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Cardiolipin from ethanol-fed rats confers tolerance to ethanol in liver mitochondrial membranes

(electron spin resonance/phospholipids/phosphatidylinositol/membrane reconstitution)

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ABSTRACT **In rats chronically consuming ethanol, the liver mitochondrial membranes develop resistance to the disordering effects of ethanol *in vitro*, so-called "membrane tolerance." To investigate the molecular basis of this tolerance in the inner mitochondrial membrane, multilamellar vesicles were produced by recombining the mitoplast phospholipids (quantitatively separated by preparative HPLC) from control and ethanol-fed animals in various combinations. The effect of *in vitro* ethanol on the physical properties of these vesicles was determined by electron spin resonance. Vesicles composed of all mitoplast phospholipids from control rats were disordered by 50–100 mM ethanol, whereas those made of the phospholipids from ethanol-fed animals were resistant. When phosphatidylcholine (46 mol %) or phosphatidylethanolamine (42 mol %) from ethanol-fed rats replaced the corresponding phospholipids of control rats, the vesicles were disordered by ethanol. By contrast, when as little as 2.5 mol % of cardiolipin (one-fourth the naturally occurring amount) from ethanol-fed rats replaced that phospholipid from control rats, vesicles were rendered entirely resistant to disordering by ethanol. The same amount of cardiolipin from ethanol-fed rats also conferred membrane tolerance to vesicles composed of bovine phospholipids, demonstrating that this effect is not restricted to rat mitoplast phospholipids. In vesicles composed of a single mitoplast-phospholipid class, only vesicles composed of cardiolipin from ethanol-fed rats resisted disordering. Phosphatidylinositol from liver microsomes of ethanol-fed rats also confers membrane tolerance and was the only microsomal phospholipid that formed tolerant vesicles. Thus, in livers of rats chronically fed ethanol, anionic phospholipids are selectively converted into potent promoters of membrane tolerance in both mitochondrial and microsomal membranes.**

Alcoholism has long been recognized as a leading contributor to liver disease, although the mechanism(s) by which chronic ethanol intake produces hepatic injury remains obscure. Among the hallmarks of ethanol-induced liver damage are structural and functional alterations of mitochondria. Chronic ethanol ingestion in experimental animals (1–3) and humans (3–5) leads to enlarged and misshapen liver mitochondria, which exhibit disoriented cristae and, occasionally, paracrystalline inclusions. These organelles also display conspicuous defects in the respiratory chain and ATP synthesis (6, 7).

Ethanol is known to disorder (or fluidize) biological membranes *in vitro* (for review, see ref. 8). A decade ago experimental chronic ethanol inhalation was shown to induce an adaptive resistance to this molecular disordering in mouse synaptosomes and erythrocytes (9). Reports from our laboratory demonstrated that such adaptive resistance, so-called "membrane tolerance," is consistently acquired by hepatic mitochondria and microsomes (10–13). We also showed that this property could be detected in membrane vesicles composed of phospholipids isolated from these organelles (10, 12, 13), a finding that clearly implicates a structural alteration of these molecules. Because the mitochondrial abnormalities, both structural and functional, all appear related to the mitochondrial inner membrane, these alterations are probably in some way involved with the phospholipid changes responsible for membrane tolerance.

We recently reported that resistance to ethanol-induced molecular disordering in hepatic microsomes is specifically conferred by phosphatidylinositol (PtdIns) in a molar concentration as low as 2.5%, or about one-third of the naturally occurring amount (12). Because the inner mitochondrial membranes contain little or no PtdIns (for review, see ref. 14), we investigated whether membrane tolerance in hepatic mitochondria reflects a general change in membrane phospholipids or whether this adaptive property also resides in a single phospholipid.

METHODS

Animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories) initially weighing 100–130 g, were fed a totally liquid diet (Bio-Serve, Frenchtown, NJ), in which ethanol composed 36% of total calories (15) for 35 days, while pair-fed littermate controls received the same diet except that carbohydrates isocalorically replaced ethanol. Ethanol consumption averaged 14–16 g per kg of body weight per day.

Preparation of Membranes. Rat liver microsomes (12) and mitoplasts (16) were prepared and characterized as previously described.

Extraction and Analysis of Lipids. Lipids were extracted by the Bligh and Dyer procedure (17). Phospholipids were separated from neutral lipids by silicic acid chromatography (18) and stored under N_2 in $CHCl_3$ at −20°C. The extraction solvents, which contained 0.01% butylated hydroxytoluene to prevent oxidation of polyunsaturated fatty acids, were flushed with N_2. Phospholipid phosphate was determined by the Bartlett procedure (19).

Compositition of the mitoplast membrane phospholipids was determined by quantitative 2-dimensional TLC, as described (18). Phospholipids were identified by comparison of their R_f values with those of standards and by the use of spray reagents (18).

Separation of the Phospholipid Classes by Preparative HPLC. Up to 200 mg of phospholipids were separated on a

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Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; Ste12, 12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]stearic acid; PtdCho12, 1-palmitoyl-2-{12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]-stearoyl}-*sn*-glycero-3-phosphocholine; PtdEtn12, 1-palmitoyl-2-{12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]-stearoyl}-*sn*-glycero-3-phosphoethanolamine.

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250 × 25-mm Merck Hibar RT preparative column packed with 7-μm LiChrosorb-Si-60 silica particles (EM Science, Cherry Hill, NJ) using the gradient procedure described (20).

Fatty Acid Analysis. Fatty acid methyl esters were prepared with BF_3 in methanol (21); they were separated on a 6-ft × 1/8-inch glass column containing GP 10% diethylene glycol succinate–H_3PO_4 on 80/100 Supelcoport (Supelco, Bellefonte, PA) at 195°C with a flow rate of 20 ml/min. A Perkin–Elmer model 3920 gas chromatograph was used, and methyl esters were measured using a Shimadzu CR3-A computing integrator. The integrator response was checked daily using methyl ester standards (Supelco). Fatty acid methyl esters were identified by comparison of retention times of standards and by equivalent chain length data.

Preparation and Spin-Labeling of Multilamellar Vesicles. Lipid bilayer vesicles made from mitoplast total lipids, phospholipids, or recombined HPLC-separated phospholipids were prepared, as described (12). They were labeled with a spin-labeled fatty acid, 12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]stearic acid (Ste12) or the spin-labeled phospholipids, 1-palmitoyl-2-{12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]-stearoyl}-*sn*-glycero-3-phosphocholine (PtdCho12) or 1-palmitoyl-2-{12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]-stearoyl}-*sn*-glycero-3-phosphoethanolamine (PtdEtn12) as described (12).

Spin-Labeling of Mitoplasts. Mitoplasts were labeled with the fatty acid or phospholipid spin probes using the techniques previously used for spinlabeling microsomes (12).

Measurement of Order Parameter by ESR. The ESR spectrum of the spin-labeled sample was recorded with an IBM Instruments ER 200D ESR spectrometer as described (22). Spectra were accumulated with an IBM 9000 computer interfaced with the spectrometer, and the molecular order parameter, *S*, was calculated by described methods (8, 12).

RESULTS

Isolation of Mitoplast Phospholipids by Preparative HPLC. The isolated phospholipids were used to reconstitute lipid bilayers of mixtures of mitoplast phospholipids or of a single phospholipid class. We previously established that the HPLC procedure quantitatively separated mitoplast phospholipids that had similar mobilities on TLC and similar reactions with specific spray reagents as standard phospholipids (18). The procedure uses no salts, buffers, acids, or bases—thereby insuring that the phospholipids are recovered in their natural states. Oxidation of the lipid acyl chains was minimized by conducting all procedures under N_2.

Ethanol-Induced Changes in Lipid Composition of Mitoplasts. Quantitative 2-dimensional TLC revealed that the phospholipid composition of the mitoplasts changed upon chronic consumption of ethanol. In mitoplasts from ethanol-treated animals, the phosphatidylcholine (PtdCho) content increased from 45.4 mol % to 49.2 mol %, and the amount of phosphatidylethanol (PtdEtn) decreased from 42 mol % to 38 mol %. The content of cardiolipin remained at 9.5 mol % (Table 1) (19.0% if based on percent of lipid phosphate; cardiolipin contains two P atoms per molecule). Phosphatidylserine (PtdSer) is not a hepatic mitochondrial inner-membrane phospholipid (for review, see ref. 14), and the small amount seen shows the presence of a minor amount of another membrane. Fatty acid composition of the individual phospholipids did not change markedly in ethanol-fed rats, except for cardiolipin. The major differences between cardiolipin in control and ethanol-fed animals was a decrease in linoleic acid from 69 to 62% and an increase in oleic acid from 19 to 25%, consistent with previous findings (10, 23).

Effects of Ethanol on the Order Parameter of Mitoplasts and Recombined Phospholipid Membranes. Membrane tolerance was determined by labeling either intact mitoplast membranes or vesicles of the extracted mitoplast phospholipids with a spin-labeled fatty acid or phospholipid probe and determining the order parameter, *S*, with and without ethanol. Use of ESR to determine the order parameter of the acyl chains in the membranes and the phospholipid vesicles has been described in detail (8, 22).

Table 1. Phospholipid composition of liver mitoplasts from ethanol-fed and control rats

Phospholipid	Control, mol %	Ethanol-fed, mol %
PtdCho	45.37 ± 1.07	49.22 ± 1.66
PTdEtn	42.01 ± 0.81	38.01 ± 1.34
Cardiolipin	9.91 ± 0.43	9.63 ± 0.48
PtdIns	1.17 ± 0.44	1.37 ± 0.28
PtdSer	0.38 ± 0.10	0.34 ± 0.10
Sphingomyelin	0.40 ± 0.28	0.71 ± 0.24
Lysocardiolipin	0.50 ± 0.13	0.41 ± 0.19

Composition is presented as mol % of phospholipid ± SD. Data are calculated from a minimum of three phosphate analyses done on each lipid preparation from four pair-fed animals.

Addition of increasing amounts (50–100 mM) of ethanol to mitoplast membranes from untreated animals decreased the order parameter from 0.384 to 0.373 (Fig. 1), which represents disordering of the membranes ($\Delta S = 0.011$). By contrast, when mitoplasts from animals chronically fed ethanol were exposed to the same amounts of ethanol, no change in the order parameter was seen (Fig. 1).

Recombined bilayer vesicles were prepared by combining HPLC-separated mitoplast phospholipids in their naturally occurring molar ratios of PtdCho, 46%; PtdEtn, 42%; PtdIns plus PtdSer, 2%; and cardiolipin, 10%. Recombined bilayers composed of all phospholipids from control rats were disordered by 50–100 mM ethanol, whereas those prepared from

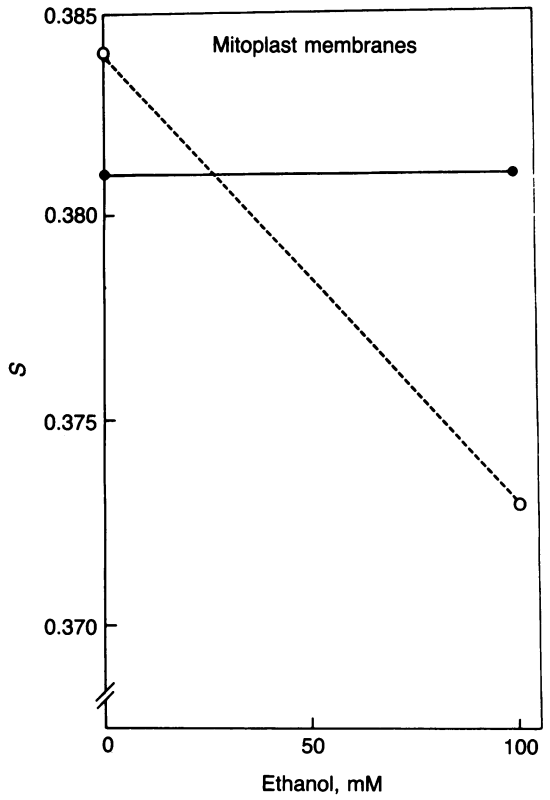

FIG. 1. Typical order parameter profile showing the effect of ethanol *in vitro* on the molecular order parameter (*S*) obtained by ESR at 37°C. Intact rat liver mitoplasts were labeled with PtdCho12. Mitoplasts were obtained from control (○) and ethanol-fed (●) rats. The same results were obtained using the Ste12 spin-label probe and three other sets of pair-fed animals.

all phospholipids from ethanol-fed rats were not disordered (Fig. 2*A*). Response of the reconstituted vesicles to *in vitro* addition of physiologically relevant amounts of ethanol was qualitatively the same as that in intact mitoplast membrane.

Ability of Individual Mitoplast Phospholipids to Confer Membrane Tolerance to Disordering by Ethanol. To identify the component responsible for membrane tolerance, bilayers were made by recombining (in their naturally occurring molar ratios) all individual HPLC-separated phospholipids from control or ethanol-fed rats, except that in each preparation one different phospholipid class was omitted; the missing phospholipid from the control preparation was then replaced by the corresponding one from ethanol-fed animals. In vesicles composed of phospholipids from ethanol-fed rats, the corresponding phospholipid from control animals replaced the omitted phospholipid from ethanol-fed rats. Recombined membrane vesicles of phospholipids from control animals were disordered by 50–100 mM ethanol when PtdCho (46 mol %) or PtdEtn (42 mol %) from the ethanol-fed preparation was substituted for its counterpart from controls (Fig. 2*B*). By contrast, when cardiolipin from ethanol-fed animals was substituted into control vesicles in its naturally occurring amount (10 mol %), bilayers were rendered resistant to disordering by ethanol (Fig. 2*C*).

The ability of cardiolipin from ethanol-fed animals to confer membrane tolerance to phospholipid bilayers was not limited to rat liver mitoplast phospholipids. Vesicles prepared with bovine liver PtdCho and PtdEtn and bovine heart cardiolipin were disordered to the same extent as were those prepared from control rat liver mitoplast phospholipids. Only cardiolipin from ethanol-fed rats conferred tolerance to the vesicles of bovine phospholipids (Fig. 3).

To determine the minimal amount of cardiolipin necessary to confer tolerance to reconstituted bilayers, vesicles composed of phospholipids from controls were prepared with increasing amounts of cardiolipin from the ethanol-fed preparation. The total cardiolipin was kept at 10 mol %—i.e., when cardiolipin from ethanol-treated rats was 4.0 mol %, the other 6.0 mol % of cardiolipin was from control rats. Results in Fig. 3 show that as little as 2.5 mol % cardiolipin from ethanol-fed rats conferred tolerance to reconstituted bilayers made from phospholipids of either rat mitoplasts or bovine tissues.

Effects of Ethanol on the Order Parameter of Vesicles Composed of a Single Mitoplast or Microsomal Phospholipid. The response of individual mitoplast phospholipids to *in vitro* addition of ethanol was measured to determine whether cardiolipin from ethanol-treated animals conferred tolerance by itself or only in combination with other lipids. Reconstituted bilayers composed of cardiolipin from ethanol-fed animals were resistant to disordering by ethanol, whereas vesicles made from each of the other mitoplast phospholipids from ethanol-fed or untreated rats were disordered (Table 2). The same response to ethanol was obtained with both PtdCho12 and Ste12 spin-label probes, showing that the results were not due to association of a specific phospholipid with a particular spin probe.

Previously, we had demonstrated PtdIns of liver microsomes from ethanol-fed animals to promote membrane tolerance also at a level of 2.5 mol % (12). To determine whether vesicles composed solely of microsomal PtdIns from ethanol-fed rats were also tolerant, we examined the response of individual phospholipids from microsomes to *in vitro* addition of 100 mM ethanol. Only vesicles of PtdIns from the ethanol-

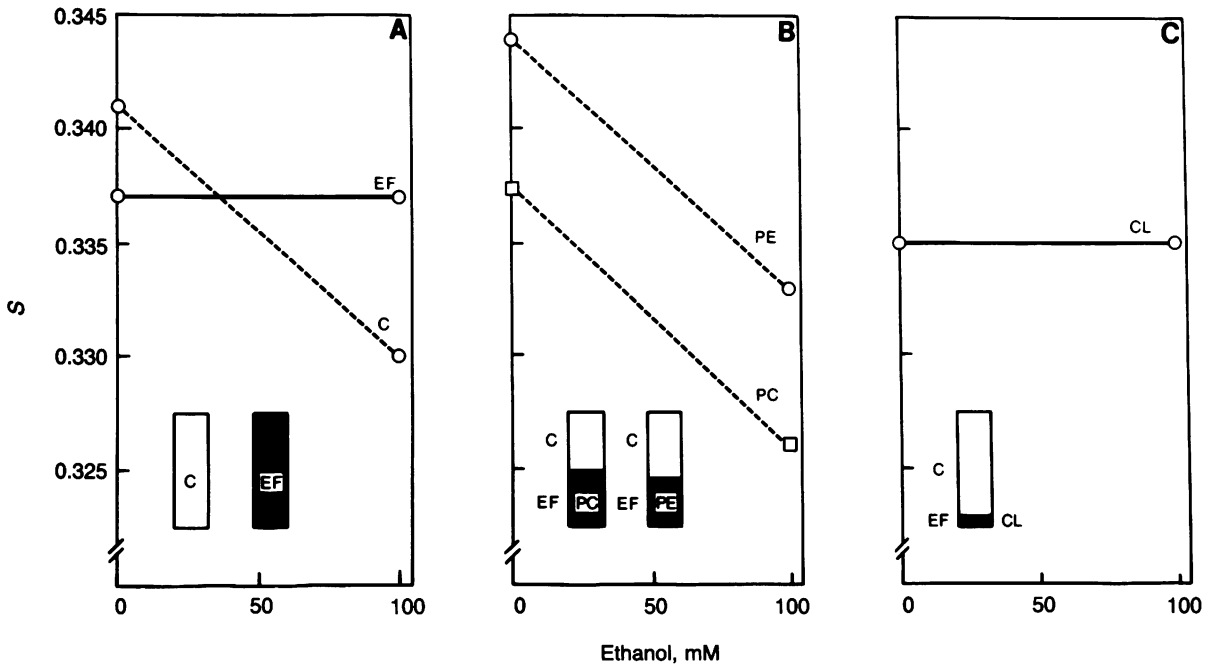

FIG. 2. Ability of individual liver inner-mitochondrial membrane phospholipids from ethanol-fed rats to confer tolerance to vesicles composed of phospholipids from control rats. Multilamellar vesicles prepared from recombined mitoplast phospholipids that had been quantitatively separated into individual classes by HPLC were labeled with PtdCho12. The order parameter (*S*) was obtained by ESR at 37°C. HPLC-separated phospholipids were recombined in their naturally occurring molar ratios to form reconstituted membrane systems of composition: PtdCho at 46 mol %, PtdEtn at 42 mol %, PtdIns plus PtdSer at 2 mol %, and cardiolipin at 10 mol %. Phospholipids used to prepare the reconstituted vesicles were extracted from mitoplasts from control and ethanol-fed rats. Composition of each reconstituted system is indicated in the bar graph insert, in which full length of the bar represents 100%. Dark areas show mole fraction of the phospholipid(s) from ethanol-fed (EF) rats, and the white area shows the mole fraction from control (C) animals. ESR order parameters determined at the indicated concentration of ethanol are shown for recombined phospholipid vesicles labeled with PtdCho12. (*A*) Results from vesicles of all control (C) and all ethanol-fed (EF) phospholipids. (*B*) Results from vesicles containing PtdCho (PC) at 46 mol % or PtdEtn (PE) at 42 mol % from ethanol-fed rats. (*C*) Results from vesicles containing 10% cardiolipin (CL) from ethanol-fed rats. Similar results were obtained with three other sets of pair-fed animals.

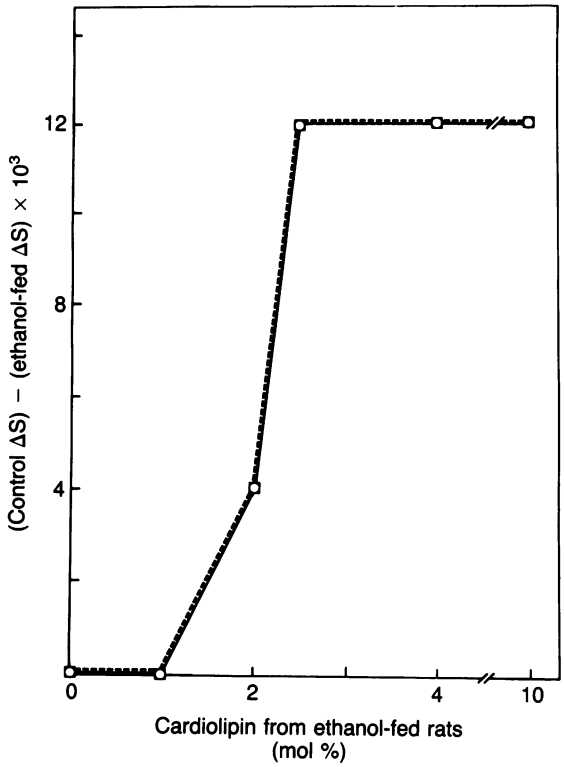

FIG. 3. Determination of the minimum amount of cardiolipin from ethanol-fed rats needed to impart tolerance to disordering by ethanol to reconstituted vesicles. Vesicles labeled with Ste12 were prepared from rat liver mitoplast phospholipids from control rats (□) or from bovine liver and heart standard phospholipids (○) as in Fig. 2. Vesicles prepared from phospholipids of control rats or bovine standard phospholipids plus 10.0 mol % of cardiolipin from control rats are referred to as control (*C*). Vesicles prepared from mitoplast phospholipids or bovine phospholipids and containing various amounts of cardiolipin from ethanol-fed rats are referred to as ethanol-fed (EF). ESR spectra were recorded without and with 100 mM ethanol at 37°C. Control ΔS represents the difference in the order parameter, S, between 0 and 100 mM ethanol in vesicles with no cardiolipin from ethanol-fed rats. Control $S \times 10^3 = 11$ because this is the maximal extent of disordering by 100 mM ethanol seen in reconstituted control mitoplast. Ethanol-fed ΔS represents percent difference in the order parameter between 0 and 100 mM ethanol in vesicles containing specified amounts of cardiolipin from ethanol-fed rats. Total amount of cardiolipin (cardiolipin from control animals plus cardiolipin from ethanol-fed rats) was 10 mol % in all vesicles. When (Control ΔS) − (ethanol-fed ΔS) $\times 10^3 = 11$, vesicles containing cardiolipin from ethanol-fed rats are completely resistant (ethanol-fed $\Delta S = 0$) to disordering by 100 mM ethanol.

treated animals were resistant to disordering by ethanol, whereas vesicles composed exclusively of any one of the other individual microsomal phospholipids were disordered (Table 2).

DISCUSSION

Increasing evidence suggests that chronic ethanol ingestion causes profound structural, compositional, and functional alterations in membranes of cells and subcellular organelles from the major organs in the body (for review, see ref. 8). Our understanding of the link between ethanol-induced membrane perturbations and the physiological effects of this compound on individual cells and tissues is unsatisfactory. Similarly incomplete is our understanding of the molecular nature of changes in membrane properties induced by long-term ethanol ingestion or of the possible connection between these membrane phenomena and the cell injury that often accompanies long-term exposure to ethanol.

Table 2. Response of individual liver mitoplast and liver microsomal phospholipids to *in vitro* addition of 100 mM ethanol

Preparation	Phospholipid	$\Delta S \times 10^3$
Mitoplast		
Control	PtdCho	11
	PtdEtn	11
	Cardiolipin	11
Ethanol-fed	PtdCho	11
	PtdEtn	11
	Cardiolipin	0 (tolerant)
Microsome		
Control	PtdCho	11
	PtdEtn	11
	PtdSer	11
	PtdIns	11
Ethanol-fed	PtdCho	11
	PtdEtn	11
	PtdSer	11
	PtdIns	0 (tolerant)

Spin-labeled (PtdCho12 or Ste12) vesicles comprised of a single phospholipid from mitoplasts or microsomes from control and ethanol-fed rats were prepared as described. Order parameters determined at 0 and 100 mM ethanol, and difference between the two values is $\Delta S \times 10^3$.

Use of recombined phospholipid bilayers has permitted us to make advances over compositional studies in defining the molecular basis of ethanol-induced alterations in membrane properties. Results obtained with the reconstituted bilayer vesicles reflect the properties of the intact membrane, in terms of response to ethanol: The response of the reconstituted vesicles to ethanol is qualitatively the same as that in intact erythrocytes, liver microsomes, submitochondrial particles, and mitoplast membranes from which the vesicles were extracted (13). When animals are withdrawn from ethanol, the rate of loss of tolerance measured in the intact erythrocytes and liver microsomal membranes is the same as that in vesicles composed of extracted phospholipids (13). The observation of tolerance to molecular disordering in membranes and phospholipids does not depend on the probe used because spin-labeled fatty acid and phospholipid probes yield similar results (12, 22). Therefore, reconstituted phospholipid vesicles provide a convenient system to identify those phospholipids that promote membrane tolerance.

The mitoplast membrane is the second membrane identified in which a specific anionic phospholipid is selectively converted into a potent promoter of membrane tolerance. Previously we showed that PtdIns in liver microsomes from ethanol-treated rats confers tolerance to those membranes (12). Thus, in both membranes a quantitatively minor anionic phospholipid selectively acquires the ability to confer membrane tolerance at a concentration of 2.5 mol % of the bilayer phospholipids, an amount one-fourth the naturally occurring amount of mitoplast cardiolipin and one-third that of microsomal PtdIns. This ability to promote tolerance is not limited to rat liver microsomal lipids, as the inclusion of 2.5 mol % into vesicles of bovine brain and liver phospholipids produces the same effect. By contrast, the considerably larger amounts of PtdCho (46 mol %) or PtdEtn (42 mol %) from mitoplasts or PtdCho (66.5 mol %) or PtdEtn (21 mol %) from microsomes did not confer tolerance to the recombined vesicles. Thus, only cardiolipin and PtdIns are such strong promoters of membrane tolerance that they confer tolerance at levels significantly lower than their own naturally occurring concentrations. The other microsomal and mitoplast phospholipids from ethanol-fed rats apparently undergo minor modifications that convert them into weak promoters of membrane tolerance. Although they cannot confer tolerance to

lipid vesicles individually at naturally occurring amounts, these phospholipids can confer tolerance when they are combined and present as 90 mol % of the vesicle phospholipids; this conclusion is based on the observation that vesicles prepared from mitoplast phospholipids from ethanol-fed rats, in which 10 mol % cardiolipin from controls replaced that from ethanol-fed rats, are tolerant to disordering (data not shown). We had previously reported that the microsomal phospholipids (other than PtdIns) from ethanol-fed rats were also converted into weak promoters of membrane tolerance and conferred tolerance only when present in a combined amount of 90 mol % of vesicle lipids (12).

In bilayer vesicles composed solely of a single class of phospholipid, only those prepared from the strong promoters of membrane tolerance, mitoplast cardiolipin or microsomal PtdIns from ethanol-fed rats, are tolerant to disordering by ethanol. By contrast, vesicles composed of any other individual phospholipid, including those from ethanol-fed animals (the weak promoters), form disordered bilayers. Thus weak promoters from microsomes or mitoplasts are individually sensitive to ethanol and tolerant only when combined in their naturally occurring molar ratios, whereas the strong promoters, PtdIns and cardiolipin, are individually tolerant and confer tolerance at levels significantly lower than their naturally occurring levels.

In the same cell—the hepatocyte—two membranes acquire the same physical property, tolerance to disordering by ethanol, by selective modification of different anionic phospholipids. Membrane tolerance probably does not arise by an identical mechanism in all membranes. The ethanol-induced membrane tolerance seen in synaptosomes (24), erythrocytes (13), and pancreatic acini (25) may be acquired by mechanisms completely different from those in the liver. Cardiolipin is synthesized on the inner mitochondrial membrane, whereas PtdIns is synthesized on the endoplasmic reticulum. Therefore, chronic ethanol consumption apparently affects different sites of phospholipid metabolism within the same cell.

The exact molecular modification(s) responsible for membrane tolerance remains to be identified. Chronic ethanol treatment does not alter the mobility of phospholipids on silicic acid HPLC or TLC in our solvent systems, indicating that the polar groups are probably not covalently modified. The anionic lipids from ethanol-fed animals could tightly bind some specific cation, thereby affecting their packing properties in the membrane (26). A modification of the hydrocarbon chains of lipids may also account for tolerance. Changes in phospholipid molecular species content could affect packing of the bilayer and alter the partition coefficient of ethanol into the membrane (27). This concept is not unreasonable in view of the fact that small changes in phospholipid molecular species have major effects on membrane properties of cilia in *Tetrahymena pyriformis* (28) and mammalian erythrocytes (29).

Even though tolerance is a bulk membrane property, selective alteration of certain lipids indicates that microregions of the membrane may be changed, thereby affecting specific membrane functions. To illustrate, besides affecting the response of the inner mitochondrial membrane to ethanol, selective alteration of fatty acid composition and the physical properties of cardiolipin may partially explain the aberrant effects of ethanol on mitochondrial respiration and ATP synthesis (6, 7), since cardiolipin is known to be required for cytochrome oxidase activity. By contrast, skeletal muscle mitochondria from ethanol-fed rats are not tolerant to disordering by ethanol and display no decrease in respiration rates (F. Cardellach, J. Hoek, J.S.E., T.F.T., and E.R., unpublished observations). Consequently, the latter represent an organelle that develops no ethanol-induced aberrent functions when phospholipids remain unmodified and membrane tolerance is absent.

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