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Phosphatidylinositol from ethanol-fed rats confers membrane tolerance to ethanol

(electron spin resonance/microsomes/membrane reconstitution)

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ABSTRACT **The presence of ethanol disorders (fluidizes) biological membranes, but its chronic administration confers resistance to this perturbation (membrane tolerance). The latter effect has been invoked as an explanation for behavioral tolerance in alcoholics, but the molecular basis for membrane tolerance is obscure. To study the molecular mechanisms of this acquired resistance to disordering, we fed rats ethanol (36% of total calories) for 35 days, after which we quantitatively separated the phospholipids of hepatic microsomal membranes by high-performance liquid chromatography. Multilamellar vesicles were prepared from the recombined phospholipid classes, and their physical properties were examined by electron spin resonance. Vesicles composed of phospholipids from untreated rats were disordered (fluidized) in the presence of ethanol, whereas those made from phospholipids of ethanol-fed rats were resistant to this effect. When phosphatidylcholine (66.5 mol %), phosphatidylethanolamine (21 mol %), or phosphatidylserine (4.0 mol %) from ethanol-fed rats replaced their corresponding phospholipids in vesicles prepared from microsomal phospholipids from untreated rats, the membranes were still disordered by ethanol. In contrast, when 2.5–8.5 mol % phosphatidylinositol from ethanol-fed rats replaced phosphatidylinositol from untreated rats, the reconstituted membranes were rendered resistant to ethanol-induced disordering. Liver microsomal phosphatidylinositol (2.5–8.5 mol %) from ethanol-fed rats also conferred membrane tolerance to vesicles composed of bovine liver and brain phospholipids, an effect which demonstrates that the ability of phosphatidylinositol to confer membrane tolerance is not restricted to the microsomal membrane.**

Chronic alcohol abuse is responsible for severe economic, social, and health-related problems, among which are diseases of major body organs. The chronic effects of ethanol consumption on the central nervous system, the liver, and a variety of other organs may be related, at least in part, to its interactions with biological membranes (reviewed in ref. 1). The presence of ethanol generally exerts a disordering (fluidizing) effect on the lipid bilayer of biological membranes. By contrast, chronic consumption of ethanol leads to altered membranes that are resistant to this disordering (membrane tolerance) (2–4). Behavioral tolerance to ethanol in chronic alcoholics has been attributed to alterations in the chemical composition and resistance to disordering of neuronal membranes (5, 6). This tolerance is not restricted to ethanol, since membranes from ethanol-fed rats also display resistance to other anesthetic agents [e.g., halothane (6)]. Membrane tolerance is also observed in multilamellar vesicles composed of phospholipids extracted from membranes of ethanol-treated animals [synaptosomes (7), hepatic mitochondria (3) and microsomes (8), and erythrocytes (8)]. Thus, the property of membrane tolerance clearly resides in the phospholipid portion of the membrane. Despite numerous studies of the effects of chronic ethanol consumption on the lipid composition of membranes (reviewed in ref. 1), no large changes have been reported, and no consistent pattern has emerged. Thus, the molecular basis of membrane tolerance remains obscure.

Since resistance to disordering by ethanol is a membrane property measured by physical, rather than chemical, techniques—e.g., electron spin resonance (ESR) and fluorescence polarization (2–4, 8, 9)—it is unlikely that compositional studies alone will reveal the molecular origin of this response to chronic ethanol consumption. We therefore embarked upon a study to assess by ESR the ability of individual liver microsomal phospholipids from ethanol-fed rats to promote membrane tolerance in membrane vesicles composed of recombined individual phospholipids of the microsomal membrane.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (Charles River Breeding Laboratories) initially weighing 100–130 g ingested 14–16 g of ethanol per kg of body weight in a nutritionally adequate, totally liquid diet for 35 days, while pair-fed controls consumed the same diet, except that carbohydrate isocalorically replaced ethanol (10).

Preparation of Membranes. Hepatic microsomes were prepared by differential centrifugation, as described (4).

Phospholipids. Bovine liver phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), and phosphatidylinositol (PtdIns) and bovine brain phosphatidylserine (PtdSer) and sphingomyelin were purchased from Avanti Polar Lipids. The lipids were analyzed by thin-layer chromatography and gas-liquid chromatography and found to be chromatographically pure.

Isolation and Analysis of Phospholipids. Total lipids were extracted according to Bligh and Dyer (11). Phospholipids were separated from neutral lipids on a silicic acid column (8) and stored in $CHCl_3$ at −20°C under N_2. All procedures were performed under N_2 in order to prevent lipid oxidation.

Separation of Phospholipids by Preparative High-Performance Liquid Chromatography (HPLC). Extracted microsomal phospholipids were separated by HPLC on a 250 × 25-mm EM Science HiBar preparative column packed with 7-μm particles of LiChrosorb-Si-60 silica gel (EM Science, Cherry Hill, NJ). A Perkin-Elmer 4B liquid chromatographic system was used, and lipids were detected at 206 nm using a

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

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Perkin-Elmer LC-95 variable wavelength detector fitted with an 18-μl flow-through cell. Microsomal phospholipids (8–10 μmol in 1 ml of $CHCl_3$) were injected onto the column and the phospholipid classes were separated using the following gradient program. The initial solvent mixture of hexane/2-propanol/water (6:8:0.75, vol/vol) was run for 8 min, followed by a linear gradient for 26 min to achieve the final solvent mixture of hexane/2-propanol/water (6:8:1.5), which was then run for an additional 45 min. The flow rate was 9 ml/min and the solvents were purged with He. Fractions containing the separated phospholipid classes were evaporated under vacuum and stored in $CHCl_3$ at −20°C. The purity of the separated phospholipids was checked by chromatography on 5 × 5-cm HPTLC silica gel 60 plates, using the solvent system methyl acetate/1-propanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, vol/vol). Phospholipids were identified by I_2 vapor, ninhydrin, and Dragendorf's and phosphate-detecting spray reagents (12) and by comparing their R_f values with those of standard phospholipids (Avanti Polar Lipids). Lipid phosphate was determined according to Bartlett (13).

Spin-Labeling of Microsomes. Microsomes were labeled with a spin-labeled fatty acid 12-[β-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)]stearic acid (12-doxylstearic acid, Ste12) as described (4). The spin-labeled phospholipid, 1-palmitoyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphocholine (PtdCho12) was synthesized and incorporated into the microsomal membrane as described (4). The final membrane/lipid probe ratio in all of the labeled membranes was 120:1 (mol/mol).

Preparation and Spin-Labeling of Multilamellar Vesicles. Multilamellar vesicles were prepared by (*i*) drying of a chloroform solution containing the desired phospholipid mixture with nitrogen, (*ii*) high-vacuum dessication of the dried lipid film for 30 min, and (*iii*) hydration with phosphate-buffered saline (PBS: 0.9% NaCl/10 mM sodium phosphate, pH 7.2). The fatty acid probe Ste12 and the phospholipid spin labels PtdCho12 and 1-palmitoyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphoethanolamine (PtdEtn12) were incorporated into the reconstituted membranes as described (4). PtdEtn12 was synthesized as reported previously (4). The ratio of membrane phospholipid/spin probe was 120:1 (mol/mol).

ESR Spectroscopy. The spin-labeled sample, following the addition of the appropriate amount of ethanol, was mixed on a Vortex for 2 min at room temperature, transferred to a 100-μl capillary tube, and flame-sealed. Capillary tubes were placed in an IBM Instruments (Danbury, CT) ER 200D ESR spectrometer cavity in a standard 4-mm quartz tube, which contained silicon oil to maintain thermal stability. All samples were equilibrated for 7 min in the cavity at 37°C prior to recording the spectrum. Temperatures were accurate to 0.5°C.

Spectra were accumulated with an IBM 9000 computer interfaced with the spectrometer. The molecular order parameter, S, was calculated as described (4). Typical spectrometer settings were as follows: spectral scan, 100 G; modulation amplitude, 1.0 G; and microwave power, 5 mW. All measurements were made at 37°C.

RESULTS

Preparative HPLC of Microsomal Phospholipids. Since the separated phospholipids isolated by the HPLC procedure (see *Materials and Methods*) were to be used to make reconstituted membranes, it was important to establish that the isolated microsomal phospholipids were not altered during this process. Usually, 10 μmol of microsomal phospholipids was separated in 75 min into the following fractions: 22 mol % PtdEtn, 8.5 mol % PtdIns, 4.0 mol % PtdSer, and 65.5 mol % of a PtdCho/sphingomyelin fraction. This composition is in agreement with other reported values for rat liver microsomes (14). No significant differences in the molar proportions of individual phospholipid classes were observed as a result of chronic ethanol feeding. Recovery of phospholipid phosphate from the column was >96%, which indicated that the column did not retain specific phospholipids. The fractions were chromatographically pure as determined by HPTLC, and their reaction with specific spray reagents was the same as those of standard phospholipids. The HPLC procedure used no salts, buffers, acids, or bases, thereby ensuring that the phospholipids were recovered in their native states. To prevent oxidation of the phospholipid fatty acid chains, the procedures were done under N_2.

Effects of Ethanol on the Order Parameter of Microsomes and Recombined Phospholipid Membranes. The molecular order parameter, S, derived from the ESR spectrum, provides a measure of membrane structural order (fluidity), since the motionally averaged spectral hyperfine splittings are influenced by the angular amplitude and rate of motion experienced by the lipid hydrocarbon chains bearing the spin-label group. Molecular disordering, characterized by an increase in the spectral splitting, $2T_\perp$, reflects the greater angular displacement of the lipid fatty acid chains from the long molecular axis of the lipid molecule in the membrane bilayer.

The order parameters in the absence of ethanol (baseline order parameter) were the same for the rat liver microsomal preparations isolated from untreated and ethanol-fed rats (Fig. 1*A*). In agreement with an earlier report from this laboratory (4), the addition of increasing amounts of ethanol (50–100 mM) to microsomes from untreated animals caused a decrease in the order parameter from 0.330 to 0.305, a change indicative of significant membrane disordering (ΔS = 7.5%). In contrast, when microsomes from ethanol-treated rats were exposed to the same range of ethanol concentrations, no change in the order parameter was observed (Fig. 1*A*). These results were not dependent on the type of spin label, since microsomes from untreated or ethanol-fed rats labeled with the phospholipid spin probes PtdCho12 and PtdEtn12 exhibited the same response to the addition of ethanol as those labeled with the fatty acid spin label Ste12 (4).

In vesicles comprising various mole fractions of microsomal phospholipids (before separation by HPLC) obtained from the livers of untreated and ethanol-fed rats, we determined that only 30–40 mol % of the phospholipids derived from the ethanol-fed animals was necessary to render the vesicles tolerant to ethanol-induced disordering. Thus, the phospholipids obtained from the ethanol-fed rats contained a component potent enough to confer membrane tolerance, even when diluted with a larger amount of phospholipids from untreated rats.

Vesicles were then prepared by recombining the HPLC-separated microsomal phospholipids in their naturally occurring molar proportions. Reconstituted membranes composed of phospholipids from untreated rats were disordered by the addition of ethanol, as evidenced by a significant decrease (4.8%) in the order parameter, from 0.314 to 0.299 (Fig. 1*B*). On the other hand, vesicles composed of microsomal phospholipids from ethanol-treated rats showed no change in the order parameter over the same range of ethanol concentrations (Fig. 1*B*). As in the intact membrane, chronic ethanol treatment did not alter the baseline order parameters of the vesicles prepared from the phospholipids derived from the ethanol-fed rats. The response of the reconstituted vesicles to ethanol *in vitro* was qualitatively parallel to that of the intact microsomal membranes, thereby providing the opportunity to identify the phospholipid(s) responsible for conferring membrane tolerance.

In an attempt to identify the component responsible for membrane tolerance, vesicles were made by recombining

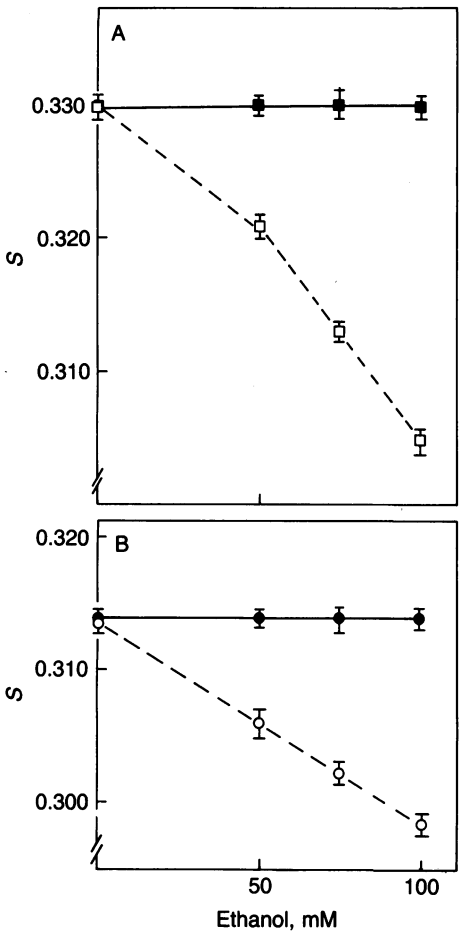

FIG. 1. Typical order-parameter profiles showing the effect of ethanol *in vitro* on the molecular order parameters (S) obtained by ESR at 37°C. Intact rat liver microsomes (*A*) and multilamellar vesicles prepared from recombined microsomal phospholipids that had been separated into individual classes by HPLC (*B*) were labeled with Stel2. Each phospholipid class in the recombined vesicles was present in its naturally occurring mole fraction. Microsomes were obtained from untreated (□) and ethanol-fed (■) rats. Microsomal phospholipids (○, control; ●, ethanol-fed) used to prepare the reconstituted vesicles were isolated from the same microsomal preparations used to obtain the data in *A*. Points and bars represent the mean ± SD from four pairs of animals for each concentration of ethanol.

either all the individual liver microsomal phospholipids from the untreated animals or all the individual phospholipids from the ethanol-fed animals (in the same molar proportions as found in the microsomal membrane), except that in each preparation one different phospholipid class was omitted. The missing phospholipid in the untreated preparation was then replaced by the corresponding phospholipid from the ethanol-fed rats, while in the preparation composed of phospholipids from the ethanol-fed rats, a phospholipid from the untreated rats replaced the phospholipid from the ethanol-fed rats. Recombined membranes prepared from the phospholipids of ethanol-fed rats retained their resistance to fluidization by ethanol after substitution of PtdCho (66.5 mol %), PtdEtn (21 mol %), PtdIns (8.5 mol %), or PtdSer (4.0 mol %) from untreated rats for the corresponding phospholipid from the ethanol fed rats (Fig. 2). Recombined "untreated" membranes were still fluidized by ethanol after substitution of PtdCho, PtdEtn, or PtdSer from the ethanol-fed rats for the corresponding phospholipid from the untreated rats. In contrast, when PtdIns from treated rats was substituted in the membranes made of phospholipids from untreated rats, in its naturally occurring amount (8.5 mol %), the membranes from the untreated rats were rendered resistant to disordering by ethanol (Fig. 2).

To rule out the possibility that these results reflected a particular association between the fatty acid probe and PtdIns, we repeated the experiments with two phospholipid spin probes, PtdCho12 and PtdEtn12. The phospholipid probes provide similar structural information about the membrane yet are chemically different from the fatty acid spin label. These probes gave results identical to those obtained with the fatty acid probe; again, PtdIns obtained from the liver of the ethanol-fed animals was the only phospholipid that imparted membrane tolerance.

To determine whether the effect of PtdIns is peculiar to rat hepatic microsomes, we prepared vesicles that had the same proportions of phospholipid classes as the rat microsomal membrane but were made from bovine liver PtdCho, PtdEtn, and PtdIns and bovine brain PtdSer and sphingomyelin. These reconstituted vesicles were disordered by ethanol to the same extent (S = 0.316 in the absence of ethanol; S = 0.301 at 100 mM ethanol; ΔS = 4.6%) as vesicles made from the microsomal phospholipids of untreated animals. Vesicles in which bovine liver PtdIns was replaced with microsomal PtdIns from untreated animals were also disordered to the same extent. However, when the bovine liver PtdIns was replaced by PtdIns from the ethanol-fed rats, the bovine vesicles became resistant to disordering. Thus, 8.5 mol % microsomal PtdIns from the treated animals also conferred membrane tolerance to vesicles composed of 91.5 mol % phospholipids from another animal species and another organ.

A series of experiments was devised to determine the minimal amount of microsomal PtdIns from the ethanol-fed rats that was needed to confer membrane tolerance to the reconstituted multilamellar vesicles. Vesicles composed of phospholipids from untreated rats were prepared with increasing substitution of PtdIns from treated rats, with the total amount of PtdIns kept at 8.5 mol %. For example, if the vesicle contained 4.5 mol % PtdIns from treated rats, the other 4.0 mol % was PtdIns from untreated rats. The results in Fig. 3 demonstrate that only 2.5 mol % PtdIns from the ethanol-fed rats was required to provide resistance to ethanol-induced fluidization. When the vesicle contained 1.5 mol % PtdIns from the ethanol-fed rats, the percent decrease in the order parameter between 0 and 100 mM ethanol was two-thirds that observed when all the PtdIns was from untreated rats (Fig. 3). When PtdIns from ethanol-fed rats was recombined in the same fashion using bovine phospholipids instead of microsomal phospholipids, 2.5 mol % PtdIns from the ethanol-fed rats also conferred membrane tolerance (Fig. 3). Identical results were obtained in both types of recombined vesicle systems labeled with either the fatty acid or the phospholipid spin labels.

DISCUSSION

Although membrane tolerance has been observed by a number of investigators in a variety of membranes from different animal species, the molecular basis of this phenomenon has not been identified. In a previous study (8), we reported that membrane tolerance in microsomes and erythrocyte membranes is not limited to the intact membranes, since reconstituted vesicles prepared from the extracted phospholipids are also tolerant to ethanol-induced disordering. In addition, after chronically ethanol-fed rats are withdrawn from ethanol, tolerance is rapidly lost both in the intact membranes and in the vesicles prepared from the extracted membrane phospholipids. Because the alterations in the physical properties of the vesicles prepared from microsomal phospholipids isolated from the ethanol-fed rats are the same as those observed in the intact membrane, the reconstituted system provides a convenient model to identify the specific

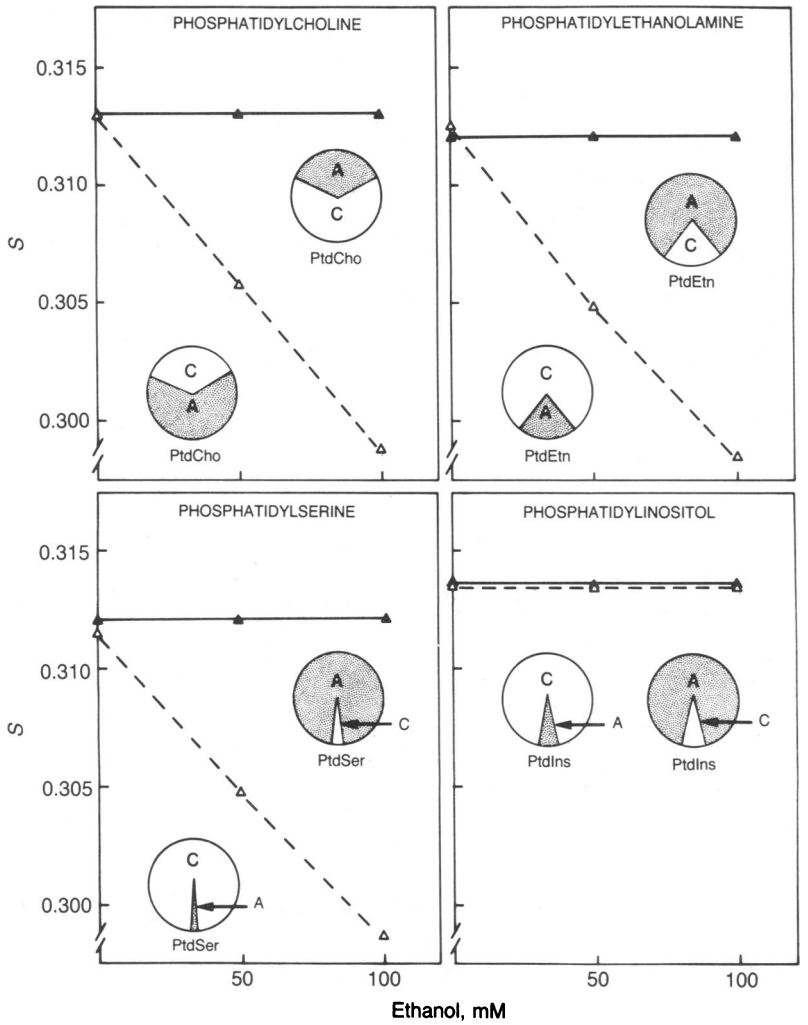

FIG. 2. Capacity of individual microsomal phospholipid classes isolated from ethanol-fed rats to produce membrane tolerance in vesicles composed of microsomal phospholipids from untreated rats. The phospholipid compositions of each of the reconstituted membrane systems are indicated in the pie charts: the individual phospholipid class tested in each experiment is given next to the corresponding section of the pie; shaded areas indicate the mole fraction of phospholipid(s) from ethanol-fed rats ("alcoholic," A), and unshaded areas, the mole fraction of phospholipid(s) from untreated animals (control, C). ESR order parameters of Ste12-labeled vesicles in the absence or presence of ethanol for recombined phospholipid vesicles composed of three phospholipid classes from ethanol-fed rats and one phospholipid class from untreated rats are shown by ▲, and vesicles composed of three phospholipid classes from untreated rats and one phospholipid class from ethanol-fed rats, as △. Results obtained for four other sets of pair-fed animals were similar: the variation in each point was always less than 0.75%.

phospholipid that promotes membrane tolerance. We have now developed a refined model of reconstituted membranes composed of well-defined mixtures of phospholipids from untreated and ethanol-fed animals. To mimic the phospholipid composition of the intact membrane in the reconstituted vesicles, the separated phospholipid classes are recombined in the same molar proportions that exist in the intact microsomal membrane. The reconstituted vesicles made of only phospholipids from untreated animals or only phospholipids from ethanol-treated animals exhibited the same response to ethanol as the intact membranes from which they were isolated (Fig. 1). It is reasonable, therefore, to extrapolate results derived from the reconstituted phospholipid membranes to the intact microsomal membrane.

PtdIns, which is a quantitatively minor (8.5 mol %) component of the microsomes, was the only phospholipid found to impart membrane tolerance. PtdCho and PtdEtn, which are present in much greater amounts (66 mol % and 22 mol %, respectively), were without effect when tested separately. Even more surprising was the finding that as little as 2.5 mol % PtdIns from ethanol-fed animals rendered the membrane resistant to fluidization. The ability of PtdIns to promote membrane tolerance is not restricted to microsomal phospholipids; the inclusion of as little as 2.5 mol % PtdIns from the ethanol-fed animals into vesicles of bovine phospholipids of brain and liver had the same effect (Fig. 3). The observation that vesicles prepared from phospholipids of ethanol-fed animals, in which 8.5 mol % PtdIns from untreated animals replaced the PtdIns from treated animals, remained resistant to disordering by ethanol (Fig. 2) suggests that minor ethanol-induced modifications in the other microsomal phospholipids make them weak promoters of tolerance and that they can confer tolerance to reconstituted membranes when they comprise 90 mol % of the vesicle phospholipids. Thus, although PtdIns is a very strong promoter of membrane tolerance and is the only individual microsomal phospholipid capable of rendering membranes tolerant to disordering by ethanol *in vitro*, combinations of other microsomal phospholipids (e.g., PtdCho plus PtdEtn) from ethanol-fed rats, when present in high concentrations, can also produce membrane tolerance.

The fatty acid spin label Ste12 can be expected to orient randomly throughout the membrane vesicle. To rule out the possibility that tolerance is a reflection of a specific interaction between Ste12 and PtdIns, the vesicles were also labeled with the phospholipid probes PtdCho12 and PtdEtn12. The

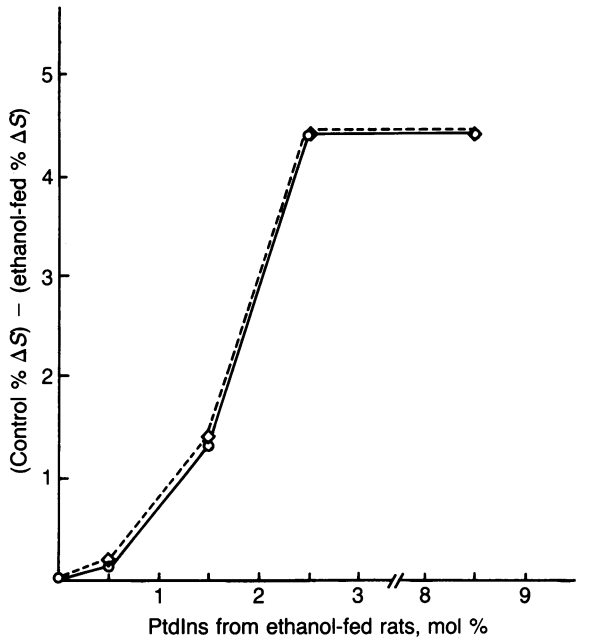

FIG. 3. Determination of the minimal amount of PtdIns from ethanol-fed rats required to impart resistance to reconstituted phospholipid vesicles. Spin-labeled (Ste12) vesicles were prepared by recombining either rat liver microsomal phospholipids from untreated rats (○) or bovine phospholipids (◇) as described in *Materials and Methods*. Vesicles prepared either from microsomal phospholipids of untreated rats or from bovine standard lipids plus 8.5 mol % microsomal PtdIns from untreated rats are referred to as "control." Vesicles prepared from microsomal phospholipids of untreated rats or from bovine standard lipids and containing various amounts of PtdIns from ethanol-fed rats are referred to as "ethanol-fed". ESR spectra were recorded in the absence and presence of 100 mM ethanol at 37°C. Control % ΔS represents the percent difference in the order parameter, S, between 0 and 100 mM ethanol in vesicles with no PtdIns from ethanol-fed rats. Control % ΔS = 4.4%, since this is the maximal extent of disordering by 100 mM ethanol observed in the reconstituted control microsome and bovine standard vesicle preparations. Ethanol-fed % ΔS represents the percent difference in order parameter between 0 and 100 mM ethanol in vesicles containing various amounts of PtdIns from ethanol-fed rats. The total amount of PtdIns (PtdIns from untreated animals plus PtdIns from ethanol-fed animals) was 8.5 mol % in all vesicles. When (Control % ΔS) − (ethanol-fed % ΔS) = 4.4%, the vesicles containing PtdIns from ethanol-fed rats are completely resistant (ethanol-fed % ΔS = 0) to disordering by 100 mM ethanol.

distribution of these probes in membranes is the same as that of unlabeled PtdCho and PtdEtn (15). The same amount of microsomal PtdIns from ethanol-fed rats that conferred membrane tolerance in the fatty acid-labeled vesicles produced tolerance in vesicles labeled with the phospholipid probes. This finding is in agreement with our previous study (4), which showed that the interaction of ethanol with intact microsomes from untreated and ethanol-fed animals labeled with fatty acid or phospholipid spin probes is qualitatively similar. It appears that the presence of small amounts of PtdIns from the ethanol-fed animals alters bulk membrane structure and that tolerance is not due to the disruption of the interaction of ethanol with a particular membrane phospholipid.

In this study, PtdIns isolated from ethanol-fed rats exhibited a small decrease in the level of arachidonic acid and a slight increase in the quantity of oleic acid. These results differ from those reported by other workers (16), but the reasons for this discrepancy are not clear. Nevertheless, minor ethanol-induced alterations in fatty acid composition do occur and may be related to the capacity of PtdIns from ethanol-fed rats to confer membrane tolerance. Chronic ethanol treatment may also cause changes in the phospholipid molecular species, although such alterations have not yet been sought. It has recently been reported that minute changes in the molecular species of erythrocyte PtdCho cause alterations in the physical properties of the membrane (17). Alterations in the phosphoinositol moiety of PtdIns may also contribute to membrane tolerance.

We have previously reported (4, 8), and confirmed in this study, that the baseline order parameter—i.e., the molecular order in the absence of ethanol—is unchanged by chronic ethanol administration. Thus, it is inappropriate to speak of a more rigid membrane; rather, the property conferred by chronic ethanol ingestion is that of resistance to disordering by ethanol. Previous work showed that chronic ethanol ingestion leads to a decrease in the partitioning of lipophilic compounds, including ethanol, into hepatic mitochondria and brain synaptosomes (6) and into erythrocyte ghosts (18). It may be that chemical alterations that occur in PtdIns as a result of ethanol ingestion change the packing properties of the microsomal phospholipids, so that the partition of ethanol into the membrane is reduced.

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