3 weeks in the presence of 0.2 mM 4-methylpyrazole alone, 10 mM ethanol alone, or both agents, were compared to control cells. Growth in 4-methylpyrazole had no effect on plasma membrane DPH polarization (control, 0.206 ± 0.004; 4-methylpyrazole, 0.204 ± 0.004; n = 4; NS). Growth in 10 mM ethanol decreased DPH polarization (0.181 ± 0.001; n = 4; p < 0.001). The presence of 4-methylpyrazole completely prevented the ethanol-induced decrease in plasma membrane DPH polarization (0.203 ± 0.001; n = 4; NS).

Chinese Hamster Ovary Cells. To determine whether the plasma membrane changes observed in ethanol-treated H35-ACl-1 cells occur in cells of extrahepatic origin, CHO-K1 fibroblasts were grown for 3 weeks in the presence or absence of 80 mM ethanol. Plasma membranes prepared from these cells showed a similar enrichment of marker enzymes as in H35-ACl-1 cells. DPH fluorescence polarization were not different in plasma membranes from the control and ethanol-treated groups (0.240 ± 0.003 vs. 0.238 ± 0.006; n = 8; NS).

Discussion

The plasma membrane preparations were residually contaminated with other intracellular membranes; as judged by marker enzyme analysis (Table I). Contamination by mitochondrial and microsomal markers was low and was not affected by growth of cells in ethanol. Therefore, changes in DPH polarization are likely to reflect true differences in the physical properties of these membranes.

The data presented indicate that chronic ethanol administration caused a decrease in the order parameter of DPH in the H35 cell plasma membrane. The order parameter of DPH is a measure of the degree to which molecular packing of membrane lipids (a static factor) hinders rotation of the probe. Thus, it appears that chronic ethanol treatment leads to a relative relaxation of these packing restraints in the hydrophobic core region of the plasma membrane.

Fluorescence polarization of DPH reflects the bulk properties of membranes, since this probe appears to partition equally into liquid-crystalline and gel-phase lipid regions (Lentz et al., 1976). Ethanol could selectively alter lipid microenvironments in the vicinity of various intrinsic plasma membrane proteins. Two plasma membrane enzymes whose activities are influenced by lipid fluidity, 5′-nucleotidase (Dipple et al., 1982) and (Na,K)-ATPase (Kimelberg, 1977; Davis et al., 1978; Sinensky et al., 1979), responded differently to chronic ethanol. The ethanol-dependent increase in 5′-nucleotidase activity noted in the H35 cell is similar to that seen in the cultured C6 glioma cell (Syapin et al., 1980). The opposite effect was reported in the liver plasma membrane of ethanol-fed rats (Nishimura & Teschke, 1982). We observed no change in (Na,K)-ATPase activity after growth of H35 cells in the presence of ethanol. In a previous study, (Na,K)-ATPase activity was reportedly increased after chronic ethanol feeding in rats, and the hypothesis was put forward that this increase could induce a hypermetabolic state in the liver leading to centrolobular necrosis (Israel et al., 1975). Other workers have disputed this result (Gordon, 1977; Schaffer et al., 1981).

Our results appear to conflict with the concept that membrane alterations induced by ethanol are necessarily governed by homeoviscous adaptation (Sinensky, 1974) to the direct membrane-disordering actions of the drug. Evidence favoring homeoviscous adaptation of microsomal membranes to chronic ethanol treatment has been reported. Treatment of cultured

CHO-K1 cells with high (0.34 M) concentrations of ethanol increased the cholesterol content of microsomal membranes due to an enhancement of 3-hydroxy-3-methylglutaryl-CoA reductase activity (Sinensky & Kleiner, 1981). Our plasma membrane purification procedure did not permit simultaneous purification of microsomal or mitochondrial membranes. However, the average DPH polarization of all intracellular membranes was similar in control and ethanol-treated H35 cells. This suggests that the response of the plasma membrane to chronic ethanol may be distinct from the responses of other cellular membranes.

In mammalian systems, most work on plasma membrane alterations induced by chronic ethanol has been done in brain. Synaptic plasma membranes from mice treated chronically with ethanol were more rigid than control membranes, whether measured by electron spin resonance (Lyon, & Goldstein, 1983) or by fluorescence polarization (Harris et al., 1984). In the latter study, extracted membrane lipids from the two groups had similar fluidities, suggesting that membrane proteins were important in the observed changes. In studies of liver plasma membranes, lipid composition was measured after ethanol treatment (Wing et al., 1982), but measurements of membrane physical properties were not reported.

In addition to direct measurements of plasma membrane physical properties, we measured the physical properties of plasma membrane lipid fractions. As judged by fluorescence polarization of DPH, cells treated chronically with ethanol had plasma membrane lipids which were less ordered than lipids from control membranes. These changes persisted after removal of neutral lipids, suggesting that membrane sterol content played no important role in altering the physical properties of ethanol-treated liver cell plasma membranes. Consistent with this conclusion, the plasma membrane cholesterol to phospholipid molar ratio was unaltered by exposure of cells to ethanol (Table II). Growth of H35 cells in ethanol decreased the phospholipid/protein ratio of the plasma membrane (Table II). While such a change would be expected to increase lipid order, it was not sufficient to restore this parameter to control levels.

The polar head-group and fatty acid composition of plasma membrane phospholipids were measured to identify biochemical changes associated with altered membrane physical properties. From model membrane studies, the increased phosphatidylcholine/sphingomyelin ratio found in membranes from ethanol-treated cells (Figure 4) would be expected to increase bilayer fluidity (Shinitzky & Barenholz, 1974). Work on erythrocytes has confirmed that this ratio is also an important determinant of fluidity in biological membrane (Cooper et al., 1977). The decrease in plasma membrane phosphatidylserine content after ethanol treatment (Figure 4) may also contribute to the change in membrane physical properties, since we have determined that 18:0 constitutes greater than 65% of the acyl chains of this lipid in the H35 cell (data not shown). A 50% increase in this phospholipid in guinea pig synaptic plasma membranes after chronic ethanol administration was previously reported (Sun & Sun, 1983).

While dramatic changes in total phospholipid fatty acid composition accompanied chronic ethanol treatment of cells (Figure 5), no shift in the saturated to unsaturated fatty acid ratio occurred. It is therefore difficult to predict the effect that altered fatty acid composition might have had on lipid order. In a previous study (Wing et al., 1982), mice were exposed to chronic ethanol. These workers reported a significant decrease in liver plasma membrane phospholipid fatty acid content of 18:0 and a significant increase in 18:1, changes quite distinct from those reported here. This differential effect of ethanol on the fatty acid composition of phopsholipids might be explained by differing degrees of plasma membrane purification (not reported in the earlier study), altered regulation of lipid metabolism in hepatoma cells, or secondary effects of ethanol on hormone levels or dietary lipid absorption in the whole animal.

Chronic ethanol feeding of experimental animals made liver mitochondrial (Waring et al., 1981), liver microsomal (Ponnappa et al., 1982), and brain synaptic plasma membranes (Lyon & Goldstein, 1983; Harris et al., 1984) more resistant to the disordering effects of ethanol in vitro. While the underlying mechanism of this resistance is unknown, it may involve a decrease in the binding of ethanol to these membranes (Rottenberg et al., 1981). We observed that plasma membranes of ethanol-treated hepatoma cells were also more resistant to ethanol disordering (Figure 1). Thus, ethanol resistance may arise by a mechanism unrelated to bulk membrane fluidity.

The decreased plasma membrane lipid order seen after chronic ethanol treatment may be unique to the liver cell. In preliminary studies, we have observed a similar effect on liver plasma membranes of C57Bl/6 mice after chronic ethanol feeding.[2] However, cultured cells of nonliver origin (CHO-K1) did not respond in a similar fashion. Furthermore, 4-methylpyrazole, a potent inhibitor of ethanol metabolism in the H35 cell (Polokoff et al., 1983), completely prevented the ethanol-dependent change in plasma membrane DPH polarization. Thus, the ability of the liver cell to metabolize ethanol may be crucial in determining the effect of chronic ethanol on the structure of its plasma membrane.

Liver plasma membrane alterations induced by ethanol do not appear to be governed by the principle of homeoviscous adaptation and may not be beneficial to the cell. We have observed that H35 cells grown in the presence of 80 mM ethanol are actually more sensitive to the cytotoxic effects of acute exposure to 320 mM ethanol than are control cells.[4] The apparent failure of its plasma membrane to undergo homeoviscous adaptation to the disordering effects of ethanol may help to explain the liver cell's sensitivity to ethanol toxicity.

ADDED IN PROOF

Following submission of this paper, two reports appeared on the effects of chronic ethanol feeding of rats on liver plasma membrane physical properties (Schuller et al., 1984; Yamada & Lieber, 1984). Both groups observed significant decreases in plasma membrane DPH polarization following ethanol treatment.

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Registry No. ATPase, 9000-83-3; 16:0, 57-10-3; 18:0, 57-11-4; 18:1, 112-80-1; 18:2, 60-33-3; 20:4, 506-32-1; 22:5, 32839-34-2; 22:6, 32839-18-2; 5′-nucleotidase, 9027-73-0; cholesterol, 57-88-5; ethanol, 64-17-5; alcohol dehydrogenase, 9031-72-5.

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[4] M. A. Polokoff and M. Iwahashi, unpublished observations.

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