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Role of Phosphatidylethanol in Membranes. Effects on Membrane Fluidity, Tolerance to Ethanol, and Activity of Membrane-Bound Enzymes†

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ABSTRACT: We investigated the effect of phosphatidylethanol (PEt) on fluidity and membrane tolerance to the fluidization induced by ethanol as well as on the activity of two membrane-bound enzymes, Na^+/K^+ ATPase and 5′-nucleotidase. PEt was synthesized from 1,2-dimyristoylphosphatidylcholine and phosphatidylcholine from bovine brain and studies were performed to determine the optimal experimental conditions for the insertion of PEt in natural bilayers. The effects of PEt, evaluated by differential scanning calorimetry or fluorescence polarization techniques, were studied in model membranes made of synthetic phospholipids or made of total lipids extracted from rat brain crude mitochondrial fraction (P_2 fraction) and from natural membranes (P_2 fraction). The presence of PEt increased the fluidity of artificial as well of natural membranes, but tolerance to the addition of ethanol, displayed by dimyristoylphosphatidylcholine vesicles and by natural membranes containing PEt, was lacking in vesicles made of dimyristoylphosphatidylethanolamine and in artificial bilayers reconstituted from total P_2 lipid extracts, suggesting an involvement of PC on PEt-induced ethanol resistance. Na^+/K^+ ATPase activity was enhanced by the addition of small amounts of ethanol (up to 50 mM) and progressively inhibited at higher concentrations, while 5′-nucleotidase was not affected up to 400 mM ethanol. The presence of PEt in the bilayer exerted the opposite effects on the two enzymes, reducing the Na^+/K^+ ATPase activation induced by ethanol and enhancing 5′-nucleotidase activity. The mechanisms of the PEt-induced modifications are discussed.

Recently, a pathway for alcohol metabolism whose product is an unusual phospholipid, phosphatidylethanol (PEt)[1] (Alling et al., 1984), has been reported. The conformation and the properties of this phospholipid could be of considerable interest since it is at the level of the polar region that the membrane interacts with its external environment. In a previous paper (Omodeo-Salè et al., 1989) we investigated by high-sensitivity differential scanning calorimetry the thermotropic behavior of PEt in vesicular dispersions of phosphatidylcholine, showing that PEt can markedly influence the physicochemical properties of the membrane where it is occasionally synthesized. However, the role of this unusual phospholipid in the structure and functions of biological membranes has not been studied. Moreover, the mechanism of membrane tolerance to the disordering effect of ethanol observed in membranes of ethanol-treated animals is still obscure (Waring et al., 1981;

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[1] Abbreviations: PC, phosphatidylcholine from bovine brain; DMPC, 1,2-dimyristoylphosphatidylcholine; PEt, phosphatidylethanol synthesized from bovine brain PC; DMPEt, 1,2-dimyristoylphosphatidylethanol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; DMPE, 1,2-dimyristoylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; HPTLC, high-performance thin-layer chromatography; DSC, high-sensitivity differential scanning calorimetry; T_m, temperature of transition from gel to liquid-crystalline phase.

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Johnson et al., 1979; Taraschi et al., 1986a). On this basis we investigated the effect of PEt in promoting membrane tolerance to ethanol and in affecting membrane fluidity. Furthermore, we studied the effects of PEt on the activity of two membrane-linked enzymes (Na^+/K^+ ATPase and 5′-nucleotidase) differently located in the bilayer (Salem et al., 1981; Franco et al., 1986; Kimelberg et al., 1977).

Experimental Procedures

Crystalline bovine serum albumin, adenosine triphosphate (ATP), adenosine monophosphate (5′-AMP), phosphoenolpyruvate (PEP), ouabain, DMPC, DMPE, PC, PE, PS, and SM from bovine brain and PI from bovine liver were purchased from Sigma (St. Louis, MO; nicotinamide–adenine dinucleotide reduced form (NADH), pyruvate kinase/lactate dehydrogenase (PK/LDH, E.C. 2.7.1.40/1.1.1.27 from rabbit muscle), and adenosine deaminase (ADA, E.C. 3.5.4.4. from calf intestine) were from Beohringer Mannheim (Germany). DPH was from Aldrich Chemical Co.

Synthesis and Purification of PEt. PEt was enzymatically synthesized from bovine brain PC and from DMPC and purified as previously reported (Omodeo-Salè et al., 1989). PEt obtained from DMPC will be referred to as DMPEt.

Preparation of Rat Brain Crude Mitochondrial Fraction ("P_2 Fraction"). Sprague-Dawley adult male rats (from Charles-River, 175–200-g body weight) were decapitated and the brains rapidly removed and washed in ice cold isotonic solution. The P_2 fraction was prepared according to Morgan et al. (1971). Membranes were suspended in 2 mM Hepes–NaOH buffer, pH 7.4, in sucrose, 0.32 M (buffer A), at a protein concentration of 10 mg/mL and kept under liquid N_2 prior to analysis. Aliquots of the membrane preparations were taken for protein analysis (Lowry et al., 1951), fluorescence studies, and enzymatic studies (see below).

Lipid Extraction and Compositional Analysis. Total lipids were isolated from an aliquot of the membrane suspension according to Folch et al. (1957). Total lipid extracts were dried under N_2, dissolved in $CHCl_3/CH_3OH$ 2:1 (by volume), and stored at −20 °C. Phospholipid phosphorus and total cholesterol were determined by the Bartlett (1959) and Pearson and Stern (1953) procedures, respectively. Compositional analysis of phospholipids was carried out by subjecting the total lipid extract to HPTLC in chloroform/methanol/acetic acid/water (60/40/4/2, by volume). Lipids were visualized by iodine staining and the spots corresponding to phospholipid standards were scraped and submitted to phosphorus determination (Dodge & Phillips, 1967).

Association of PEt to Membranes. Membranes (P_2 fraction) were diluted in 2 mM Hepes–sucrose buffer to a final phospholipid concentration of 3×10^{-3} M. PEt was dissolved in ethanol (2–10 mM final concentration) and an aliquot of 50 μL slowly injected with a microsyringe under stirring at 37 °C into 1 mL of the membrane suspension. An aliquot of the same membrane preparation was injected with 50 μL of pure ethanol and used as a control. After 30 min of incubation under gentle stirring at 37 °C, the solution was diluted with 6 volumes of buffer A and centrifuged at 80.000*g* for 25 min at 4 °C. The pellet was homogeneously suspended in the same buffer by use of a manually driven Teflon glass homogenizer and the centrifugation step repeated. Protein recovery in the pellet generally exceeded 95%. The pellet was resuspended with the same buffered solution and employed for the enzymatic or fluorescence assays. Control experiments performed by adding ethanolic PEt to a buffer solution showed that 99% PEt was recovered in the supernatant after the centrifugation step.

The amount of PEt associated to the membranes was determined by extraction of the total lipids from the pellet (Folch et al., 1957) and fractionation by DEAE-Sephadex A-25 ion-exchange column chromatography as previously reported (Omodeo-Salè et al., 1989). The fraction containing PEt was subjected to HPTLC in chloroform/methanol/acetone/acetic acid/water (50:15:15:10:5, by volume). Spots corresponding to PEt were visualized and quantitated as described for total lipid extract.

Preparation of Aqueous Lipid Dispersions. An aliquot of pure lipids, lipid mixtures, or total lipids extracted from P_2 membranes, dissolved in chloroform/methanol 2:1 (by volume) was dried to a thin film with a gentle flow of N_2. After lyophilization the samples were resuspended in a proper volume of Tris-HCl buffer, 50 mM, pH 7.4, above their T_m (28 °C for DMPC and DMPC/DMPEt, 55 °C for DMPE and DMPE/DMPEt, 37 °C for total lipid extracts and total lipid extracts/PEt) and vortexed for 5 min. In the case of fluorescence samples, a prefixed volume of DPH solution (10^{-3} M in tetrahydrofuran) was mixed to the chloroform/methanol lipid extract. The final lipid concentration was 10^{-3} M for the calorimetric experiments and 1.25×10^{-4} for the fluorescence experiments. In the fluorescence samples the final DPH concentration was 10^{-6} M. Vesicles formed under these conditions are mostly multilamellar structures (Bangham et al., 1967).

Fluorescence Spectroscopy. A suspension containing 50 μg of protein per milliliter of Tris-HCl buffer, 50 mM, pH 7.4, was used for studies on intact membranes. P_2 membranes injected with pure ethanol were used as the 0% PEt sample. The fluorescent probe molecule DPH was added to the membrane suspension at 10^{-6} M final concentration. Light scattering was corrected by using a blank containing all lipid components but DPH.

Membrane or lipid suspensions with and without DPH were incubated in the dark under stirring for 45 min at a temperature above their T_m and were used for fluorescence polarization studies immediately after preparation. An FP-550 polarization spectrofluorometer (Jasco) 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 & Schroeder, 1982). Excitation and emission wavelengths were 360 and 430 nm, respectively. Fluorescence anisotropy, was calculated as $r(I_\parallel - I_\perp/I_\parallel + 2I_\perp)$. After the sample reached the desired temperature in the fluorometer (35 °C for intact membranes or total lipids extracts and 28 °C for DMPC) the polarization was determined by using a grating correction factor to compensate for the polarization artifacts of the optical system. After the addition of ethanol (400 mM final concentration) to the blank and to the test cuvette, the sample was allowed to equilibrate (ca. 2 min) and the fluorescence anisotropy was again noted. Ten or 20 determinations of each parameter were averaged for each membrane preparation. The sample was continously stirred with a microstirrer and the temperature was monitored by a thermistor in the cuvette.

Ethanol Determination in P_2 Membranes and Aqueous Lipid Dispersions. After the fluorescence experiments, an aliquot of the sample corresponding to 1 μmol of phospholipids was centrifuged at 100.000*g* for 30 min and the pellet, resuspended in 200 μL of the experimental buffer, was used for the determination of the ethanol incorporated in the bilayer. Ethanol was determined by measurement of NADH increase after enzymatic dehydrogenation with alcohol dehydrogenase (Boehringer-Mannheim Test Combination). The enzymatic

assay on P_2 membranes was performed on the supernatant obtained after treatment with 0.33 M perchloric acid and centrifugation. The standard curve was performed by using membrane preparations containing known amounts of ethanol.

Calorimetric Measurements. DSC measurements were performed by using a Microcal (Amherst, MS) MC 2D calorimeter. The calorimetric data were automatically recorded and processed by an interfaced IBM PC computer and digitized by a Data Translation DT 805 A/D converter. The measurements were performed as previously reported (Omodeo-Salè et al., 1989).

Na^+/K^+ ATPase Assay. Na^+/K^+ ATPase activity was measured spectrophotometrically according to Wallick et al. (1974). The reaction mixture consisted of 50 mM sucrose, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2 mM PEP, and 0.2 mM EDTA in 30 mM Hepes–NaOH buffer, pH 7.4. After addition of 50–70 μg (as protein) of P_2 fraction to 1.9 mL of the mixture, the solution was incubated for 1 min at 37 °C and added to 45 milliunits of PK/LDH. Half of the sample was pipetted in a tube containing 6 μL of 5 mM ouabain (3×10^{-5} M final). The reaction was started by the addition of 50 μL of 6.6 mM NADH and recorded for about 5 min at 340 nm. Na^+/K^+ ATPase activity was calculated by the difference between total ATPase and ATPase measured after the addition of ouabain.

5'-Nucleotidase Assay. The activity was assayed spectrophotometrically according to McIntosh and Plummer (1976); the reaction was coupled to the deamination of the adenosine formed in the presence of adenosine deaminase. A mixture of 1.2 mL of Tris-HCl buffer, pH 7.4 (43 mM), $MgCl_2$ (0.3 M), and sodium β-glycerophosphate (0.2 M) containing 80–100 μg of membranes (as protein) was preincubated at 37 °C for 20 min. Adenosine deaminase (25 μL of 1 unit/mL) was added to the mixture and the reaction started with 50 μL of 2 mM 5'-AMP. The decrease in extinction at 265 nm was followed for 3–5 min.

Na^+/K^+ ATPase and 5'-nucleotidase were assayed in P_2 membranes, in P_2 membranes injected with ethanol, and in P_2 membranes enriched with PEt.

Statistical Analysis. Statistical analysis was carried out by using the Student's *t* test. All the results are expressed as mean plus or minus standard deviation.

Results

Fluorescence Spectroscopy and Differential Scanning Calorimetry of a Lipid Dispersion. Ethanol incorporated during the fluorescence experiments in either DMPC or total lipid extract vesicles and in P_2 membranes was respectively 29, 22, and 40 mM. DMPE vesicles incubated with ethanol under the same conditions incorporated 23 mM ethanol. These values were not modified by the presence in the bilayer of PEt at the tested concentrations (up to 15 mol %).

The effect of PEt on fluorescence polarization of DPH in DMPC vesicles was at first evaluated. Moreover, the effect of ethanol addition to the same experimental system was also investigated. Fluorescence anisotropy (r) was lower ($p < 0.01$) for aqueous dispersions of DMPC containing 2 mol % DMPEt ($r = 0.147 \pm 0.008$) or 10 mol % DMPEt ($r = 0.137 \pm 0.006$) than for pure DMPC ($r = 0.155 \pm 0.010$) (Figure 1A). This result indicates that the presence of PEt increases the fluidity of the bilayer, in agreement with our previous calorimetric data (Omodeo-Salè et al., 1989). The change of r values following ethanol addition was higher in pure DMPC than in DMPEt/DMPC vesicles. As shown in Figure 1A, the addition of 400 mM ethanol caused a 10% decrease of the fluorescence polarization values in DMPC ($p < 0.01$) and a 4% decrease

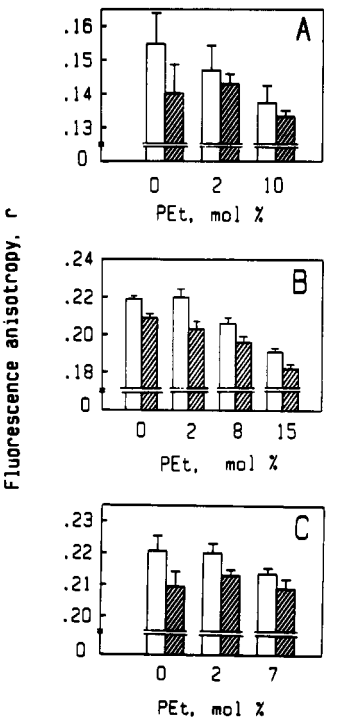

FIGURE 1: Fluorescence anisotropy values of DPH before (open bars) and after (filled bars) the addition of 0.4 M ethanol. (A) DMPC and DMPEt/DMPC (2 and 10 mol %). Assay performed at 28 °C. $p < 0.01$, DMPC versus DMPC + ethanol, DMPC versus DMPEt/DMPC 10 mol %; $p < 0.05$, DMPC versus DMPEt/DMPC 2 mol %, DMPEt/DMPC 2 mol % versus DMPEt/DMPC 2 mol % + ethanol, DMPEt/DMPC 10 mol % versus DMPEt/DMPC 10 mol % + ethanol, DMPC + ethanol versus DMPEt/DMPC 10 mol % + ethanol. (B) Aqueous dispersions of total lipids extracted from P_2 membranes and containing PEt (2%, 8%, or 15% of the total phospholipids). Assay temperature 35 °C. $p < 0.001$, 0% PEt versus 8% PEt and 15% PEt, 0% PEt + ethanol versus 8% PEt + ethanol and 15% PEt + ethanol, 0% PEt versus 0% PEt + ethanol, 2% PEt versus 2% PEt + ethanol, 8% PEt versus 8% PEt + ethanol, 15% PEt versus 15% PEt + ethanol; $p < 0.05$, 0% PEt + ethanol versus 2% PEt + ethanol. (C) P_2C membranes (0% PEt) and P_2 PEt membranes (2% or 7% of the total phospholipids). Assay performed at 35 °C. $p < 0.001$, 0% PEt versus 7% PEt, 0% PEt versus 0% PEt + ethanol, 2% PEt versus 2% PEt + ethanol; $p < 0.01$, 7% PEt versus 7% PEt + ethanol; $p < 0.05$, 0% PEt + ethanol versus 7% PEt + ethanol. Values are the mean ± SD of 20 samples.

in DMPEt/DMPC vesicles ($p < 0.05$).

Aqueous dispersions of lipids extracted from P_2 membranes showed a mean fluorescence polarization value of 0.219 ± 0.003. This value was not affected by 2% PEt ($r = 0.220 \pm 0.004$) but was lowered by higher PEt amounts (8% PEt, $r = 0.206 \pm 0.003$; 15% PEt, $r = 0.191 \pm 0.002$, $p < 0.001$) (Figure 1B).

However, in contrast with the results observed with DMPC vesicles, the presence of PEt did not decrease the sensitivity of the bilayer to the disordering effects of ethanol.

On DMPE vesicles, 10 mol % DMPEt decreased the r values from 0.246 ± 0.005 to 0.231 ± 0.004, while 2 mol % DMPEt was ineffective. No tolerance to the effects of 400 mM ethanol was observed either with 2% or 10% DMPEt. According to previous findings (Omodeo-Salè et al., 1989), the T_m of DMPEt was 21.6 °C. The responsiveness of dispersions of pure phospholipids containing or not 10 mol % DMPEt to the addition of 750 mM ethanol was investigated by DSC (Figure 2). The addition of ethanol to pure DMPE (Figure 2A) decreased the gel to liquid-crystalline temperature transition (T_m) of the phospholipid by 1.8 °C (from 50.0 to 48.2 °C) and the cooperative unit size from 94 to 88 molecules. The addition of the same amount of alcohol to DMPEt/

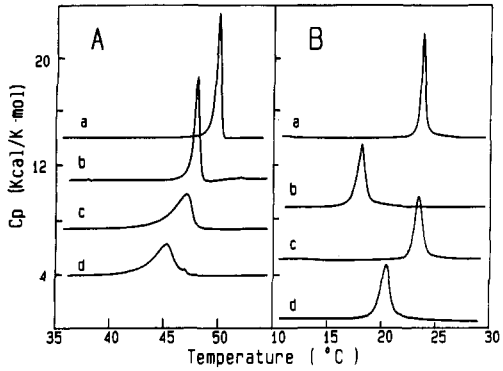

FIGURE 2: Heat capacity versus temperature for phospholipid dispersions in the absence (a, c) or in the presence of 750 mM ethanol (b, d). (A) DMPE (a, b) and DMPEt/DMPE (10 mol %) (c, d). (B) DMPC (a, b) and DMPEt/DMPC (10 mol %) (c, d).

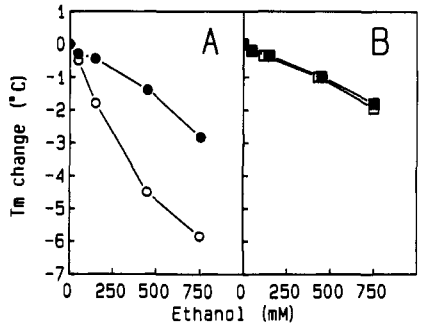

FIGURE 3: Change of the gel to liquid-crystalline temperature transition (T_m) of phospholipid dispersions in the presence of increasing ethanol concentrations. (A) DMPC (open circle); DMPEt/DMPC (10 mol %) (filled circle). (B) DMPE (open square); DMPEt/DMPE (10 mol %) (filled square).

DMPE (10 mol %) mixtures caused the same decrease of T_m (1.8 °C, from 47.0 to 45.2 °C), but there was no change in the cooperative unit size (24 molecules). The ΔH of the transition (5.7 kcal/mol) remained constant within the experimental error. When 750 mM ethanol was added to vesicles of pure DMPC, the temperature, centered at 23.8 °C, decreased by 5.8 °C to a value of 18.0 °C, while the cooperative unit size decreased from 232 to 115 molecules. When 10% DMPEt was present in the DMPC bilayer, the addition of an identical amount of ethanol caused a decrease in the T_m of only 2.8 °C (from 23.3 to 20.5 °C) and no change in the cooperative unit size (105 molecules). The ΔH of the transition (5.4 kcal/mol) remained constant within the experimental error.

In Figure 3 is reported the different responsiveness of DMPE and DMPC lipid dispersions containing or not 10 mol % DMPEt to increasing amounts of ethanol. The calorimetric data show that (a) DMPE is less sensitive than DMPC to the addition of identical amounts of ethanol and (b) the presence of DMPEt significantly decreases the effects exerted by ethanol on DMPC, but not on DMPE.

Lipid Composition of Rat Brain Crude Mitochondrial Fraction (P_2 Fraction). P_2 membranes contained 880 nmol of phospholipid/mg of protein and 329 nmol of total cholesterol/mg of protein and were characterized by high contents of PC and PE (41.3% and 46% of total phospholipids, respectively); PS and PI represented 8.5% and SM 4.4% of total phospholipids.

Association of PEt to Membranes. Studies were performed to determine the optimal experimental conditions for the insertion of PEt (synthesized from bovine brain PC) in the P_2 fraction. After incubation of the P_2 fraction with the ethanolic

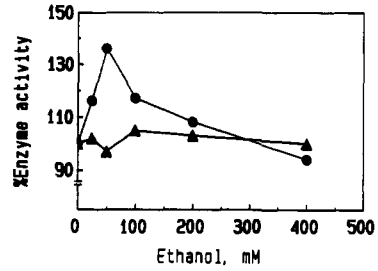

FIGURE 4: Effects of increasing amounts of ethanol on the Na^+/K^+ ATPase (●) and 5′-nucleotidase (▲) activity of P_2 membranes. Membranes were preincubated with ethanol at 37 °C for 20 min in stoppered tubes before the enzymatic assay.

solution of PEt and washing of the membranes, the final membrane suspension (10 mg of protein/mL) contained 30 mM ethanol. The same amount of alcohol was inserted in the control membranes injected with pure ethanol. The extent of association of PEt was proportional to the phosphatidylethanol concentration used in the incubation medium and did not show saturation up to 10 mM PEt in ethanol. Washing of the membranes resulted in almost complete maintenance of bound PEt. With 2 mM PEt in ethanol, the amount of inserted phospholipid approximated 1.5–2.0% of the total phospholipids.

Fluorescence Spectrometry of Rat Brain Crude Mitochondrial Fraction (P_2 Fraction). In view of the effects exerted by PEt in artificial membranes we investigated the PEt influence on the fluidity of natural bilayers (P_2 membranes) either in the absence or in the presence of ethanol. In Figure 1C we show the anisotropic values of DPH inserted into P_2 membranes before and after the addition of 400 mM ethanol. The mean fluorescence polarization value was 0.223 ± 0.005 and decreased to 0.211 ± 0.005 in the presence of ethanol. A fluidizing effect of PEt on membranes is present only at the higher concentration (7% of the total phospholipids, $r = 0.215 \pm 0.002$). Tolerance to the fluidizing effect exerted by ethanol ($r = 0.210 \pm 0.003$) is also marked at this concentration of PEt.

Enzymatic Studies. Since the amount of PEt found in organs of rats exposed to ethanol (Alling et al., 1984) sums up to 1–2% of the total phospholipids, we decided to investigate the enzymatic activities of membranes enriched with this amount of PEt.

Preliminary assays of Na^+/K^+ ATPase and 5′-nucleotidase were performed by adding increasing ethanol amounts directly to the assay mixture. Na^+/K^+ ATPase activity was enhanced by ethanol concentrations ranging from 5 to 50 mM and progressively inhibited at higher concentrations, while 5′-nucleotidase was not affected up to 400 mM ethanol (Figure 4). Na^+/K^+ activity of the rat brain P_2 fraction was 0.477 ± 0.02 (SD) IU/mg of protein (data not shown). In membrane injected only with ethanol the specific activity increased to 0.681 IU, corresponding to 43% activation. However, in membrane treated with the ethanolic solution of PEt (leading to 2% PEt incorporation) the specific activity increased to 0.572 IU, corresponding to an activation of only 20%. The Lineweaver–Burk double-reciprocal plot (Lineweaver & Burk, 1934) showed that the enzyme of untreated membranes and membranes injected with ethanol or with PEt in ethanol had the same apparent K_m (0.42 mM). In untreated P_2 membranes the K_m and V_{max} for 5′-nucleotidase were 59.3 and 13.1 ± 0.2 nmol (mg of protein)$^{-1}$ min^{-1}, respectively. The two values did not vary in the ethanol-injected membranes, while the PEt-enriched membranes showed the same K_m value but a 13% increase of the apparent V_{max} value (15.1 ± 0.3).

The enzymatic determinations in membranes enriched with

higher PEt concentrations (5% and 10% of the total phospholipids) confirmed the effects on the V_{max} of the two enzymes even with a large variability of the experimental data.

Discussion

To our knowledge no data are reported in the literature concerning the biological effects of PEt synthesis in cell membranes consequent to ethanol intoxication. In the present investigation we show that PEt induces an increase of fluidity of artificial and natural bilayers and that it is able to confer membrane tolerance to the fluidizing effects of ethanol.

The increase of fluidity caused by PEt confirms data previously obtained by differential scanning calorimetry (Omodeo-Salè et al., 1989). The r value of P_2 membranes is markedly decreased by the presence of 7 mol % PEt, but physiological PEt concentrations (1–2% of total phospholipids) seem to exert little effect on membrane fluidity or on the tolerance to ethanol. However, it is worthwhile to note that our P_2 fraction is a crude preparation containing more than one type of membrane and that the fluorescence data represent the average behavior of the fluorophore. We cannot exclude that in some membrane microenvironments these effects might be amplified by a local increase of PEt concentration. Moreover, it should be pointed that no effect was exerted by 2% PEt in DMPE vesicles and in vesicles reconstituted from the total lipid extract, while significant effects were observed in vesicles of pure DMPC. The PEt-induced fluidization does not seem dependent on membrane asymmetry of phospholipids or on the presence of proteins of natural membranes: the same effect is in fact exerted in artificial bilayers reconstituted from the total lipid extracts that, as presumed by literature data (Curtain et al., 1988), have a symmetrical distribution of phospholipids in the two leaflets of the bilayer. On the other hand, data obtained with DMPEt/DMPC and DMPEt/DMPE vesicles, homogeneous in fatty acid composition, seem to exclude that the fluidizing effects might be due to the lipid portion of the molecule. It can be speculated that the hydrophobic head group of PEt might perturb the membrane architecture by disrupting the hydrogen or ionic bond lattice extending over the surface of the membrane (Boggs, 1987).

Membrane "tolerance" has been detected in membrane preparations isolated from rats intoxicated with ethanol for prolonged periods. Ethanol concentrations causing significant membrane disordering in control preparations have little effect on membranes prepared from ethanol-treated rats (Harris et al., 1984; Goldstein & Chin, 1981; Lyon & Goldstein, 1982). However, despite the considerable body of work of the last years, the molecular bases of the membrane tolerance phenomenon are still debated. Recently, the molecular mechanism of this acquired resistance has been attributed to minor ethanol-induced alterations in fatty acid composition of phosphatidylinositol or cardiolipin (Taraschi et al., 1986b; Ellingson et al., 1988). However, we can exclude that the tolerance herein shown in P_2 membranes might be due to a bulk-phase interaction among the lipid portions since, in our experiments, model membranes of PEt/PC, homogeneous in fatty acid composition (dimyristoyl form), still retain the same property. The tolerance to the fluidizing effect of ethanol displayed by DMPEt/DMPC vesicles and not by DMPEt/DMPE vesicles suggests that the interaction between PEt and the polar head groups of PC or PE might be different. For instance PEt could form with PC or PE ionic interactions of different strength, due to the different pKs of their positive head groups (Davenport, 1971). However, other possible hypotheses such as the different extent of hydrogen bonding in PC and PE bilayers or hydrophobic interactions between the ethyl head group of PEt and the methyl groups of PC must be taken into account.

An involvement of PC in the phenomenon of tolerance could explain the different resistance to ethanol displayed by P_2 membranes and reconstituted bilayers. In reconstituted bilayers, because of the loss of phospholipid asymmetry, PC and PE are "diluted" among the other lipids of the bilayer and uniformly distributed in the two leaflets of the membrane. These compositional features reduce the possibility of interaction between PEt and PC head groups and could explain the loss of resistance to the disordering effect of ethanol of these dispersions.

Previous works showed that chronic ethanol ingestion, leading to membrane tolerance, is accompanied by a decreased partitioning of ethanol into membranes (Rottenberg et al., 1981). However, from our data concerning the alcohol determination in synthetic vesicles and in natural membranes, we can exclude that the presence of PEt intercalating among the other phospholipids in the bilayer could influence the ethanol partitioning in the membrane.

Ethanol affects a wide variety of membrane-bound enzyme activities and many of its effects may be associated with its action on the membrane bilayer. However, it cannot be excluded that, in some cases, ethanol-induced perturbations could be due to the synthesis of PEt, which would represent a key to understanding some of the biological effects of alcohol. Therefore, the possibility of varying the lipid composition of the brain P_2 fraction intercalating PEt in the bilayer has been exploited to investigate the effects of this unusual phospholipid. It is generally agreed that the phospholipid composition of membranes is important for their biological activity. Properties such as K_m and V_{max} (Demel et al., 1972; Zachim & Vessey, 1976) or the degree of cooperativity (Esfahani et al., 1977) of some enzymes are sensitive to their lipid environment. Rat brain Na^+/K^+ ATPase and 5′-nucleotidase, in spite of both being intrinsic membrane proteins, have a different localization in the bilayer (Salem et al., 1981): Na^+/K^+ ATPase, spanning the width of the bilayer, is located in a hydrophobic domain and its sensitivity to the lipid environment as well as to changes in lipid viscosity has been well characterized (Kimelberg, 1977; Sinensky et al., 1979), while 5′-nucleotidase seems located in a more polar microenvironment. Our results show that the activities of Na^+/K^+ ATPase and 5′-nucleotidase are differently affected by ethanol. In agreement with data reported by Sun and Seaman (1980), Na^+/K^+ ATPase exhibits a biphasic response to alcohol; at low alcohol concentration the increase in activity is presumably due to the major capability of the enzyme to undergo conformational changes necessary to its catalytic cycle. It is of particular interest that we found increases in the activity at ethanol concentrations (50–100 mM) that are present after chronic ethanol treatment (Ritzmann & Tabakoff, 1976). As shown also by other authors (Akera et al., 1973; Sun & Seaman, 1980), ethanol concentrations above 200 mM, usually lethal, are inhibitory.

Resistance to ethanol of rat brain 5′-nucleotidase suggests that alcohol does not affect the enzymatic protein either directly or through the increase of fluidity of the bilayer. A similar resistance of 5′-nucleotidase to ethanol was observed by Rao et al. (1985) both in acute and in short-term ethanol toxicity.

The incorporation of small amounts of PEt (2% of the total phospholipids) results in significant changes in the activity of both enzymes. The presence of PEt reduces the Na^+/K^+ ATPase activation induced by ethanol with a decrease in the apparent V_{max}. The origin of this phenomenon could be related either to the resistance to ethanol-induced fluidization acquired

by the membranes enriched with PEt or to a more specific influence of PEt on the catalytic site of the enzyme or on some lipid–lipid or lipid–protein interactions important for its activity. The PEt-induced increase in 5′-nucleotidase activity could be determined by a direct interaction of the apolar head group of PEt with the catalytic portion of the enzyme at the membrane surface where it is located (Franco et al., 1986).

In conclusion, our findings show that PEt may have important functional implications with regard to intoxication by and tolerance to ethanol. On the one hand, low, but critical, amounts of exogenously incorporated PEt can markedly modify the physicochemical properties of the bilayer, increasing the fluidity as well as the tolerance to the addition of ethanol in model and natural membranes; on the other hand, depending upon the localization of the protein in the bilayer, the influence exerted by PEt on the enzymatic activity could be related to its effects on the physicochemical properties of the membrane or on the protein itself. Moreover, the extent to which an enzyme is or is not affected by PEt and the selectivity of PEt influence would be properties peculiar to the protein and to its relationship in the bilayer with the annular phospholipids.

Registry No. DMPC, 18194-24-6; DMPEt, 998-07-2; DMPE, 20255-95-2; ATPase, 9000-83-3; 5′-nucleotidase, 9027-73-0; ethanol, 64-17-5.

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