Phospholipid Spin Probes Measure the Effects of Ethanol on the Molecular Order of Liver Microsomes†

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ABSTRACT: Ethanol, in vitro, is known to perturb the molecular order of the phospholipids in biological membranes, while chronic ethanol exposure, in vivo, leads to resistance to disordering. Such changes have usually been measured by electron spin resonance, utilizing fatty acid spin probes. The use of such probes is controversial, since their orientation in the membrane may not accurately represent that of individual phospholipids. We, therefore, compared ethanol-induced structural perturbations in the membranes of rat hepatic microsomes measured with the spin probe 12-doxylstearic acid (SA 12) with those assayed with various phospholipid spin probes. With SA 12, the addition of increasing amounts of ethanol (50–250 mM) in vitro caused a progressive decrease in the membrane molecular order, as measured by electron spin resonance (ESR). By contrast, microsomes obtained from rats chronically fed ethanol were resistant to the disordering effect of ethanol. Microsomes labeled with the phospholipid spin probes 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine, -phosphatidylethanolamine, or -phosphatidic acid also exhibited increased disordering with the addition of increasing amounts of ethanol. However, the effect noted with phospholipid spin probes was less than that observed with the fatty acid probe. Microsomes obtained from the livers of chronically intoxicated animals labeled with the phospholipid probes were also resistant to the disordering effects of ethanol in vitro. These results suggest that (1) fatty acid spin probes are qualitatively valid for measuring membrane perturbations in biological membranes, (2) ethanol affects all microsomal phospholipids, regardless of chemical dissimilarities (e.g., head-group structure), in a qualitatively similar fashion, and (3) the fluidization of fatty acyl chains in microsomal membranes is comparable in different membrane phospholipids.

Increasing evidence from this (Rottenberg et al., 1981; Waring et al., 1981, 1982) and other laboratories (Chin & Goldstein, 1977a, 1981; Harris & Schroeder, 1981) suggests that the deleterious effects of chronic ethanol intoxication may be related to changes that occur at the level of cell membranes. Incubation with ethanol in vitro has been shown by electron spin resonance (ESR)[1] to cause molecular disordering of brain synaptic membranes (Harris & Schroeder, 1981; Chin & Goldstein, 1977b), liver mitochondria (Waring et al., 1981, 1982) and microsomes (Ponnappa et al., 1982), and red blood cell membranes (Chin & Goldstein, 1977b). Similar changes have been reported for synaptic plasma membranes in studies utilizing fluorescence polarization (Harris & Schroeder, 1981).

In contrast to the fluidizing effect of ethanol on biological membranes in vitro, chronic ethanol intoxication causes resistance to the disordering effects of ethanol and, in some cases, has been reported to increase membrane molecular order, as first reported by Chin & Goldstein (1977a) for synaptic membranes. The resistance to disordering has subsequently also been observed in liver mitochondria (Rottenberg et al., 1981; Waring et al., 1981, 1982) and microsomes (Ponnappa et al., 1982), erythrocyte ghosts (Chin & Goldstein, 1977a; Kelly-Murphy et al., 1984), and pancreatic plasma membranes (Ponnappa et al., 1982). Recent studies from this laboratory have demonstrated that chronic ethanol treatment decreases the partitioning of ethanol and other lipophilic compounds into a variety of biological membranes (Waring et al., 1981; Kelly-Murphy et al., 1984).

The resistance to disordering by ethanol in the various membranes obtained from animals chronically consuming ethanol presumably results from adaptive changes in the phospholipid composition of the membranes, since a similar resistance is demonstrated in vesicles formed from lipid extracts of these membranes (Waring et al., 1981; Johnson et al., 1979). Changes in the lipid composition of synaptic membranes (LaDroitte et al., 1984; Littleton et al., 1980), hepatic mitochondria (Waring et al., 1981; Miceli & Ferrell, 1973; Cunningham et al., 1982) and microsomes (Miceli & Ferrell, 1973; Cunningham et al., 1982), and red blood cells (LaDroitte et al., 1984; Wing et al., 1984) have been reported. These include an increased proportion of saturated fatty acyl chains in membrane phospholipids (LaDroitte et al., 1984; Wing et al., 1984) and in some instances an increase in the cholesterol content (Chin et al., 1978).

Spin-labeled stearic acid has been commonly used to investigate structural perturbations in model and biological membranes in response to acute and chronic ethanol treatment. A common assumption in many of these studies is that the fatty acid spin-labels randomly distribute throughout the membrane and therefore report an average membrane environment. However, this premise is controversial. The site and depth of interaction of the fatty acid labels with the membrane phospholipids are to a large extent determined by the charge of the fatty acid (Sanson et al., 1976). It has been shown that pH, salt, and temperature can affect the degree of ionization

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[1] Abbreviations: SA 12, 12-doxylstearic acid; PC 12, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine; PE 12, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylethanolamine; PA 12, 1-palmitoyl-2-(12-doxylstearoyl)phosphatidic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoylphosphatidylcholine; NMR, nuclear magnetic resonance; ESR, electron spin resonance.

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of fatty acid probes and, consequently, their partition coefficient (Sanson et al., 1976); Brotherus et al., 1980). Several studies have reported that membrane proteins [e.g., (Na,K)-ATPase (Brotherus et al., 1980)] exhibit a preference for negatively charged fatty acids. In light of the possible complications and uncertainties which are associated with the use of fatty acid spin-labels, we synthesized a number of phospholipid spin-labels with the following objectives: (1) they can be compared to fatty acid spin-labels in order to determine whether the latter reliably and representatively measure membrane structural perturbations; (2) they provide information which compliments that obtained with the fatty acid probes, since they sample different portions of the membrane; and (3) one can obtain localized structural information at the single phospholipid level. Such information will assist in determining whether certain lipid species are selectively perturbed by ethanol treatment.

In the current study, the phospholipid probes used represent the major classes of microsomal membrane phosholipids and presumably provide data relating to the microenvironment of particular membrane phosholipid species. We chose liver microsomes for this study because there is considerable information about the biochemical alterations caused by chronic ethanol consumption in these membranes (Michaelis & Michaelis, 1983), the physical changes are highly reproducible, and the membranes are easily accessible.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories), initially weighing 100–130 g, were fed a totally liquid diet (Bioserv, Inc.) in which ethanol compromised 36% of total calories. Pair-fed, littermate controls received the same diet, except that carbohydrates isocalorically replaced ethanol. Rats were maintained on the diet for 35–45 days. Ethanol consumption averaged 14–16 g (kg body weight)$^{-1}$ day^{-1}.

Preparation of Microsomes. Rats were decapitated, and the livers were placed in 5 volumes of buffer [0.25 M sucrose, 10 mM 4-morpholinepropanesulfonic acid (pH 7.0), and 2 mM benzamidine] and homogenized with a motor-driven Potter-Elvehjem homogenizer at 1000 rpm. The homogenate was then sequentially centrifuged at 1500g for 10 min and 15000g for 15 min to remove the nuclear and mitochondrial fractions, respectively. The postmitochondrial supernatant was centrifuged at 125000g for 45 min to obtain the microsomal pellet. This pellet was resuspended in buffer at a concentration of 10 mg of protein/mL. All steps were carried out at 4 °C. Protein was determined according to Lowry et al. (1951).

Spin Probes. The stearic acid spin-label β-12-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)stearic acid, 12-doxylstearic acid (SA 12), was purchased from Molecular Probes (Junction City, OR). The phosphatidylcholine spin-label 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine (PC 12) was synthesized according to the method of Boss et al. (1975). The phoshatidylethanolamine spin-label (PE 12) was synthesized from PC 12 by the transphosphatidylation reaction of phospholipase D (Calbiochem-Behring, San Diego, CA) and purified on a silicic acid column. The phosphatidic acid spin-label (PA 12), which is a byproduct of this reaction, was also purified on a silicic acid column. The purity of spin-labeled phospholipids was determined by thin-layer chromatography against corresponding non-spin-labeled phospholipids.

Spin-Labeling of Membranes. (A) Phospholipid Probes. A suspension of PC 12 in 100 mM potassium phosphate buffer, pH 7.5, with 20% glycerol was sonicated 5 times for 30-s intervals under N_2, at 4 °C, with a Branson tip sonicator, followed by centrifugation at 38 000 rpm for 30 min. The supernatant was added to the microsomes in the same buffer (5 mg of membrane protein/mL) and mixed for 1 h at 20 °C. The initial ratio of microsomal lipid to spin-label was 20:1 (mol/mol). After 1 h, unincorporated spin-label was removed by diluting the suspension 1:60 with buffer, and the spin-labeled microsomes were pelleted at 100000g for 1 h. The microsomal pellet was rehomogenized in an excess of buffer and again pelleted. With the use of this procedure, no broad component, which would arise from pure spin-labeled vesicles, was present in the EPR spectrum. The final microsomal lipid to phosholipid probe ratio was 150:1 to 100:1. Microsomes were labeled with PA 12 in an identical fashion.

Labeling of microsomes with PE 12 required a minor modification. Since phoshatidylethanolamines do not form stable vesicles in the pure state, we premixed PE 12 with an equal amount of bovine liver lecithin (Avanti Polar Lipids, Inc., Birmingham, AL) prior to sonication. Inclusion of an equal amount of bovine lecithin in the PC 12 vesicles did not alter their ESR spectrum.

(B) Stearic Acid. Microsomes were labeled with SA 12 (Molecular Probes, Junction City, OR) by adding the probe in a minimal volume of ethanol (final ethanol concentration 5 mM) to the microsomal membrane suspension in buffer [0.25 M sucrose, 10 mM 4-morpholinepropanesulfonic acid (pH 7.0), and 2 mM benzamidine] in a ratio of 150:1 (moles of lipid to moles of probe). The sample was flushed with N_2 and shaken for 30 min at 25 °C to ensure that the label was incorporated into the membrane.

Spin-Labeled Liposomes. Total membrane lipids were isolated by extraction from the microsome preparation essentially according to Bligh & Dyer (1959). Spin-labels were incorporated into the total lipid liposomes by drying under N_2 the organic solvents containing the lipid extract and the spin probe (150:1, moles of lipid to moles of probe), followed by high vacuum desiccation to remove traces of solvents and hydration with 0.25 M sucrose, 10 mM 4-morpholinepropanesulfonic acid (pH 7.0), and 2 mM benzamidine. The solution was vortexed, a procedure which resulted in the spontaneous formation of lipid vesicles which contained the desired probe. Spin-labeled dimyristoylphosphatidylcholine (DMPC) vesicles were similarly prepared, except that they were hydrated in phospate-buffered saline (pH 7.2).

ESR Spectroscopy. The spin-labeled sample, following the addition of the desired amount of ethanol, was vortexed, transferred to a 100-μL capillary tube, and flame-sealed. Capillary tubes were placed in a Varian E-109B ESR spectrometer cavity Dewar linear in a standard 4-mm quartz ESR tube, which contained silicone oil to maintain thermal stability. All samples were equilibrated for 10 min in the cavity prior to recording of the spectra.

ESR spectra were accumulated with an SD Systems S-100 microcomputer interfaced to the ESR spectrometer. The order parameter, calculated from the inner hyperfine splitting ($2T_{\perp}$), was measured according to Gaffney (1974) and is illustrated in Figure 1. Typical spectrometer settings were spectral scan 100 G, modulation amplitude 1.0 G, and microwave power 5 mW. All measurements were made at 37 °C.

Results

Effect of Ethanol in Vitro on the Molecular Order of Microsomal Membranes. The order parameters calculated for microsomal membranes labeled with the fatty acid spin-label SA 12 in the absence and presence of increasing amounts of ethanol are shown in Figure 2. The addition of 50 mM ethanol to the microsomal suspension resulted in a substantial

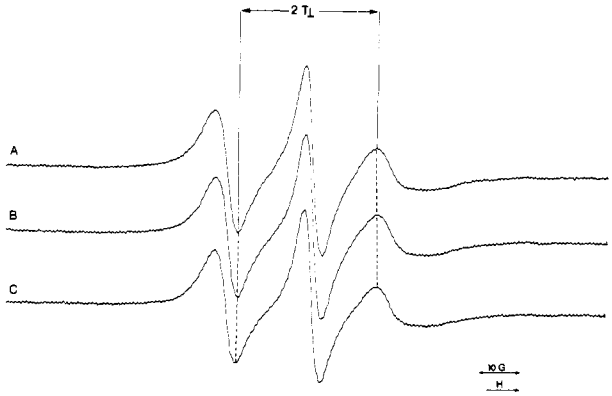

FIGURE 1: Typical ESR spectra of control microsomes labeled with PC 12 in the absence (A) and presence of 100 (B) or 250 mM (C) ethanol at 37 °C. Conditions: spectral scan, 100 G; midfield line, 3250 G; modulation amplitude, 1.6 G; microwave power, 5 mW. Spectra are the result of one scan.

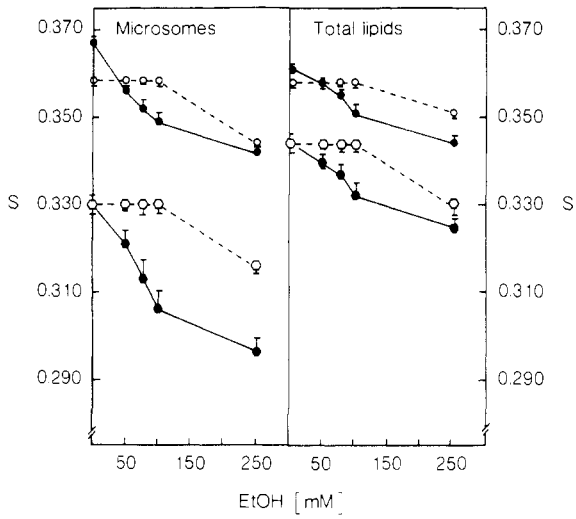

FIGURE 2: Effect of ethanol, in vitro, on the order parameter, S, of microsomes (and vesicles prepared from their total lipid extracts) obtained from the livers of ethanol-fed rats labeled with PC 12 (○) and SA 12 (○) and their pair-fed controls labeled with PC 12 (●) and SA 12 (●) at 37 °C. Points and bars represent the mean ± SD from four pairs of animals. The differences in base-line order parameters for the PC 12 labeled control and alcoholic microsomes are not statistically significant.

decrease in the order parameter (from 0.330 to 0.321). Further titration with ethanol, up to 250 mM, resulted in extensive decreases in the molecular order of the membrane (250 mM ethanol, $S = 0.296$). The overall decrease in the order parameter was 10.3%, a value indicative of considerable membrane disordering. A qualitatively similar change, although of lesser magnitude, has been observed previously in hepatic mitochondrial (Waring et al., 1982) and microsomal (Ponnappa et al., 1982) membranes labeled with 5-doxylstearic acid.

In contrast to the changes in the molecular order of control microsomes, the addition of 100 mM ethanol to microsomes from chronically treated rats had no effect on the order parameter of the membrane (Figure 2). Further addition of ethanol (250 mM) induced a decrease in the order parameter. In four pairs of rats, no statistically significant difference in the base-line order parameter (no ethanol) was observed between control microsomes and microsomes from chronically treated rats.

Control microsomes labeled with the phospholipid spin probe PC 12 and exposed to increasing amounts of ethanol exhibited a response qualitatively similar to that observed with SA 12.

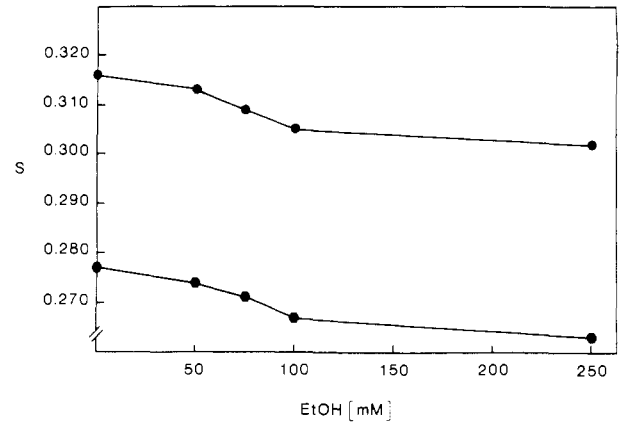

FIGURE 3: Effect of ethanol, in vitro, on the order parameter, S, of DMPC vesicles labeled with PC 12 (●) and SA 12 (●) at 37 °C. Vesicles were labeled as described under Materials and Methods.

Addition of 50 mM ethanol decreased the order parameter (0.367 to 0.357), and further titration with ethanol up to 250 mM produced a larger effect (0 mM ethanol, 0.367; 250 mM ethanol, 0.342). This change (over the range 0–250 mM) represents a 6.8% decrease in the order parameter. The results for membranes labeled with PC 12 were qualitatively similar but quantitatively different from those obtained for SA 12. The decrease in the order parameter of the phosholipid spin-labeled microsomes, expressed as a percent of the control value, was about two-thirds that of SA 12 labeled membranes, in the range of 50–250 mM ethanol, respectively.

To investigate the reason for this difference, vesicles containing either PC 12 or SA 12 were prepared from DMPC and titrated with increasing amounts of ethanol at 37 °C. As in intact microsomes, vesicles labeled with PC 12 had a base-line order parameter 12% higher than SA 12 labeled vesicles. By contrast, DMPC vesicles labeled with PC 12 and SA 12 were disordered by ethanol to the same extent over the range 50–250 mM (Figure 3). It seems that SA 12 and PC 12 report from different environments in the microsomal membrane, SA 12 being in a domain which is more susceptible to the fluidizing effect of ethanol.

As in SA 12 labeled microsomes obtained from chronically treated rats, PC 12 labeled membranes were resistant to the disordering effect of ethanol up to 100 mM (Figure 2). Higher concentrations of ethanol (250 mM) did disorder the membranes ($S = 0.358$ to $S = 0.344$), an effect similar to that seen in SA 12 labeled membranes.

It is apparent that, qualitatively, a fatty acid spin probe and a phospholipid spin probe measure the same changes in membrane order produced by ethanol in vitro and by chronic ethanol feeding. It is interesting to note that the base-line order parameters obtained for PC 12 labeled membranes (control and ethanol treated) were considerably higher than those in SA 12 labeled membranes (Figure 2). In the case of the control membranes, the order parameter calculated for PC 12 in the absence of ethanol was 10% higher than for the same membrane preparation labeled with SA 12. This difference in base-line order parameters is also seen in microsomes from chronically treated animals (PC 12 is 8% higher). To gain insight into this observed difference in order parameters measured by the PC 12 and SA 12 probes, two experiments were performed. Vesicles prepared from DMPC were labeled with PC 12 and SA 12, respectively, and the order parameter was calculated as a function of temperature. Above the gel to liquid-crystalline phase transition of DMPC (24 °C), the order parameter calculated for PC 12 labeled vesicles was

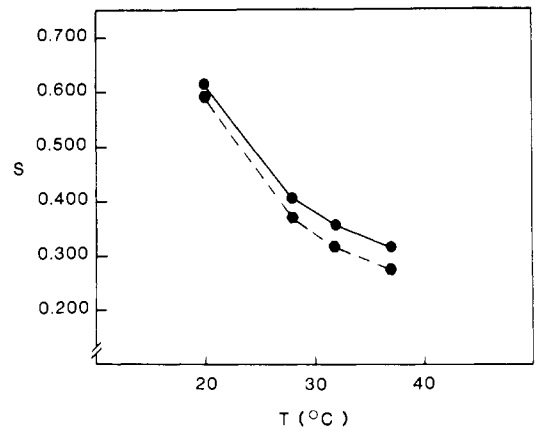

FIGURE 4: Temperature-dependent behavior of the order parameter, S, obtained for DMPC vesicles labeled with PC 12 (●) and SA 12 (●).

8–13% higher (more ordered) than a corresponding DMPC sample labeled with SA 12 (Figure 4). This result suggests that the observed differences in the order parameter in the microsomal membranes reflect chemical dissimilarities in the probes, one being a phospholipid and the other a fatty acid. Owing to the size of the phospholipid probe, it is likely to experience more restricted mobility than the fatty acid probe.

To address this point further, we monitored the change in the order parameters of vesicles made from the total lipid extracts of microsomes to ethanol in vitro (Figure 2). Membrane disordering by ethanol in vitro, and resistance to ethanol disordering by microsomes obtained from ethanol-fed animals, was noted in vesicles labeled with either PC 12 or SA 12. No significant differences in base-line order parameters between the two preparations were observed for either probe. The extent of disordering caused by ethanol in vitro was reduced in the total lipid extracts compared to the intact membranes for PC 12 (6.8% vs. 4.7%) as well as for SA 12 (10.3% vs. 5.5%). Similar observations have been made for 1,6-diphenyl-1,3,5-hexatriene (DPH) labeled total lipid extracts of brain synaptosomal membranes by fluorescence polarization (Harris & Schroeder, 1981; Johnson et al., 1979).

As was observed in the model DMPC system, the SA 12 labeled control microsomal extract was disordered to the same extent as the PC 12 labeled control extract (Figure 2). The base-line order parameters obtained for the same preparation labeled with SA 12 were higher than those in the intact membrane. By contrast, those measured for the PC 12 labeled samples were similar. In the case of the SA 12 labeled control membranes, the base-line order parameter of the extract was 4% higher than that of the intact membrane. We found a similar difference between the two SA 12 labeled preparations from the alcoholic rats.

Effect of Ethanol in Vitro Measured with Phospholipid Spin Probes. The effects of the addition of ethanol to the same microsomes labeled with phospholipid probes are shown in Figure 5. The addition of ethanol to control microsomes, labeled with PC 12, PE 12, or PA 12, leads to comparable reductions in the order parameter. The base-line order parameters were not significantly different. Thus, the disordering action of ethanol appears not to be specific for phospholipid head groups in microsomal membranes. The phospholipid probes gave rise to higher order parameters than those obtained from fatty acid spin-labeled membranes (compare Figures 2 and 5).

The resistance to disordering by ethanol in microsomes obtained from treated rats was also evident from the order

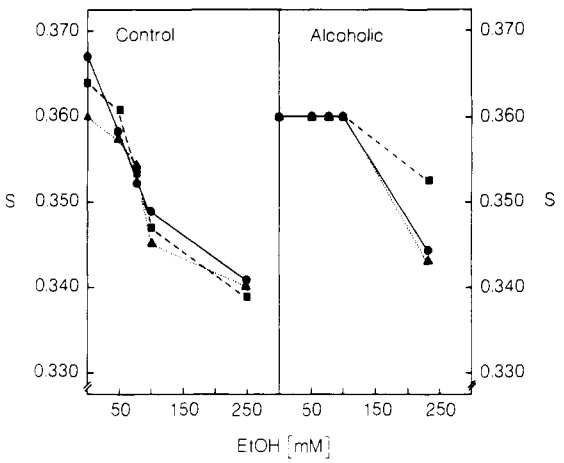

FIGURE 5: Effect of ethanol, in vitro, on the order parameter, S, of microsomes obtained from the livers of ethanol-fed rats and their pair-fed controls labeled with PC 12 (●), PE 12 (▲), and PA 12 (■) at 37 °C. Each point represents the mean obtained from four pairs of animals. The differences in base-line order parameters between control microsomes labeled with PC 12, PE 12, and PA 12 are not statistically significant. The differences in base-line order parameters between control and alcoholic microsomes labeled with the phospholipid spin probes are not significantly different.

parameter profile depicted in Figure 5. From 0 to 100 mM ethanol, the order parameters were the same for membranes labeled with PC 12, PE 12, and PA 12. Higher concentrations of ethanol (250 mM) induced some membrane disordering (Figure 5). There was no statistically significant difference in the base-line order parameters with any of the three phospholipid spin-labels in membranes obtained from treated animals compared to their controls. Thus, although the fatty acid composition of PC and PE has been reported to be modified by chronic alcohol treatment (Cunningham et al., 1982), these changes seem not to affect the structural order of these lipids in the membrane, at least within the limits of the sensitivity of ESR. We realize, however, that any labeling method relies on probe molecules which are chemically similar to but not identical with the natural molecule that they are intended to represent. The phospholipid probes used in this study, while having the same head-group structure as the major microsomal membrane phospholipids they minic, have different fatty acyl chain compositions.

Discussion

Acute and chronic ethanol treatment alters the chemical and structural properties of a variety of biological membranes (Michaelis & Michaelis, 1983). Using fatty acid spin probes, it has been demonstrated that the presence of ethanol causes a decrease in membrane molecular order, whereas chronic ethanol treatment results in an acquired resistance to this disordering effect. Chronic ethanol intoxication has also been reported to produce changes in phospholipid fatty acid composition of membrane phospholipids, mainly in the form of an increase in the number of saturated acyl chains. Chronic ethanol administration also results in decreased protein content and enzyme activity in biological membranes isolated from liver (Thayer & Rubin, 1979, 1981) and brain (Sun & Sun, 1983).

In the current study, we sought to answer a number of questions relating to the observations mentioned above. These include the following: (1) Does a fatty acid spin probe reliably measure membrane perturbation by external agents? (2) Does the presence of ethanol and chronic ethanol administration affect membrane phospholipid classes similarly? (3) Do changes in fatty acid composition of microsomal PC and PE

differentially affect the molecular order of these components of the microsomal membrane?

Ethanol titration of control microsomes labeled with the phospholipid probes or the fatty acid probe SA 12 produces qualitatively similar results, namely, a decrease in the order parameter S. However, membranes labeled with SA 12 are disordered considerably more than microsomes labeled with PC 12, PE 12, or PA 12. Interestingly, this difference is not present in total lipid extracts. These results suggest that SA 12 resides in membrane environments which are disordered by ethanol to a greater degree than those domains in which the phospholipid probes are located. This difference is not caused by chemical dissimilarities of the probes, since in model membranes labeled with both SA 12 and PC 12 the order parameters were decreased to the same extent by ethanol (Figure 3).

Using fatty acid and phospholipid spin probes in a reconstituted membrane system comprised of major microsomal phospholipids and cytochrome P-450, it has been demonstrated that fatty acids have a high affinity for the protein (Bosterling et al., 1981). By contrast, microsomal phosholipids which interact with proteins undergo rapid exchange, and the protein shows no preference for any particular phospholipid, at least in the time domain of physiological reaction (Bosterling et al., 1981). This observation may have some relevance to the more pronounced disordering in microsomes labeled with SA 12, but since little is known about the effects of ethanol on fatty acid–protein or lipid–protein interactions, such an association is highly speculative. It does seem that the fatty acid probes and the phospholipid probes reside in different environments within the microsomal membrane.

Through the use of the spin-labeled phospholipids PC 12, PE 12, and PA 12, we were able to examine the effect of ethanol in vitro on the structural organization of single phospholipid classes in a biological membrane. As is the case for SA 12 labeled membranes, ethanol decreases molecular order when measured with phospholipid probes (Figure 2). The base-line order parameter of PC 12, PE 12, and PA 12 are comparable, as is the extent of fluidization by ethanol. These results suggest that the action of ethanol is not specific for any particular class of membrane lipid, at least in the microsomal membrane. Microsomes obtained from ethanol-fed animals and labeled with the phospholipid probes are also resistant to the disordering effects of ethanol.

The base-line order parameters for PC 12, PE 12, and PA 12 in control and chronic alcohol treated microsomes are very similar. Thus, although some phospholipid classes (e.g., PC and PE) were reported to be more saturated following chronic ethanol administration, we find no alteration in their molecular order in microsomal membranes obtained from alcoholic animals. Recently, however, we have found that the molecular order of phosphatidylethanolamine, reported by PE 12, is significantly (5%) reduced in rat liver submitochondrial particles prepared from alcoholic rats compared to their pair-fed controls (T. F. Taraschi and E. Rubin, unpublished results).

The origin of the increased order parameter in the total lipid extracts of the control and experimental microsomal membranes labeled with SA 12, compared to the intact membranes, is not clear. Previous studies of microsomal membranes by ^{31}P NMR have shown that the lipids of the intact membrane exhibit considerably more motional freeedom than do those in the lipid extract (De Kruijff et al., 1980; Stier et al., 1978; Bayerl et al., 1984). To date, this observation is unique to the microsomal membrane. Red cell ghosts and submitochondrial particles have considerably higher ESR order parameters than their respective lipid extracts (T. F. Taraschi, unpublished results).

In summary, the use of phospholipid spin probes provides a technique for studying the structure of individual phospholipids in intact biological membranes. Fatty acid spin probes, while apparently residing in different membrane environments than the phospholipid probes, are qualitatively reliable for measuring membrane perturbation by external agents, such as ethanol. The disordering effect of ethanol seems to involve all phospholipids of the membrane.

Registry No. SA 12, 29545-47-9; PC 12, 55402-86-3; PE 12, 81991-55-1; PA 12, 98760-19-1; EtOH, 64-17-5.

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Penetration of a Cardiotoxin into Cardiolipin Model Membranes and Its Implications on Lipid Organization†

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ABSTRACT: The interaction of cardiotoxin II of *Naja mossambica mossambica* with cardiolipin model membranes was investigated by binding, fluorescence, resonance energy transfer, fluorescence quenching, ^{31}P NMR, freeze–fracture, and small-angle X-ray experiments. An initially electrostatic binding appeared to be accompanied by a deep penetration, most likely into the acyl chain region of the phospholipids, indicating a hydrophobic contribution to the strong interaction ($K_D \simeq 5 \times 10^{-8}$ M). This binding results in a fusion of unilamellar vesicles as indicated by a fluorescence-based fusion assay, freeze–fracture, and X-ray diffraction. In these fused structures freeze–fracture electron microscopy reveals the appearance of particles, which is accompanied by the induction of an isotropic component in ^{31}P NMR. The well-defined particles are interpreted as inverted micelles, and the localization of the cardiotoxin molecule in these structures is discussed.

Proteins and phospholipids are the major components of biological membranes and for a proper functioning of the membranes lipid–protein interactions appear to be essential. One aspect of these interactions is the modulation by proteins of local lipid structure. Protein-induced nonbilayer lipid structures (lipidic particles, short inverted H_{II} cylinders) have been suggested to play a role in essential processes as transbilayer transport of lipids, membrane fusion, and translocation of newly synthesized proteins across membranes (Nesmeyanova, 1982; de Kruijff et al., 1981; Cullis & Hope, 1978).

Model membrane experiments revealed that lipid polymorphism can be modulated by the hydrophobic peptide gramicidin and the membrane spanning part of the intrinsic protein glycophorin. Whereas gramicidin displays a strong bilayer destabilizing action, for instance in the typical bilayer system of dioleoylphosphatidylcholine (Van Echteld et al., 1981, 1982), even when added through the aqueous phase (Killian et al., 1984), glycophorin in contrast stabilizes the bilayer structure in H_{II} phase preferring lipid systems of dioleoylphosphatidylethanolamine (Taraschi et al., 1982) and Ca^{2+}–cardiolipin (Taraschi et al., 1983). Extrinsic proteins too are able to affect macroscopic lipid structure. In the beef heart cardiolipin system, poly(L-lysine) with a polymerization degree of $n = 120$ down to $n = 5$ was found to stabilize bilayer structure in the presence of Ca^{2+}, a strong inducer of H_{II} formation in pure cardiolipin (de Kruijff & Cullis, 1980a; B. de Kruijff, unpublished observations). On the other hand, cytochrome *c*, a basic protein with eight net positive charges, was observed to induce inverted structures in cardiolipin-containing systems (de Kruijff & Cullis, 1980b) and must in this respect be marked a bilayer destabilizer.

These results suggest that the effects of proteins and polypeptides on lipid polymorphism cannot directly be correlated to overall positive charge or hydrophobicity but that more subtle features, for instance, the spacial distribution of the charged amino acids and the three-dimensional structure of the protein, are important. For a better understanding of the relative importance of these features it is fundamental to study the interaction of well-defined peptides with lipid model membranes.

Among the natural peptides having a high affinity for certain membrane lipids are the cardiotoxins from snake venoms. The cardiotoxins form a group of highly basic proteins consisting of 60–61 amino acid residues, giving the protein a molecular weight of about 7000 (Dufton & Hider, 1983). Besides positively charged residues, the toxins contain some hydrophobic stretches, especially the sequence 6–11. The tertiary structure, which is believed to contain three loops forming an extended β-sheet (Lauterwein & Wüthrich, 1978), is firmly stabilized by four disulfide bridges. On the basis of the high amount of cardiotoxin that was observed to be bound to biological membranes, it was proposed that lipids were involved in this binding (Vincent et al., 1976), and this opened the way for studies of cardiotoxin–phospholipid interactions (Bougis et al., 1983). It was established that the cardiotoxins bind only to negatively charged phospholipids (Dufourcq & Faucon, 1978; Vincent et al., 1978), indicating a strong electrostatic component in the interaction. On the other hand, the sequence around tryptophan 11 is believed to leave its aqueous environment upon binding, explaining the blue shift and increased intensity of the tryptophan fluorescence (Du-

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