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Phospholipid Asymmetry in Large Unilamellar Vesicles Induced by Transmembrane pH Gradients†

Michael J. Hope,*,‡,§ Tom E. Redelmeier,‡ Kim F. Wong,‡ Wendi Rodrigueza,‡ and Pieter R. Cullis‡,§

Biochemistry Department, The University of British Columbia, Vancouver, BC, Canada V6T 1W5, and The Canadian Liposome Company Ltd., Suite 308, 267 West Esplanade, North Vancouver, BC, Canada V7M 1A5

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ABSTRACT: The influence of membrane pH gradients on the transbilayer distribution of some common phospholipids has been investigated. We demonstrate that the transbilayer equilibrium of the acidic phospholipids egg phosphatidylglycerol (EPG) and egg phosphatidic acid (EPA) can be manipulated by membrane proton gradients, whereas phosphatidylethanolamine, a zwitterionic phospholipid, remains equally distributed between the inner and outer monolayers of large unilamellar vesicles (LUVs). Asymmetry of EPG is examined in detail and demonstrated by employing three independent techniques: ion-exchange chromatography, ^{13}C NMR, and periodic acid oxidation of the (exterior) EPG headgroup. In the absence of a transmembrane pH gradient (ΔpH) EPG is equally distributed between the outer and inner monolayers of LUVs. When vesicles composed of either egg phosphatidylcholine (EPC) or DOPC together with 5 mol % EPG are prepared with a transmembrane ΔpH (inside basic, outside acidic), EPG equilibrates across the bilayer until 80–90% of the EPG is located in the inner monolayer. Reversing the pH gradient (inside acidic, outside basic) results in the opposite asymmetry. The rate at which EPG equilibrates across the membrane is temperature dependent. These observations are consistent with a mechanism in which the protonated (neutral) species of EPG is able to traverse the bilayer. Under these circumstances EPG would be expected to equilibrate across the bilayer in a manner that reflects the transmembrane proton gradient. A similar mechanism has been demonstrated to apply to simple lipids that exhibit weak acid or base characteristics [Hope, M. J., & Cullis, P. R. (1987) *J. Biol. Chem. 262*, 4360–4366].

Phospholipid asymmetry is now well established for many biological membranes (Op den Kamp, 1979; Zwaal, 1978; Michaelson et al., 1983; Higgins & Pigott, 1982; Herbette et al., 1984; Houslay & Stanley, 1982). The most commonly studied systems are mammalian plasma membranes in which the amino-containing phospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are observed to be predominantly located in the cytoplasmic side of the bilayer [for review see Op den Kamp (1979)].

The mechanism whereby phospholipid asymmetry is generated and maintained is not understood. However, several recent papers provide compelling evidence for the existence of a transport mechanism that exhibits specificity for PE and

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‡ The University of British Columbia.
§ The Canadian Liposome Co. Ltd.

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PS resulting in the movement of these two lipids from the outer monolayer (OM) to the inner monolayer (IM) of the erythrocyte membrane (Zachowski et al., 1986; Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit, 1988). In model membrane systems, a curvature-induced asymmetry is often observed for small vesicles. For example, sonicated vesicles (SUVs) of phosphatidylcholine (PC) containing low mole fractions of PE display an asymmetry in which PE favors the OM (Litman, 1973; Lentz & Litman, 1978; Nordlund et al., 1981). According to some papers the acidic phospholipids PS and phosphatidylglycerol (PG) also favor the OM of SUVs at low mole fraction (Lentz et al., 1982; Nordlund et al., 1981; Massari et al., 1978), whereas other investigators suggest these phospholipids are preferentially located on the IM (Kumar & Gupta, 1984; Berden et al., 1975; Barsukov et al., 1980). However, there is general agreement that curvature-induced asymmetry is only relevant to small vesicles and that large unilamellar vesicles (LUVs) with diameters >40 nm (Hope et al., 1987) exhibit an equal distribution of lipids across the membrane (Kumar & Gupta, 1984; Hope et al., 1985; Hope & Cullis, 1987; Nordlund et al., 1981).

In recent work we have investigated the effect of ion gradients on the transbilayer distribution of lipids and amino-containing drugs in LUVs. Such ion gradients can act to drive the accumulation of a variety of lipophilic cations into the vesicle interior (Bally et al., 1985; Mayer et al., 1985a,b). Recently, we described the effect of transmembrane pH gradients on the transbilayer distribution of simple lipids that are weak acids or weak bases (Hope & Cullis, 1987). It was demonstrated that fatty acids could be sequestered to the IM of LUVs if the vesicle interior was basic with respect to the external medium. Similarly, the amino lipids stearylamine and sphingosine accumulated at the IM when the vesicle interior was acidic.

In this paper we describe the effect of transmembrane pH gradients (ΔpH) on the distribution of phospholipids between the OM and IM of LUVs. We demonstrate that an asymmetric distribution of PG and phosphatidic acid (PA) can be induced by a ΔpH.

Materials and Methods

Lipids. Egg phosphatidylcholine (EPC), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), egg phosphatidylglycerol (EPG), beef heart cardiolipin, egg phosphatidic acid (EPA), and soya phosphatidylinositol (SPI) were obtained from Avanti Polar Lipids and used without further purification. [^{3}H]Dipalmitoylphosphatidylcholine ([^{3}H]DPPC) and [^{14}C]DPPC were purchased from New England Nuclear (NEN, Quebec).

Preparation of Large Unilamellar Vesicles. Large unilamellar vesicles were prepared by the technique of Hope et al. (1985) employing a 10-mL capacity Lipex extruder (Lipex Biomembranes Inc., Vancouver). All LUVs used here were extruded through polycarbonate filters (Nuclepore) with a 0.1-μm pore size, resulting in a vesicle population with a mean diameter of approximately 100 nm as determined by quasi-elastic light scattering and freeze–fracture. Lipids were mixed in chloroform to give the desired composition. Solvent was removed under a stream of nitrogen followed by exposure to high vacuum for 1 h. The lipid was hydrated at 25 °C by adding the desired volume of buffer and vortexing for approximately 2 min. The resulting liposomes were subjected to five cycles of freezing and thawing (Mayer et al., 1986) in liquid nitrogen and warm water prior to extrusion. Typically, vesicles would be prepared at lipid concentrations in the range 20–100 mg/mL, depending upon the experiment and desired specific activity.

Preparation of ^{13}C-Labeled Egg Phosphatidylcholine. [1,3-^{13}C]Glycerol was purchased from ICN Biochemicals Canada (Quebec). Phospholipase D was isolated from Savoy cabbage according to published procedures (Comfurius & Zwaal, 1977). [^{13}C]Glycerol was exchanged for the choline headgroup of EPC as follows. A reaction mixture was prepared consisting of 100 mg of [^{13}C]glycerol dissolved in 1 mL of 100 mM sodium acetate buffer (pH 5.6) containing 100 mM $CaCl_2$ plus 2 mL of washed diethyl ether containing 100 mg of EPC. Lyophilized phospholipase D (40 mg) was suspended in 1 mL of buffer and sonicated in a bath sonicator for approximately 2 min to obtain an even dispersion. The enzyme solution was added to the buffer mixture and shaken vigorously in a stoppered tube at 40 °C for 10–15 min. The reaction was stopped and the ether layer allowed to phase separate; this was then collected and the reaction mixture extracted twice with 2 mL of diethyl ether. The remaining aqueous half of the mixture was filtered through glass wool to remove denatured protein and used again with fresh lipid and fresh enzyme as described above. This reaction cycle was repeated five times, the ether fractions were pooled, and the ether was removed under vacuum. The remaining lipid was dissolved in chloroform:methanol (2:1 v/v) and washed with 0.2 volumes of 150 mM NaCl solution. The yield of ^{13}C-labeled egg phosphatidylglycerol (EPG) was approximately 20% with 20% phosphatidic acid and 60% remaining as phosphatidylcholine. The EPG was purified by CM-cellulose chromatography according to the procedure of Comfurius and Zwaal (1977).

Ion-Exchange Chromatography. LUVs were prepared in either 200 mM HEPES and 5 mM K_2SO_4, pH 8.0, or 10 mM glutamic acid and 5 mM K_2SO_4, pH 4.5. [^{3}H]- or [^{14}C]DPPC was included in all the lipid mixtures; lipid compositions are given for each experiment in the text and figure legends. Following extrusion, vesicles were passed down a 15 × 1.5 cm Sephadex G-50 (Pharmacia or Sigma) gel filtration column to exchange the external buffer for the low ionic strength glutamic acid buffer (pH 4.5) described above. Only vesicles prepared in pH 8.0 buffer exhibit a ΔpH following gel filtration. Vesicles were loaded onto 15 × 1.5 cm DEAE-Sephacel columns equilibrated with glutamic acid buffer at pH 4.5. The ion-exchange columns were run at a flow rate of 1 mL/min, and 1-mL fractions were collected. After 11 fractions had been collected, the elution buffer was stopped and the columns washed with 0.5 M NaCl. After a total of 30 fractions, elution was stopped and the fractions transferred to 10 mL of aqueous scintillant, and radioactivity was counted by using a Packard 2000CA scintillation counter.

^{13}C NMR. [^{13}C]EPG (3 mg) was added to EPC (57 mg) and dried under a stream of nitrogen followed by exposure to high vacuum for 1 h. The lipid was hydrated in 200 mM phosphate and 10 mM citrate adjusted either to pH 8.0 or to pH 4.5. The liposomes were freeze–thawed and extruded as described above, and the external buffer was exchanged for 5 mM citrate, pH 4.5, by using Sephadex G-50. The low ionic strength external citrate buffer was used because it did not affect the interaction of Mn^{2+} with the vesicles, the natural abundance ^{13}C spectrum of citrate did not superimpose on the [^{13}C]EPG spectrum, and it also buffers well at pH 4.5. By use of a Bruker WH400 spectrometer operating at 100 MHz, the T_1 of the terminal carbon of glycerol was determined to be approximately 0.6 s from standard inversion recovery techniques and proton decoupling. The measurements reported

here were obtained on a Bruker WP200 NMR spectrometer operating at 50.3 MHz with proton decoupling conditions. A 45° pulse with a 0.41-s interpulse delay was used. Each spectrum is the result of 20 000 acquisitions obtained at 20 °C and referenced to external TMS.

Oxidation of Phosphatidylglycerol Using Periodate. Vesicles with or without transmembrane pH gradients were prepared as described above. The concentration of EPG present in the OM was determined by periodate oxidation of the terminal vicinal hydroxyl groups on the glycerol headgroup as described by Lentz et al. (1980, 1982). The assay procedure was as follows: 0.8 mL of vesicles (10–20 mg/mL phospholipid) was added to a mixture containing 5.0 mL of 100 mM acetate, 100 mM citrate (pH 6.0), and 3.2 mL of H_2O. Oxidation was initiated by the addition of 1 mL of freshly prepared 100 mM sodium periodate. The reaction was quenched, at the indicated time intervals, by adding a 1-mL aliquot of the reaction mixture to a Pyrex tube containing 100 μL of 1 M sodium arsenite in 0.5 M sulfuric acid. The formaldehyde produced from the oxidation of the terminal vicinal hydroxyls was determined by the Hantzsch reaction (Nash, 1953). At least 15 min after quenching, 50 μL of 200 mL sodium cholate was added to disrupt the vesicles and 1 mL of Nash reagent (15 g of ammonium acetate, 200 μL of glacial acetic acid, and 200 μL acetyl acetone in 100 mL of H_2O) was added. Samples were capped and heated at 60 °C for 10 min, and the absorbance was read at 412 nm. Total EPG was determined by adding sufficient sodium cholate to disrupt vesicles prior to the oxidation reaction.

Results

ΔpH and the Transbilayer Distribution of Phosphatidylethanolamine. Previously we have demonstrated that simple lipids which exhibit weak base or acid characteristics can equilibrate across the bilayer of LUVs in a manner that reflects the transmembrane ΔpH (Hope & Cullis, 1987). A mechanism consistent with this observation postulates that the uncharged form of the lipid undergoes transbilayer movement at a greater rate than the charged species, resulting in an equilibrium that is dependent upon the ionization state of the lipid at each interface and thus reflects the proton gradient across the membrane. This mechanism should apply to all lipids that ionize into species which exhibit considerably different transbilayer mobilities. Our initial experiments concerned phosphatidylethanolamine, which is zwitterionic and therefore possesses a charged headgroup over the whole pH range. However, the headgroup can be negatively charged, zwitterionic, or positively charged depending upon the pH. If these ionized states were to exhibit different rates of transbilayer mobility, a ΔpH applied across the bilayer should result in an asymmetric distribution of PE between the IM and OM. LUVs produced by extrusion were employed because these vesicles have been shown to exhibit a symmetrical distribution of phospholipid across the bilayer and are not subject to the curvature-induced asymmetry experienced by SUVs (Mayer et al., 1986; Hope et al., 1985). DOPC was chosen as the lipid matrix because the relatively unsaturated nature of this lipid would be expected to facilitate transbilayer movement and thus enhance the development of an asymmetric lipid distribution. Finally, DOPE was present at a low mole fraction in order that any packing constraints experienced by lipid accumulating in one monolayer would be minimized and less likely to limit redistribution.

At an external pH >8.0 a large proportion of the PE molecules in the OM will be net negatively charged due to deprotonation of the amine. If this species were able to flip-flop at a greater rate than the net neutral (zwitterionic) species, then maintaining the IM at pH 4–6 should result in the accumulation of DOPE at this interface. On the other hand, if the zwitterion underwent transbilayer movement at a greater rate than the negatively charged molecule, then PE would favor the OM. We carried out many experiments in which vesicles were incubated under both conditions (inside acidic or basic with respect to the outside pH) but could not detect any deviation from an equal distribution of DOPE between the IM and OM. In each case LUVs were incubated at an elevated temperature (40 °C) to enhance transbilayer redistribution of phospholipid. Furthermore, a membrane potential (resulting from the proton gradient in vesicles with acidic interiors) was detectable throughout the incubation period (maximum 50 h), demonstrating that the ΔpH was maintained for the duration of the incubation. Clearly either the rate of PE movement between monolayers does not vary significantly between the different charged species of PE or the rates are too slow to give rise to a redistribution during the experiment. Similar results were found for vesicles containing PS. This agrees with previous papers that phospholipid flip-flop is slow in protein-free model membrane systems, a process reported to take many days for phosphatidylcholine (van Deenen, 1981).

ΔpH and Transbilayer Distribution of Phosphatidylglycerol. It is reasonable to conclude from the observations discussed above that the presence of a charge in the phospholipid headgroup results in very slow transbilayer movement. We therefore examined the acidic phospholipid phosphatidylglycerol (PG). The single charge present on this molecule arises from the ionization of the phosphate group, which might be expected to exhibit an apparent pK_a of approximately 3 (Watts et al., 1978). Consequently, it is possible to choose a pH at which a significant proportion of the PG molecules will be uncharged due to protonation of the phosphate. Moreover, the neutral form of PG might be expected to undergo flip-flop at a faster rate than the charged species. This is because diglyceride is known to undergo rapid transbilayer movement (Allan et al., 1976; Pagano & Longmuir, 1985) and membranes tend to be permeable to glycerol, and therefore the charged phosphate moiety is likely to be the rate-determining factor for transbilayer movement. This is supported by studies in which the relative flip-flop rates of phosphatidylthioglycerol and dioleoylthioglycerol were examined in a model membrane system (Ganong & Bell, 1984).

EPC vesicles containing 5 mol % EPG were prepared with an internal pH of 8.0. The external medium was exchanged for buffer at pH 4.5, and the vesicles were incubated at 60 °C for 10 min. These conditions do not result in any detectable hydrolysis as determined by thin-layer chromatography. If the neutral species of PG is able to undergo transbilayer movement, it will move from the OM to the IM, become charged, and thus be unable to flip back to the OM. The net result should be an accumulation of EPG at the IM. The experiment was performed at 60 °C to enhance transbilayer movement, and ion-exchange chromatography was employed to monitor any decrease in negative charge at the exterior surface (Hope & Cullis, 1987). The data in Figure 1 demonstrate that LUVs with a ΔpH = 0 (inside pH 4.5; external pH 4.5) remain associated with the DEAE-cellulose column until eluted with 0.5 M buffer (Figure 1A). This is consistent with negatively charged vesicles binding to the support material via ionic interactions that are disrupted at high ionic strength. However, vesicles with an internal pH of 8.0 (ΔpH = 3.5) do not bind to DEAE and elute in the void volume, indicating a reduced surface charge (Figure 1B). It is important to

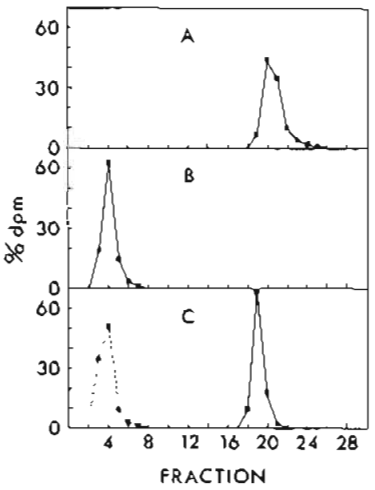

FIGURE 1: Effect of ΔpH on the surface charge of vesicles composed of EPC/EPG (5 mol %). Each panel represents elution profiles from a DEAE-Sephacel ion-exchange column. In all cases the external buffer was 10 mM glutamic acid and 5 mM K_2SO_4, pH 4.5. This buffer was used to elute the columns until fraction 11, at which point elution was continued with 10 mM glutamic acid and 0.5 M K_2SO_4, pH 4.5. (A) LUVs were prepared with an internal buffer that was the same as the external buffer (10 mM glutamic acid and 5 mM K_2SO_4, pH 4.5), ΔpH = 0. Vesicles were heated for 30 min at 60 °C prior to being applied to the column. (B) Vesicles were prepared with an internal buffer composed of 200 mM HEPES and 5 mM K_2SO_4, pH 8.5 (ΔpH = 4.0) and heated for 30 min at 60 °C. (C) Vesicles were prepared with an internal buffer of 200 mM HEPES and 5 mM K_2SO_4, pH 8.5 (ΔpH = 4.0) and heated for 30 min at 60 °C, and half were applied to the DEAE column (broken line). The inonophores nigericin and valinomycin were added to the remaining vesicles and heated at 60 °C for a further 30 min (solid line).

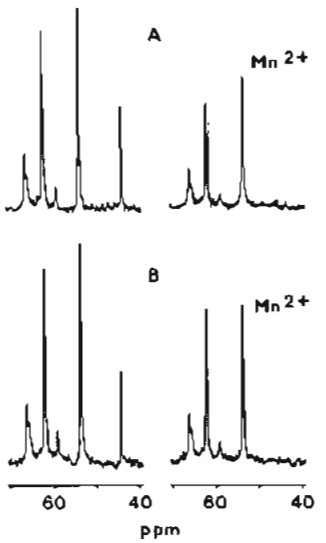

FIGURE 2: Proton-decoupled ^{13}C NMR (50.3 MHz) spectra of LUVs composed of EPC/^{13}C-enriched EPG (5 mol %). Vesicles were prepared in 200 mM phosphate and 10 mM citrate adjusted to pH 8.0 or to pH 4.5. The external buffer was exchanged for 5 mM citrate, pH 4.5, by gel filtration. (A) Vesicles with no ΔpH (inside and outside pH 4.5) were heated 15 min at 60 °C. The ^{13}C NMR spectrum was recorded before and after the addition of Mn^{2+}. (B) Vesicles with a ΔpH of 3.5 units (inside pH 8.0, outside pH 4.5). The LUVs were incubated at 60 °C for 15 min, and the NMR spectrum was taken before and after the addition of Mn^{2+}. The peaks highlighted by the shaded areas represent the ^{13}C-enriched CH_2OH moiety of the glycerol headgroup.

establish that the reduced surface charge is due to the movement of EPG from the OM to the IM and not the result of chemical alterations to the PG headgroup. For example, if PG equilibrated across the bilayer according to the mechanism proposed above, then incubation of asymmetric vesicles in the absence of a ΔpH should restore the normal symmetric distribution and hence the surface charge. Chemical modifications would not be expected to be reversible. The experiment shown in Figure 1C demonstrates that the effect can be reversed. LUVs with a ΔpH of 3.5 units (inside basic) were incubated for 10 min at 60 °C and give rise to the same reduction in negative surface charge (broken line) as was shown in Figure 1B. However, when the vesicles, which now elute from the column in the void volume, are incubated with the ionophores nigericin and valinomycin in the presence of K^+ for a further 10 min at 60 °C, the surface charge is restored and the vesicles bind to the DEAE (solid line). This combination of ionophores has been shown to dissipate the ΔpH across LUV membranes (Hope & Cullis, 1987). The ionophores themselves do not change the elution profile of DOPC vesicles alone and so are not responsible for retention of LUVs in the column (results not shown). These results support the proposal that PG equilibrates across the bilayer in a manner that reflects the transmembrane proton gradient.

To firmly establish that these results reflect the transbilayer movement of EPG, we employed two additional techniques that enabled us to assay OM PG directly. In the first, ^{13}C NMR was used to measure the transbilayer distribution of ^{13}C-enriched EPG prepared from EPC and [1,3-^{13}C]glycerol (see Materials and Methods). LUVs composed of DOPC/[^{13}C]EPG (9.5:0.5) were prepared with a ΔpH = 0 (pH 4.5 inside and outside) or with a ΔpH = 3.5 (pH 8.0 inside and pH 4.5 outside). Both sets of vesicles were heated for 15 min at 60 °C, and the ^{13}C NMR spectrum was recorded at 20 °C before and after the addition of 5 mM Mn^{2+}. In the presence of Mn^{2+} the ^{13}C signal from EPG in the OM is broadened beyond detection, and consequently the remaining signal intensity is directly proportional to EPG located at the IM. Figure 2A shows that in the absence of a ΔpH the signal intensity remaining is approximately 50% of the initial intensity, indicating an equal distribution of EPG between the OM and IM. However, for vesicles with an interior pH of 8.0 and a ΔpH of 3.5 units, 80% of the ^{13}C signal intensity remains in the presence of Mn^{2+} (Figure 2B), indicating that a pronounced movement of EPG to the IM has taken place.

The NMR data clearly demonstrate an asymmetric distribution of EPG in LUVs that exhibit a ΔpH, but complete asymmetry is not achieved. Approximately 20% of the EPG in a DOPC/5 mol % EPG LUV system remained in the OM under the conditions used to generate the asymmetry. The ion-exchange techniques described above are not quantitative, and it was only possible to determine that vesicles eluting in the void volume had an OM charge density equivalent to vesicles containing <2 mol % EPG. To obtain quantitative information on the amount of EPG in the OM, a second specific assay was employed in which EPG is oxidized to formaldehyde by using periodate (Lentz et al., 1980, 1982). The amount of formaldehyde produced is proportional to the amount of EPG in the OM of vesicles because periodate does not permeate across the membrane during the time course of the experiment. Total EPG is assayed by dissolving vesicles in sodium cholate and exposing all the glycerol hydroxyl groups to the oxidizing agent. Figure 3 demonstrates a typical time course for this reaction, which is complete after approximately 12 min at 25 °C. Under conditions that do not give rise to an asymmetric distribution of EPG (ΔpH = 0), 50% of the available hydroxyl groups are oxidized. This is consistent with the 50:50 distribution of phospholipid between the OM and IM. However, when vesicles with an internal pH of 8.0 are incubated at pH 4.0 and 60 °C for 30 min, only 14% of the

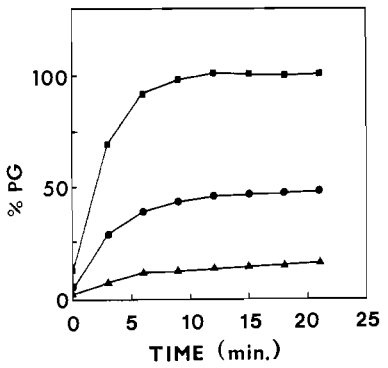

FIGURE 3: Oxidation of EPG in the OM of LUVs composed of EPC/EPG (5 mol %). The amount of EPG in the OM of vesicles was assayed by oxidation of the primary hydroxyl group of EPG to formaldehyde, which was then assayed colorimetrically. Total EPG in the LUVs was determined by dissolving vesicles in cholate prior to the oxidation procedure (■). The plateau in absorbance at 535 nm was taken as 100%. Vesicles were prepared with a ΔpH of 4 units (300 mM phosphate, pH 8.0 inside, and 100 mM Na_2SO_4, 10 mM citrate, pH 4.0 outside). The amount of EPG detected in the OM without heating was approximately 50% after 20 min (●). After heating at 60 °C for 30 min, 14% of the total EPG could be detected in the OM (▲).

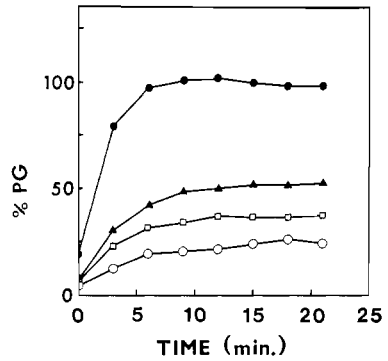

FIGURE 4: Time-dependent change in the OM EPG of vesicles composed of EPC/EPG (5 mol %) with a ΔpH = 4.0, incubated at 37 °C. LUVs (300 mM phosphate, pH 8.0 inside, and 100 mM Na_2SO_4, 10 mM citrate, pH 4.0 outside) were assayed for EPG in the presence of sodium cholate (●), which represents 100%. (▲) At t = 0, 50% of the total EPG could be detected in the OM, (□) at t = 30 min, 36% of the total EPG could be detected in the OM, and (○) at t = 120 min, 22% of the total EPG could be detected in the OM.

total hydroxyl groups are oxidized by periodate. This indicates that more than 80% of EPG in these DOPC/5 mol % EPG vesicles have been sequestered to the IM, supporting the results obtained by using ^{13}C NMR and ion-exchange chromatography.

The rate at which EPG moves from the OM to the IM is slower than that observed for fatty acids and stearylamine (Hope & Cullis, 1987). We have demonstrated that at 60 °C the maximum amount of EPG asymmetry is achieved after approximately 10 min, and this level is stable for at least 1 h (data not shown). However, at 37 °C, the rate at which PG equilibrates to the IM is reduced. The results presented in Figure 4 demonstrate a gradual loss of EPG from the OM of vesicles that exhibit a ΔpH of 3.5 units (inside basic). After 30 min, approximately 30% of the OM PG has moved to the IM and after 2 h more than 50%.

The transbilayer equilibrium of EPG in the presence of a ΔpH (inside basic) is toward the IM. A reverse pH gradient should reverse this equilibrium. This is shown in Figure 5, which demonstrates the movement of EPG to the OM when vesicles are incubated in the presence of an exterior pH of 8.0 and an interior pH of 4.0.

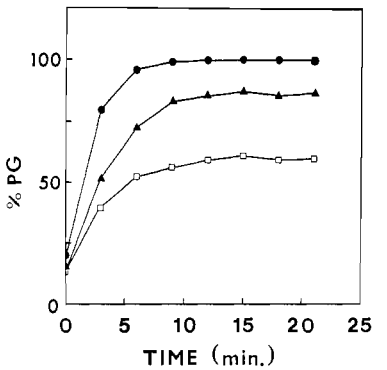

FIGURE 5: Accumulation of EPG detected in the OM induced by ΔpH (acidic inside). The amount of oxidizable EPG was determined in the OM of vesicles with a ΔpH of −4 units (300 mM citrate, pH 4.0 inside, and 100 mM Na_2SO_4, 20m mM HEPES, pH 8.0 outside): (●) in the presence of cholate 100%; (□) at t = 0, 58%; and (▲) after 30 min at 60 °C, 83%.

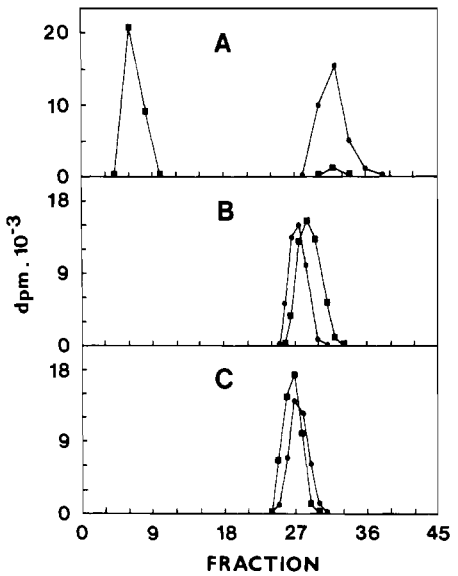

FIGURE 6: Elution profiles of vesicles composed of EPC and 5 mol % PA, PI, or cardiolipin from DEAE. Vesicles were prepared with a ΔpH of 3.5 units (300 mM phosphate, pH 8.0 inside, and 10 mM phosphate, 10 mM citrate, pH 4.5 outside), and the elution profiles determined before and after heating at 60 °C for 30 min. The elution buffer was 10 mM phosphate and 10 mM citrate, pH 4.5, until fraction 11, when elution with 300 mM phosphate, pH 8.0, was started. (A) EPC/EPA (5 mol %) vesicles before (●) and after (■) heating at 60 °C for 30 min; (B) EPC/SPI (5 mol %) vesicles before (●) and after (■) heating at 60 °C for 30 min; (C) EPC/cardiolipin (5 mol %) vesicles before (●) and after (■) heating at 60 °C for 30 min.

ΔpH and the Transbilayer Distribution of Other Acidic Phospholipids. There are several acidic phospholipids that might be expected to equilibrate across the bilayer in response to a transmembrane proton gradient. Phosphatidic acid (PA) is the most obvious, as this lipid does not have a headgroup moiety esterified to the phosphate and should undergo transbilayer movement at pHs close to the pK_a of the phosphate group. This prediction is supported by a recent paper (Homan & Pownall, 1988) in which it is noted that the rate at which a fluorescent analogue of PA is able to traverse the bilayer increases 500-fold when the headgroup is titrated from pH 7.4 to pH 4.0. By use of the same experimental conditions described above for EPG, an asymmetric distribution of dioleolylphosphatidic acid (DOPA) was achieved. The elution profiles from DEAE-Sephacel columns of vesicles containing 5 mol % DOPA are shown in Figure 6A. DOPA-containing vesicles (basic inside) eluted in the void volume following an incubation at 60 °C, indicating a reduced surface charge.

Phosphatidylinositol resembles PG, but the inositol moiety is heavily hydroxylated and less likely to cross the bilayer than glycerol. Cardiolipin, on the other hand, which consists of two PA molecules esterified to glycerol, might be expected to respond to a ΔpH more readily. As shown in Figure 6B,C, vesicles containing these acidic phospholipids did not demonstrate a reduction in surface charge. It is important to note, however, that the ion-exchange technique is limited because it cannot differentiate small changes in surface charge. A more detailed examination of the response of these lipids to transbilayer pH gradients requires a quantitative assay.

Discussion

To our knowledge this is the first report that membrane asymmetry of phospholipids can be induced by ion gradients in LUVs. Phospholipid asymmetry in model membrane systems to date has previously been restricted to that induced by curvature (Lentz et al., 1982; Nordlund et al., 1981) and to asymmetry created by exchange of OM lipids using exchange proteins (De Kruijff & Wirtz, 1977) or the enzymatic conversion of OM lipid to phosphatidic acid using phospholipase D (De Kruijff & Baken, 1978).

Previous observations (Hope & Cullis, 1987) and the results presented here suggest that the rate at which the uncharged, protonated species of EPG can undergo transbilayer movement is much faster than that of the negatively charged species. Thus, the equilibrium transmembrane distribution of EPG should obey the relationship

$$\frac{EPG^-_{in}}{EPG^-_{out}} = \frac{[H]^+_{out}}{[H]^+_{in}}$$

However, it is clear that this equilibrium is not fully realized. Given a ΔpH of 3 units, the ratio of EPG between the inner and outer monolayers should be 1000:1 to satisfy the Henderson–Hasselbach relation shown above. At 5 mol % EPG in LUVs composed of EPC or DOPC the maximum asymmetry obtained was consistently on the order of 9:1. It is interesting to speculate as to why complete asymmetry is not achieved. Stearylamine, for example, exhibits complete asymmetry at membrane concentrations in excess of 20 mol % when incorporated into LUVs with an acidic interior and a ΔpH of 3 units (S. Eastman, unpublished data). EPG occupies a greater surface area in the bilayer than stearylamine, and it is possible that an equilibrium transmembrane redistribution of EPG is inhibited due to physical packing constraints on the amount of transported EPG that can be accommodated in the absence of a compensating movement of other phospholipids in the opposite direction. Movement of PC in the reverse direction to that of EPG is unlikely to occur since the flip-flop rates of PC in protein-free model membranes have been shown to be extremely slow (van Deenen, 1981). This may be attributed to the choline headgroup of PC, which is zwitterionic and exhibits a permanently charged quaternary amine. Experiments with lysophosphatidylcholine (lyso PC) have shown that in DOPC LUVs lyso PC located at the IM cannot be detected in the OM, even after 5.5 days of incubation at 25 °C (M. J. Hope, unpublished data). It should also be noted that EPG is membrane associated, and consequently the Henderson–Hasselbach equilibrium will reflect the proton concentration at the bilayer surfaces, which might differ from the measured bulk pH.

The biological significance of these results is not clear. It has been reported (Johnson & Goldfine, 1985) that PG is asymmetrically distributed in *Clostridium butyricum*. A proton gradient (interior basic) of approximately 2 units exists across these membranes (H. Goldfine, personal communication) and thus could be implicated as a driving force for PG asymmetry. In general, the transbilayer movement of phospholipid occurs more readily in biological membranes than it does in protein-free model membrane systems. PC, for instance, undergoes rapid flip-flop in microsomal membranes (Bishop & Bell, 1985), and PE and PS pass from the OM to the IM of erythrocyte membranes within hours (Zachowski et al., 1986; Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986). The present work shows that for phospholipids which are weak acids the equilibrium transmembrane distribution could be modulated by transmembrane pH gradients.

Registry No. [1,3-^{13}C]Glycerol, 86318-32-3; dioleoylphosphatidic acid, 14268-17-8; dioleoylphosphatidylcholine, 10015-85-7.

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Rapid Isolation of OmpF Porin–LPS Complexes Suitable for Structure–Function Studies†

A. Holzenburg,‡ A. Engel,*,‡ R. Kessler,§ H. J. Manz,§ A. Lustig,‖ and U. Aebi‡

Maurice E. Müller Institute for High Resolution Electron Microscopy and Department of Biophysical Chemistry, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, and Central Function Research, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland

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ABSTRACT: A gentle and rapid isolation procedure is described yielding fractions containing better than 95% pure OmpF porin of *Escheria coli* B^E with different amounts of bound lipopolysaccharide (LPS). The procedure employs continuous free-flow electrophoresis (FFE) in the presence of detergent above its critical micelle concentration. Total yields of around 45% were typically obtained when porin-enriched membrane extracts were processed. By use of analytical ultracentrifugation a molecular mass of 114 000 and a sedimentation coefficient $s_{20,w}$ of 5.0 S were determined for porin trimers containing approximately 1 mol of tightly bound LPS. This porin readily formed 3D crystals suitable for high-resolution X-ray diffraction analysis. Three other porin–LPS isoforms isolated by FFE revealed molecular masses of 120 000, 124 000, and 151 000, suggesting that, in addition to the tightly bound LPS, 1, 2, and 8 mol of loosely bound LPS were present per mole of porin trimer. Each of the four different isoforms was suitable for reconstitution into highly ordered protein–lipid membrane arrays. The membrane crystals obtained with the 151-kDa isoform exhibited a unit cell polymorphism similar to that previously reported.

A major function of the outer membrane of Gram-negative bacteria is to serve as a permeability barrier. To this end, various pore-forming integral membrane proteins such as OmpC, OmpF (porin), LamB (maltoporin), and PhoE (phosphoporin) have been demonstrated to play an important role in the outer membrane of *Escherichia coli* (Lugtenberg & Van Alphen, 1983; Benz, 1985; Nikaido & Vaara, 1985). *E. coli* B^E porin has been the subject of extensive structural (Dorset et al., 1983; Garavito et al., 1983; Engel et al., 1985) and functional (Schindler & Rosenbusch, 1978, 1981) investigations. The functional porin unit is a trimer consisting of three 37.2-kDa polypeptides (Chen et al., 1982) forming three separate transmembrane channels at the outer surface of the cell that merge into a single channel at the periplasmic side of the outer membrane as revealed by three-dimensional (3D) electron microscopy of two-dimensional (2D) porin crystals (Engel et al., 1985). These hydrophilic water-filled pores with a physical diameter of approximately 1 nm facilitate diffusion of ions and hydrophilic solutes below a molecular mass of approximately 600 (Nikaido & Nakae, 1979). In addition to their size, the hydrophilicity (Nikaido et al., 1983) and the charge (Benz et al., 1979) of the solutes have been reported to affect the permeability coefficients of the pores. In contrast to phospho- (Korteland et al., 1982; Overbeeke & Lugtenberg, 1982) and maltoporin (Ferenci et al., 1980; Luckey & Nikaido, 1980), as yet no substrate-binding proteins located on the outer periphery of the porin channels have been identified. Furthermore, porin trimers are reported to exhibit at least two functional states—an open and a closed one—that are inducible upon application of a membrane potential

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* To whom correspondence should be addressed.
‡ Maurice E. Müller Institute for High Resolution Electron Microscopy.
§ Ciba-Geigy Ltd.
‖ Department of Biophysical Chemistry, University of Basel.

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