Rapid Transbilayer Movement of Phosphatidylethanol in Unilamellar Phosphatidylcholine Vesicles†,‡,§

Alexander V. Victorov, Nathan Janes,* Gisela Moehren, Emanuel Rubin, Theodore F. Taraschi, and Jan B. Hoek

Contribution from the Department of Pathology and Cell Biology, Medical College of Thomas Jefferson University, 1020 Locust Street, Philadelphia, Pennsylvania 19107

Received November 17, 1993®

Abstract: Phosphatidylethanol (PtdEth) is a rare anionic phospholipid formed as a metabolite of ethanol through a transphosphatidylation reaction which is catalyzed by phospholipase D (PLD). The transbilayer distribution of dilute (0.5–2%) levels of PtdEth in small and large unilamellar phosphatidylcholine vesicles (SUVs and LUVs, respectively) was monitored by ^{13}C nuclear magnetic resonance spectroscopy of PtdEth specifically ^{13}C-labeled at its headgroup methylene. Discrimination of interleaflet sites was based on shifts in the resonance frequencies of the headgroup methylene induced by differential packing in SUVs and induced by the shift reagent Pr^{3+} in LUVs. PtdEth is shown to undergo a rapid and reversible transbilayer redistribution ($t_{0.5} \leq 1$ h at 26 °C) in response to mM concentrations of added external multivalent cations (Ca^{2+}, Pr^{3+}, Mn^{2+}, and Yb^{3+}). The pH of the vesicle suspension vastly exceeds the pK of PtdEth (ca. 1.43), based on the pH dependence of the labeled site's chemical shift in LUVs. The rate of transbilayer transfer greatly exceeds, by 1–3 orders of magnitude, the transfer rates reported for other naturally occurring phospholipids near physiological pH.

Phosphatidylethanol (PtdEth) is a rare anionic phospholipid formed in a wide variety of tissues after exposure to ethanol.[1] Ethanol replaces water as a substrate for transphosphatidylation catalyzed by phospholipase D (PLD).[2] PtdEth possesses a small hydrophobic ethyl headgroup that might confer unusual properties to this phospholipid and perhaps play a role in ethanol-induced tissue injury. In this article, the transbilayer distribution of dilute (0.5–2%) levels of PtdEth in small and large unilamellar vesicles (SUVs and LUVs, respectively) of phosphatidylcholine (PtdCho) is monitored by high-resolution ^{13}C nuclear magnetic resonance spectroscopy (NMR) of PtdEth specifically ^{13}C-labeled at the headgroup methylene ($-PO^{13}CH_2CH_3$). Discrimination of the interleaflet location of PtdEth is based on chemical shift differences of the headgroup methylene induced by the differential packing constraints between leaflets in SUVs and chemical shift differences induced by the shift reagent Pr^{3+} in LUVs.

Results

Shown in Figure 1A is the headgroup region of a 90.6 MHz ^{13}C NMR spectrum of SUVs, containing 0.5 mol fraction percent (mf%) of dioleoylphosphatidylethanol (DOPtdEth; a species found in lung surfactant[3]) within a homologous lecithin matrix (dioleoylphosphatidylcholine; DOPtdCho). The PtdEth resonance is split into two well-resolved signals of approximately equal peak intensity but slightly different line widths. The downfield resonance is further deshielded upon addition of the shift reagent Pr^{3+} to the external medium (not shown); consequently, this peak is assigned to the outer leaflet. Similarly, using the Pr^{3+}-induced shifts of the outer PtdCho *N*-methyl and choline-OCH_2 resonances, the outer leaflet was noted to contain 1.9 times more PtdCho than the inner leaflet, as expected for these highly curved structures;[4] consequently, the PtdEth concentration in the inner leaflet on a mol fraction basis was approximately 2-fold higher than in the outer leaflet. A preference for the inner leaflet at this low concentration is consistent with the small headgroup size.[5]

An outward transbilayer redistribution of PtdEth was initiated by the addition of 4 mM Ca^{2+} to the external medium. The intensity of the downfield PtdEth resonance from the outer leaflet increased gradually ($t_{0.5} \leq 1$ h at 26 °C) at the expense of the upfield resonance from the inner leaflet (Figure 1A–D), reaching a steady state after several hours with a reduced preference for the inner leaflet. Approximately 15% of the PtdEth that resided in the inner monolayer was transferred to the outer monolayer (net transfer 12–18%, $n = 3$). Subsequent addition of the calcium chelator EGTA (Figure 1E,F) rapidly returned the PtdEth distribution to the initial state. Spin–lattice relaxation times and nuclear Overhauser enhancements (NOEs) of the labeled resonances were nearly identical throughout (ca. 600 ms, $\eta = 1.0$, respectively) indicating that the ratio of peak areas quantitatively reflects the interleaflet distribution.

In order to preclude the possibility that the transbilayer migration was unique to highly curved small vesicular structures, similar experiments, shown in Figure 2, were performed in LUVs in which packing constraints and lipid populations are similar in both leaflets.[6] Many of the PtdCho resonances were markedly broadened, as expected in these larger vesicles. A single and surprisingly narrow resonance was observed for the labeled PtdEth resonance, suggesting facile motion (Figure 2A). Addition of 2 mM Pr^{3+} to the external medium shifted the PtdEth outer leaflet signal downfield, leaving the signal from the inner leaflet unshifted, and split the choline *N*-methyl into two equal components (Figure

† This work was supported by U.S. Public Health Service Grants AA07186, AA00088, AA07215, AA07463, and AA08714.

‡ Keywords: phosphatidylethanol, flip-flop, transbilayer movement, ^{13}C nuclear magnetic resonance, liposomes, calcium, shift reagents, ethanol, phospholipid pK.

§ Abbreviations: DOPtdCho, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPtdEth, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanol; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; EGTA, ethlenebis-(oxyethylenenitrilo)tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

® Abstract published in *Advance ACS Abstracts*, April 1, 1994.

(1) (a) Alling, C.; Gustavsson, L.; Mansson, J.-E.; Benthin, G.; Anggard, E. *Biochim. Biophys. Acta* **1984**, *793*, 119–122. (b) Gatalica, Z.; Moehren, G.; Hoek, J. B. *Biochim. Biophys. Acta* **1993**, *1177*, 87–92.

(2) Heller, M. *Adv. Lipid Res.* **1978**, *16*, 267–326.

(3) Gatalica, Z.; Hoek, J. B. manuscript in preparation.

(4) (a) Bystrov, V. F.; Shapiro, Y. E.; Victorov, A. V.; Barsukov, L. I.; Bergelson, L. D. *FEBS Lett.* **1972**, *25*, 337–338. (b) Shapiro, Y. E.; Victorov, A. V.; Volkova, V. I.; Barsukov, L. I.; Bystrov, V. F.; Bergelson, L. D. *Chem. Phys. Lipids* **1975**, *14*, 227–232. (c) Barsukov, L. I.; Victorov, A. V.; Vasilenko, I. A.; Evstigneeva, R. P.; Bergelson, L. D. *Biochim. Biophys. Acta* **1980**, *598*, 153–168. (d) Lepore, L. S.; Ellena, J. F.; Cafiso, D. S. *Biophys. J.* **1992**, *61*, 767–775.

(5) Lee, Y.-C.; Taraschi, T. F.; Janes, N. *Biophys. J.* **1993**, *65*, 1429–1432.

(6) MacDonald, R. C.; MacDonald, R. I.; Menco, B. Ph. M.; Takeshita, K.; Subbarao, N. K.; Hu, L-r. *Biochim. Biophys. Acta* **1991**, *1061*, 297–303.

0002-7863/94/1516-4050$04.50/0

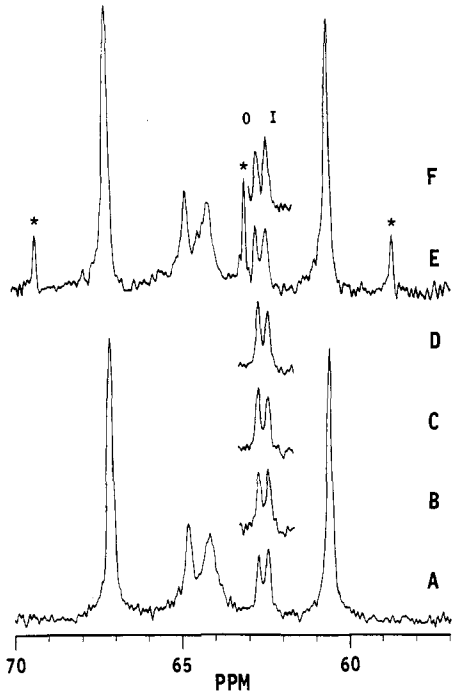

Figure 1. Effects of Ca^{2+} and EGTA on PtdEth distribution in SUVs. The headgroup region of 90.6 MHz ^{13}C NMR proton decoupled spectra of a DOPtdCho containing 0.5% $-O^{13}CH_2CH_3$ labeled DOPtdEth before (A) and after incubation with 4 mM $CaCl_2$ for 15 min (B), 45 min (C), 90 min (D); after the addition of 5 mM EGTA to sample (D) and incubation for 15 (E), and 90 min (F). The times shown represent the average of a 30-min scan. Outer "o" and inner "i" leaflet PtdEth signals are marked. Asterisk indicates resonances from EGTA.

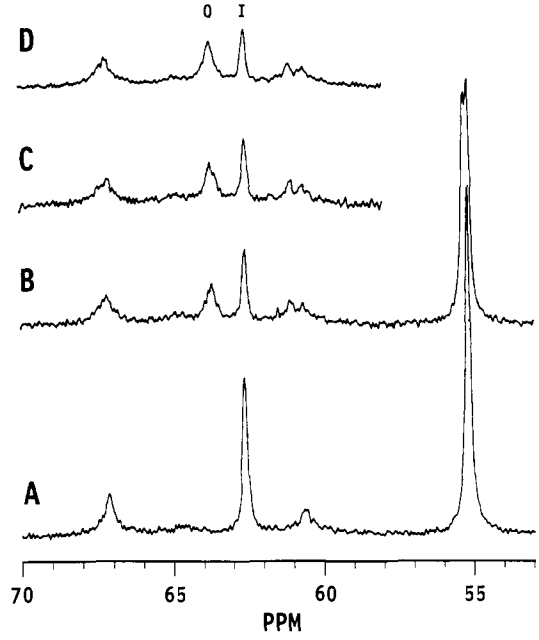

Figure 2. Effect of Pr^{3+} on PtdEth distribution in LUVs. ^{13}C NMR spectra of 100 nm LUV of dioleoylphosphatidylcholine–2% $^{13}CH_2$-ethyl labeled dioleoylphosphatidylethanol: (A) no shift reagent; (B) 2 mM $Pr(NO_3)_3$ added to the external medium, 30-min incubation; (C) 90-min incubation; and (D) 5.5-h incubation. Spectra A–C represent 1700 scans; D, 3000 scans. The signal from the acyl chain terminal methyl was used as an internal concentration standard, and "i" and "o" denote the inner and outer PtdEth resonances.

2B). As was observed for the addition of Ca^{2+} in SUVs (and Pr^{3+}, not shown), the intensity of the outer leaflet PtdEth resonance gradually increased, whereas the intensity of the inner signal diminished by approximately 30% (27–35%, $n = 3$; Figure 2C,D)

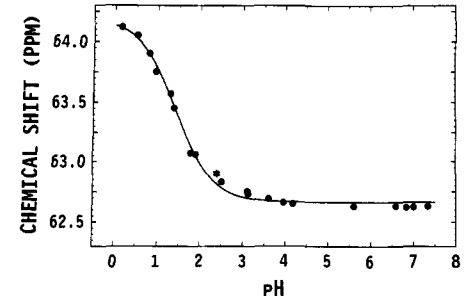

Figure 3. The dependence of the PtdEth $-O^{13}CH_2CH_3$ chemical shift as a function of pH in LUVs along with best least-squares fit. The acetate buffer equalizes the pH throughout the compartments.

relative to the chain methyl, reaching a new steady-state distribution after 5–7 h. Mn^{2+} (4–10 mM) and Yb^{3+} (2 mM) also promoted the transmembrane migration of PtdEth. Thus, a variety of multivalent cations are able to initiate facile transbilayer migration ($t_{0.5} \leq 1$ h at 26 °C) of PtdEth in bilayers of high and low curvature.

Previous reports of transbilayer movement of phospholipids indicate that the process is exceedingly slow.[7] Reported halftimes range from hours to days, compared to the tens of minutes observed here for PtdEth. There are two exceptions to this generalization. Rapid transbilayer motion is reported in saturated lecithin vesicles at the main melting phase transition temperature.[8] The vesicles examined here are in the fluid state at temperatures far above this transition. Rapid transbilayer movement is also reported at low pH for the protonated (neutral) forms of phosphatidic acid and phosphatidylglycerol (i.e., $t_{0.5} \simeq 25$ s is calculated for the protonated form of phosphatidic acid, but $t_{0.5} \simeq 12.6$ days at pH 6).[9]

The pH-dependent chemical shift of the labeled PtdEth headgroup methylene provides a novel means for the phosphate pK determination. The pH titration shown in Figure 3, with least-squares Henderson–Hasselbalch fit, indicates a phosphate pK of 1.43. This pK is similar to the phosphate pK of 1.22 reported for the model phosphodiester, dimethyl hydrogen phosphate,[7] and the intrinsic phosphate pK of 1.75[10] and 1.9[11] reported for liposomes of anionic phosphatidylmethanol, deduced from the pH dependence of the main phase transition after correction for electrostatic factors. Therefore, the rapid transbilayer migration observed for PtdEth near neutral pH is an unexpected and unprecedented finding that may arise from the hydrophobicity of the ethyl headgroup.

Discussion

The results are consistent with a steady-state transbilayer flux of anionic PtdEth, whose interleaflet distribution is sensitive to the status of the leaflets. A selective alteration in the energetic state of PtdEth in one of the leaflets, e.g., by the addition of multivalent ions to the outer leaflet, perturbs the steady state and induces net transport. Complexation of those cations with EGTA restores the original state, indicating that the cations are not a necessary prerequisite for a net transbilayer transfer. A limiting constraint on (or potential driving force for) the magnitude of net transfer may be the transmembrane potential generated by transbilayer transfer of the anion.

Multivalent cations may perturb the flux by sequestering PtdEth in the apposed leaflet and thereby induce a net transbilayer

(7) Tabulated in Marsh, D. *Handbook of Lipid Bilayers*; CRC Press: Boca Raton, FL, 1990.
(8) de Kruijff, B.; van Zoelen, E. J. J. *Biochim. Biophys. Acta* **1978**, *511*, 105–115.
(9) (a) Eastman, S. J.; Hope, M. J.; Cullis, P. R. *Biochemistry* **1991**, *30*, 1740–1745. (b) Redelmeier, T. E.; Hope, M. J.; Cullis, P. R. *Biochemistry* **1990**, *29*, 3046–3053.
(10) Trauble, H.; Teubner, M.; Woolley, P.; Eibl, H. *Biophys. Chem.* **1976**, *4*, 319–342.
(11) Copeland, B. R.; Andersen, H. C. *Biochemistry* **1982**, *21*, 2811–2820.

migration. Our data indicate that there is no significant inward transfer of the PtdEth–cation complex. In both SUVs and LUVs, the shifts of the PtdCho resonances remain stable for more than 24 h, indicating that no Pr^{3+} is transferred to the interior. The Pr^{3+}-induced shift of the PtdEth headgroup methylene resonance significantly exceeds that of the analogous choline methylene in both SUVs and LUVs (e.g., Figure 2), suggesting stronger Pr^{3+} binding to PtdEth than PtdCho. In most other anionic lipids the binding of multivalent cations induces a lateral phase separation or clustering effect; however, no phase separation is observed for PtdEth.[12] Changes in the membrane surface potential induced by the multivalent cations should also favor the outward migration observed.

Other properties of PtdEth are also distinct from more polar phospholipids. PtdEth is the most potent phospholipid promoter of membrane curvature studied to date.[5] The labeled methylene resonance in LUVs is much narrower than the analogous choline resonance (Figure 2), and the ^{31}P powder pattern in multilamellar vesicles is substantially reduced from other phospholipids,[13] suggesting facile motion or an unusual conformation. The headgroup conformation is labile and quite sensitive to steric constraints at the surface.[13] These dynamic features may facilitate a reduction in the energy barrier of transport.

If the rapid transbilayer redistribution observed for PtdEth in these lecithin vesicles as a response to calcium also occurs in plasma membranes *in vivo*, then transbilayer migration, driven by the transplasmalemmal Ca^{2+} gradient, may provide a route for the external expression of PtdEth synthesized in the inner monolayer of the plasma membrane and thereby expose an exotic phospholipid to the circulation.

Experimental Methods

Synthesis of PtdEth. One hundred milligrams of DOPtdCho (Avanti Polar Lipids) in $CHCl_3$ was dried to a film under N_2. Peanut phospholipase D (5 mg or 1300 units, EC 3.1.4.4; Sigma), 0.1 mL of buffer (0.2 M sodium acetate and 0.1 M calcium chloride at pH 5.6), 20 μL of 1-^{13}C ethanol (Cambridge Isotope Laboratories), and 0.5 mL of diethyl ether (water-washed to remove traces of unlabeled ethanol) were added to the lipid film in a 50-mL flask, which was sealed and incubated for 15 h with shaking at ambient temperature. The reaction was stopped with 0.2 mL of 0.6 M EDTA-Na, pH 7.6, followed by 0.8 mL of water. The lipids (PtdCho, PtdEth, and phosphatidic acid) were extracted by addition of 4 mL of chloroform/methanol 1:2 and vortexing for 20 min at 4 °C. Subsequently, 0.8 mL of water, 0.2 mL of the EDTA solution, and 1 mL of chloroform was added and the organic phase collected, dried, and purified on a 250 × 25 mm Merck Hibar preparative high pressure liquid chromatography column packed with 7 μm LiChrosorb-Si-60 silica particles (EM Science, Cherry Hill, NJ) using the gradient procedure described previously.[14] The yield was 30–40%. The purity was greater than 99% by HPTLC.

Vesicle Preparation. SUVs were prepared by ultrasonic irradiation of a handshaken lipid dispersion (1.65 mL in 16 × 100 mm glass tube) under ice to constant optical clarity (450 nm), using a MedSonic W-225 sonicator operating at 20 kHz and a power setting of 3 with a 3.2 mm microtip, typically 5–6 five min cycles (3 min on /2 min pause). Metal particles and any remaining multilamellar vesicles were removed by high speed centrifugation. Lyso species were not detected by high-performance thin layer chromatography (<1%) under these conditions.

LUVs were prepared by extrusion using 29 passes through two stacked 100 nm polycarbonate filters using a LiposoFast extrusion apparatus (Avestin) and the procedure described elsewhere.[6] The stability of both vesicle preparations, based on the inside/outside ratios and spectral features exceeded 2 days.

Nuclear Magnetic Resonance. ^{13}C NMR noise decoupled spectra (90.6 MHz) of DOPtdCho containing 0.5 or 2% $-O^{13}CH_2CH_3$ labeled DOPtdEth (40 mg/mL, 10 mM Na acetate buffer with 25% D_2O, pH 6.6 without ^2H isotope correction, 295 K) were obtained on a Bruker AM 8.5T WB spectrometer in a 10-mm double resonance probe without sample rotation under the following typical conditions: 45–55° flip angle, 20.1 kHz broadband decoupling power, 3.6 kHz broadband irradiation between acquisitions for NOE generation, 1.2 s between acquisitions, 20 kHz spectral window, 8 K data points, zero filled to 16 K, 1 Hz exponential filter. Quantitation in the presence of shift reagents was based on the area of the unshifted signal relative to the acyl methyl. Peak assignments (referenced to the acyl methyl at 15.05 ppm from TMS) are as follows: PtdCho glycerol C-1, 64.17; glycerol C-3, 64.81; O_3POCH_2, 60.62; $O_3POCH_2CH_2$, 67.15; $N(CH_3)_3$; 55.18; PtdEth CH_2CH_3, LUVs, 62.63; SUV inner, 62.41; SUV outer, 62.70.

(12) Omodeo-Sale, M. F.; Cestaro, B.; Mascherpa, A.; Monti, D.; Masserini, M. *Chem. Phys. Lipids* **1989**, *50*, 135–142.
(13) Browning, J. L. *Biochemistry* **1981**, *20*, 7123–7133.
(14) Ellingson, J. S.; Zimmerman, R. *J. Lipid Res.* **1987**, *28*, 1016–1018.