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Articles

Transbilayer Transport of Phosphatidic Acid in Response to Transmembrane pH Gradients†

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Received August 13, 1990; Revised Manuscript Received November 8, 1990

ABSTRACT: Preliminary studies have shown that asymmetric transbilayer distributions of phosphatidic acid (PA) can be induced by transmembrane pH gradients (ΔpH) in large unilamellar vesicles [Hope et al. (1989) *Biochemistry 28*, 4181–4187]. Here the mechanism of PA transport is examined employing TNS as a fluorescent probe of lipid asymmetry. It is shown that the kinetics of PA transport are consistent with the transport of the uncharged (protonated) form. Transport of the neutral form can be rapid, exhibiting half-times for transbilayer transport of approximately 25 s at 45 °C. It is also shown that PA transport is associated with a large activation energy (28 kcal/mol) similar to that observed for phosphatidylglycerol. The maximum induced transbilayer asymmetry of PA corresponded to approximately 95% on the inner monolayer for vesicles containing 5 mol % PA.

It is generally accepted that many biological membranes exhibit asymmetric transbilayer distributions of lipid (Op den Kamp, 1979). However, the reasons for such asymmetry and the means by which it is generated and maintained remain relatively obscure. In the case of phosphatidylethanolamine (PE) and phosphatidylserine (PS), there is strong evidence for a protein-dependent transport mechanism which results in translocation of these lipids to the inner monolayer of plasma membranes such as the erythrocyte (Zachowski et al., 1986; Seigneuret & Devaux, 1984; Connor & Schroit, 1988) although a protein exhibiting such activity has yet to be isolated. Alternatively, in protein-free liposomal systems, asymmetry of lipids such as PE, PS, and phosphatidylglycerol (PG), in mixed phospholipid systems with phosphatidylcholine (PC), has been observed for small (sonicated) vesicles (Lentz et al., 1980; Massari et al., 1978). However, it is generally agreed that these asymmetries are related to curvature effects arising from the small radius of the sonicated vesicles, and have limited implications for lipid asymmetry in biological membranes.

Research in this laboratory has been focused on the influence of ion gradients, particularly pH gradients, on the transbilayer distributions of lipids in large unilamellar vesicles (LUVs).[1] It has been shown for lipids which are simple weak bases or weak acids such as stearylamine or fatty acids that the presence of a pH gradient (ΔpH) can dramatically affect equilibrium transbilayer distributions (Hope & Cullis, 1987). The presence of a ΔpH (inside acidic) results in the rapid migration of stearylamine to the inner monolayer of LUVs, for example, whereas oleic acid migrates to the inner monolayer in LUVs exhibiting a basic interior. Phospholipids which are weak acids can exhibit similar behavior. In the case of PG, for example, the presence of a ΔpH (interior basic) results in the migration of PG to the inner monolayer (Hope et al., 1989); however, the rate of PG transport is considerably slower than observed for oleic acid. A more detailed kinetic analysis (Redelmeier et al., 1990) of PG transport in response to ΔpH reveals that PG is transported in the neutral (protonated) form which can exhibit half-times for transbilayer movement on the order of seconds.

A preliminary study of the influence of ΔpH on the transbilayer distribution of phosphatidic acid (PA) revealed that PA can also be accumulated into the inner monolayer of LUVs with a basic interior (Hope et al., 1989). However, a quantitative analysis of this transport was precluded by the lack of an appropriate assay for PA asymmetry. In this investigation, we have developed a fluorescent assay for PA asymmetry utilizing the probe 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid (TNS), which reports on the surface potential of membranes (McLaughlin & Haray, 1976; Eisenberg et al., 1979; Searle & Barber, 1979). This assay should be of general utility for detecting asymmetry of acidic lipids in LUV systems. In the case of DOPA, we show that ΔpH-induced transport

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[1] Abbreviations: LUV, large unilamellar vesicle; MLV, multilamellar vesicle; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid, sodium salt.

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proceeds via the neutral form with an activation energy similar to that observed for PG.

Materials and Methods

Lipids and Chemicals. All phospholipids were obtained from Avanti Polar Lipids (Pelham, AL) and were used without further purification. These included dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidic acid (DOPA), dioleoylphosphatidylserine (DOPS), bovine liver phosphatidylinositol (PI), and bovine heart cardiolipin. All lipids were in the Na^+ salt form. TNS [2-(*p*-toluidinyl)naphthalene-6-sulfonic acid] and all buffers were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Large Unilamellar Vesicles. Lipids were mixed in chloroform and dried down under a stream of nitrogen, and residual chloroform was removed under high vacuum for 1 h. The resulting lipid film was hydrated by the addition of the appropriate buffer followed by vortexing to form multilamellar vesicles (MLVs). These MLVs were freeze–thawed 5 times, employing alternating liquid nitrogen/warm water cycles, in order to obtain equilibrium transmembrane solute distributions (Mayer et al., 1986). The freeze–thawed MLVs were then extruded 10 times through two stacked polycarbonate filters with 100-nm pore sizes, employing an extrusion device (Lipex Biomembranes, Vancouver, BC, Canada) to produce a homogeneous population of large unilamellar vesicles (LUVs) with an average diameter of 90 nm as determined by quasi-elastic light-scattering and freeze–fracture (Hope et al., 1985).

Induction of Transbilayer Transport of Acidic Phospholipids. Vesicles were prepared in 300 mM EPPS, pH 9.0, and passed down Sephadex G-25 columns (Pharmacia) equilibrated in 150 mM Na_2SO_4/1 mM EPPS, pH 9.0. At this point, phosphate assays (Fiske & Subbarow, 1925) were performed on the vesicle preparations, and the vesicles were diluted to a concentration of 10.5 mM total phospholipid. Vesicles were then placed in test tubes and preheated to an appropriate temperature. At time $t = 0$, an equal volume of a preheated 100 mM citrate buffer, pH 4.0, was added to each tube to obtain the desired ΔpH. The tubes were then incubated for appropriate times, and PA transport was quenched by placing 200 μL of the sample into test tubes containing 500 μL of ice-cold 100 mM ammonium acetate/100 mM sodium citrate, pH 6.0, and the sample was then stored on ice until assayed for asymmetry. The transbilayer movement of PA or PG under these conditions is negligible as the pH gradient is reduced by the presence of acetate and transport is extremely slow at 0 °C (see Results). For "zero time" time points, 100-μL samples were removed from the test tubes before the citrate (pH 4.0) was added and placed into the ice-cold acetate and citrate mixture (500 μL).

To measure PA movement to the outer monolayer, the same procedure was followed except that the vesicles were hydrated in 300 mM citrate, pH 4.0. Untrapped buffer was exchanged for 150 mM Na_2SO_4/1 mM citrate, pH 4.0, employing column chromatography, and the external pH was subsequently adjusted by the addition of 100 mM EPPS, pH 9.0.

Detection of Phosphatidylglycerol Asymmetry by Periodate Oxidation. The transmembrane asymmetry of PG was assayed by two methods. The first method involved periodate oxidation as previously described (Lentz et al., 1980; Hope et al., 1989). Briefly, the PG on the external monolayer of the vesicles was oxidized by the addition of 100 μL of freshly prepared 100 mM sodium periodate to each sample. To assay the total amount of PG in the vesicles, 50 μL of 200 mM sodium cholate was added to the sample before the addition of the periodate. The oxidation of the PG was quenched after 11 min by the addition of 100 μL of 1 M sodium arsenite in 1 N H_2SO_4. The formaldehyde resulting from the oxidation of the glycerol was detected by the Hantzsh reaction (Nash, 1953).

Detection of Asymmetry Using TNS. Following the establishment of PG or PA asymmetry, two samples (200 μL) were removed and placed into test tubes. To each of these samples was added 3 mL of 3 μM TNS in 5 mM ammonium acetate/5 mM HEPES (pH 7.0) and mixed. The fluorescence of the samples was then measured by employing either an SLM Aminco SPF 500C or a Perkin Elmer LS-50 fluorometer using an excitation wavelength of 321 nm and an emission of 445 nm.

To determine asymmetry, two sets of vesicles were utilized. The first contained only PC while the second contained a well-defined mixture of PC and the acidic lipid being assayed. The acidic lipid made up a maximum of 5% of the total lipid since it was found that the TNS fluorescence varied linearly with acidic lipid content over the range 0–6% PA or PG (see Figure 1). Due to this linearity, the percentage of acidic lipid in the outer monolayer could be calculated according to $x = \{[f - f(\mathrm{PC})]/[f_0 - f(\mathrm{PC})]\}X_0$, where f is the TNS fluorescence for the sample for which asymmetry is assayed, $f(\mathrm{PC})$ is the fluorescence of the sample containing no acidic phospholipid, f_0 is the fluorescence associated with the sample prior to induction of asymmetry, and X_0 is the mole percent of acidic phospholipid in the LUVs.

Measurement of the Internal pH of LUVs. The internal pH was monitored employing entrapped pyranine according to the method of Rossignol et al. (1982). This first required construction of a standard curve, utilizing the LUVs with an external buffer of 150 mM Na_2SO_4/1 mM EPPS (pH 9.0) containing 1 mM pyranine and diluted to a concentration of 10 mM total lipid. To this dispersion was added an equal volume of a buffer with a pH in the range of 5.0–9.0. This buffer was capable of buffering over the range pH 5.0–9.0, containing 150 mM Na_2SO_4, 20 mM MES, 20 mM PIPES, 20 mM HEPES, and 20 mM EPPS. In order to ensure that the internal pH was the same as the external pH, 1 μM nigericin and 10 μg/mL gramicidin was also present. The fluorescence was then monitored (at 45 °C) by employing excitation wavelengths of 405 and 463 nm (emission wavelength 511 nm), and the ratio of I_{463} to I_{405} vs pH was employed to produce the pH titration curve. This was utilized to obtain the internal pH of vesicles with a transmembrane pH gradient (external pH 4.0) by monitoring I_{463}/I_{405} as a function of incubation time at 45 °C.

Kinetic Analysis of Phosphatidic Acid Transport. We follow the analysis presented in Redelmeier et al. (1990). Briefly, this model assumes that only the neutral (protonated) form of the acidic phospholipid is able to move across the membrane. In the case of phosphatidic acid, we then obtain the relation:

$$[\mathrm{PA}(t)]_0 = [\mathrm{PA}(0)]_0 e^{-kt} + [\mathrm{PA}(\mathrm{eq})]_0 \qquad (1)$$

where $[\mathrm{PA}(t)]_0$, $[\mathrm{PA}(0)]_0$, and $[\mathrm{PA}(\mathrm{eq})]_0$ represent the exterior surface concentrations of PA at times, t, zero, and infinity (equilibrium), respectively. The rate constant k obeys the relation $k = [\mathrm{H}^+]_0 K/K_a$ where $[\mathrm{H}^+]_0$ is the external proton concentration at the lipid/water interface, K is the rate constant for transport of the neutral form, and K_a is the dissociation constant for PA. From eq 1, it follows that

$$\ln \{[[\mathrm{PA}(t)]_0 - [\mathrm{PA}(\mathrm{eq})]_0]/[\mathrm{PA}(0)]_0\} = -kt \qquad (2)$$

and thus a plot of $\ln \{[[\mathrm{PA}(t)]_0 - [\mathrm{PA}(\mathrm{eq})]_0]/[\mathrm{PA}(0)]_0\}$ vs time should yield a straight line with slope k. A best fit to the data

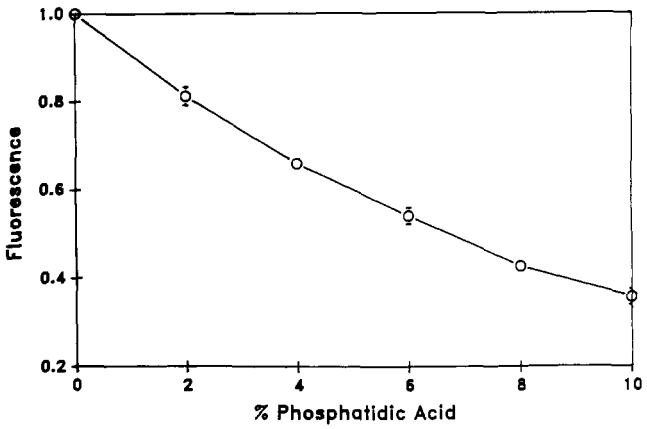

FIGURE 1: TNS fluorescence as a function of DOPA content in DOPA/DOPC LUV vesicles (100-nm diameter) was produced from lipid dispersions containing 0–10 mol % DOPA employing a protocol similar to that employed for inducing asymmetry (see Materials and Methods), with the exceptions that the heating step was omitted and the ammonium acetate buffer (pH 6.0) was added prior to the citrate buffer (pH 4.0) to avoid generating a pH gradient. Fluorescence is expressed as a percentage of the fluorescence observed for pure DOPC LUVs.

was achieved by using k and $[PA(eq)]_0$ as variables. As indicated previously (Redelmeier et al., 1990), this analysis assumes that $K_a \gg [H^+]_0$ and $[H^+]_i \ll [H^+]_0$.

RESULTS

TNS Fluorescence Assay of Asymmetry. Asymmetry of acidic phospholipids in LUVs composed of PC/acidic lipid mixtures can be detected by ion-exchange chromatographic techniques (Hope & Cullis, 1987; Hope et al., 1989). Unfortunately, this technique does not provide quantitative measures of asymmetry. In the case of PG, ^{13}C NMR studies on ^{13}C-labeled varieties (Hope et al., 1989) or chemical assays specific for PG (Redelmeier et al., 1990) provide more quantitative information. In order to facilitate asymmetry studies on PA and other acidic phospholipids, a more general and flexible assay for the presence of such lipids in the outer monolayer of LUV systems was required. An obvious approach is to monitor the surface potential of the outer monolayer, which will reflect the presence of negatively charged phospholipids. We were therefore led to investigate the utility of TNS, a probe of membrane surface potential introduced by McLaughlin and co-workers (McLaughlin & Haray, 1976; Eisenberg et al., 1979) as a probe of asymmetry. In this regard, it should be noted that TNS is a fluorescent lipophilic weak acid ($pK_a \approx 4$) which exhibits enhanced fluorescence when associated with a lipid bilayer. Thus, under the assay conditions employed here, the presence of acidic lipids in the outer monolayer of the LUVs will result in decreased partitioning of the negatively charge probe into the bilayer and correspondingly reduced fluorescence intensity. This effect is illustrated in Figure 1 for 100-nm DOPC/DOPA LUVs containing 0–10 mol % DOPA. It may be noted that the decrease in fluorescence intensity with PA content is linear over the range 0–6 mol % DOPA, and thus most asymmetry experiments were restricted to this range for ease of analysis.

Additional control experiments to establish the utility of the TNS assay were required, however. This is due to the weak acid characteristics of TNS, which would suggest that it could be accumulated into LUVs exhibiting a basic interior due to permeation of the neutral form. Such accumulation would be expected to result in enhanced fluorescence intensity arising from increased partitioning of the probe into the interior lipid monolayer due to the small aqueous to lipid volume ratio in the LUV interior. Behavior corresponding to this effect is shown in Figure 2 for 100 nm DOPC LUVs with an interior pH of 9.0 (300 mM EPPS) and an exterior pH of 4.0. However, it is also shown in Figure 2 that dissipating the transmembrane pH gradient by raising the exterior pH and adding 100 mM ammonium acetate to the exterior medium eliminated such effects.

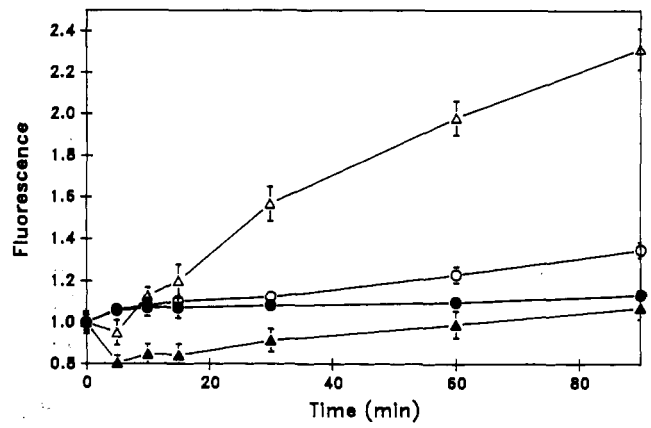

FIGURE 2: Influence of a transmembrane pH gradient (interior basic) on the fluorescent response of TNS in DOPC LUVs. Vesicles (100-nm diameter) were prepared in EPPS buffer (pH 9.0), and the exterior buffer was exchanged for the Na_2SO_4 buffer (see Materials and Methods). A transmembrane pH gradient was then generated by addition of 100 μL of the citrate buffer (pH 4.0) to 100 μL of the vesicle solution (10.5 mM phospholipid). The pH gradient was then either quenched or maintained by addition of either 500 μL of the ammonium acetate buffer (pH 6.0) or 500 μL more of the citrate buffer (pH 4.0). The TNS response was then monitored following addition of 10.5 mL of a 3 μM TNS solution. (△) indicates the response for the unquenched system at 45 °C; (▲) the quenched system at 45 °C; (○) the unquenched system at 22 °C; (●) the quenched system at 22 °C. The fluorescence is expressed as a ratio of that observed at $t = 0$ to that obtained at time t.

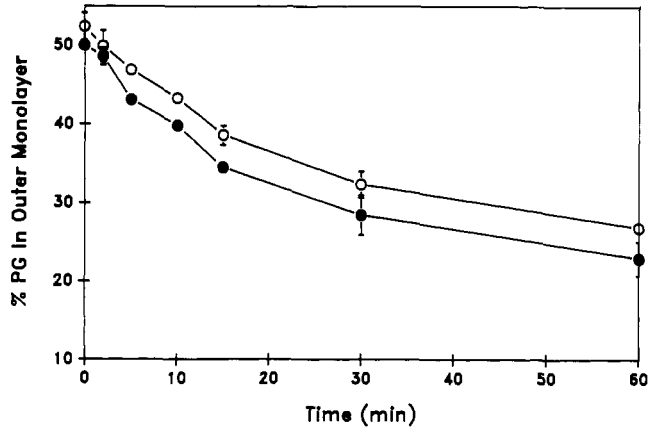

FIGURE 3: DOPG asymmetry assayed by TNS and periodate assay procedures. Vesicles (100-nm diameter) containing 5 mol % DOPG (in DOPC) were prepared as indicated under Materials and Methods to exhibit a transmembrane pH gradient ($pH_i = 9.0$; $pH_0 = 4.0$) and subsequently incubated at 45 °C for the times indicated. For details of the TNS and periodate assay procedures, see Materials and Methods.

In order to further validate the TNS asymmetry assay for acidic phospholipids, a direct comparison with the periodate assay for PG asymmetry was performed in DOPC/DOPG (95:5, mol/mol) LUV systems. As shown in Figure 3, a very similar rate and extent of PG asymmetry were reported by both procedures.

Kinetic Analysis of PA Transport. Initial experiments on PA transport in response to ΔpH were designed to monitor the time course of ΔpH-induced PA asymmetry as assayed

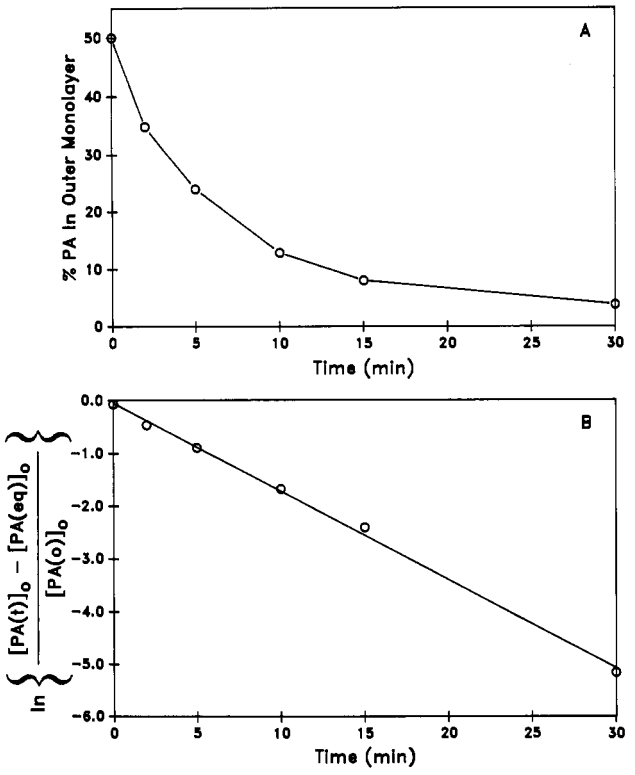

FIGURE 4: (A) Influence of a pH gradient (interior basic) on the transbilayer distribution of DOPA in DOPC/DOPA (95:5, mol/mol) LUVs. Vesicles were prepared as indicated under Materials and Methods (pH_i = 9.0; pH_0 = 4.0) and incubated at 45 °C for the times indicated prior to quenching transport. The amount of DOPA remaining in the outer monolayer was assayed employing TNS as described under Materials and Methods. (B) Best fit to these data employing the kinetic analysis summarized under Materials and Methods. From the slope of this plot, the rate constant k can be determined to be 1.67×10^{-1} min^{-1}.

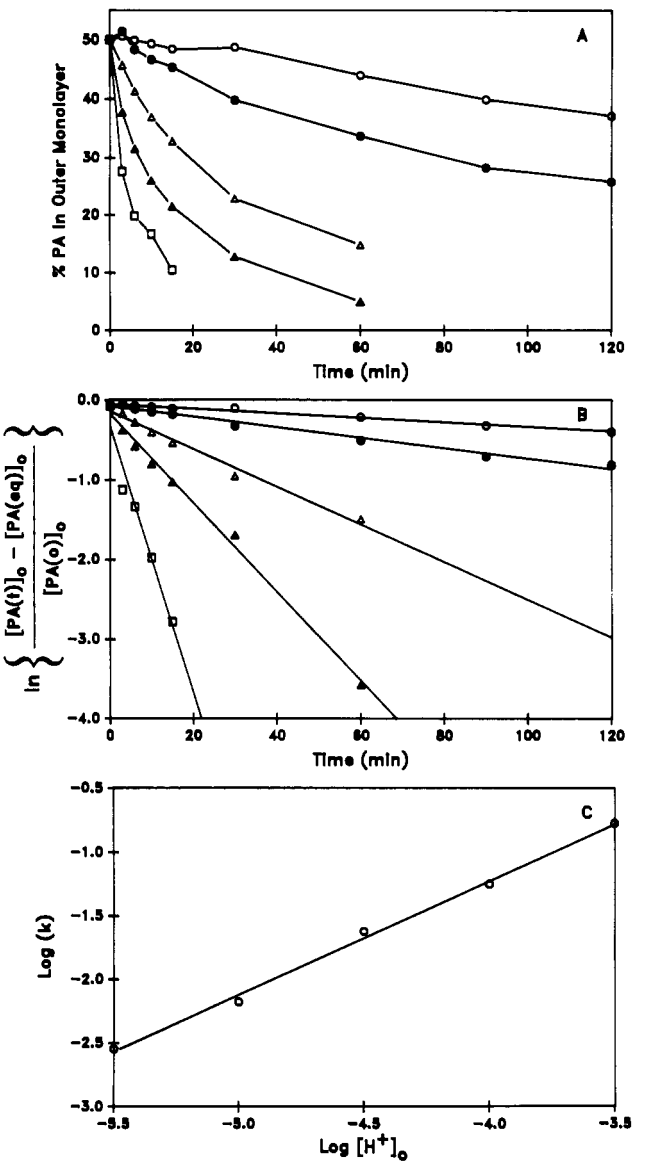

FIGURE 5: Influence of the exterior pH on the rate of transbilayer transport of DOPA in DOPC/DOPA (95:5, mol/mol) LUVs. (A) Vesicles were prepared in 300 mM NaEPPS, pH 9.0, buffer, and the exterior buffer was exchanged for 150 mM Na_2SO_4/1 mM EPPS, pH 9.0, as indicated under Materials and Methods. The vesicles were then introduced into citrate solutions with varying pH: (○) pH_0 = 5.5; (●) pH_0 = 5.0; (△) pH_0 = 4.5; (▲) pH_0 = 4.0; (□) pH_0 = 3.5. After incubation at 40 °C for the indicated times, transport was quenched and the amount of DOPA remaining in the outer monolayer assayed employing TNS. (B) Best fits to this data employing the kinetic analysis summarized under Materials and Methods, which allows determination of the rate constants, k. (C) Plot of log k vs the log of the external proton concentration.

by TNS and to test the applicability of the kinetic analysis employed elsewhere [see Materials and Methods and Redelmeier et al. (1990)]. As shown in Figure 4A, the presence of a ΔpH, interior basic (pH_0 = 4.0, pH_i = 9.0), in 100-nm DOPC/DOPA (95:5 mol/mol) LUVs results in the depletion of DOPA in the outer monolayer to approximately 5% of the original content after a 30-min incubation at 45 °C. This corresponds to a DOPA content in the inner monolayer of 9.3% and an exterior DOPA content of 0.26 mol %, which is nearing the detection limits of the TNS assay. It is interesting to note that the maximum ΔpH-induced PA asymmetry detected ($[PA]_i/[PA]_0$ = 39) is considerably greater than that detected for DOPG under similar conditions, where a maximum inside:outside ratio of 3 was obtained (Redelmeier et al., 1990).

The kinetic analysis employed here assumes that $[PAH]_0 \gg [PAH]_i$, where PAH represents the neutral form of PA. It is therefore important that the pH_i remains sufficiently high to satisfy this condition. Employing entrapped pyranine, as described in the last part of this section, the pH_i at 30 min was measured to be 7.3, indicating that $[PAH]_0/[PAH]_i \approx 50$, which satisfies this demand. It may be noted that the decrease in pH_i from pH 9 to pH 7.3 during PA transport cannot be accounted for by the import of associated protons. It may be suggested that packing problems resulting from the import of PA may result in shape changes and partial lysis, resulting in release of buffer. However, freeze–fracture studies revealed no difference in shape induced by the lipid asymmetry (results not shown), and the lack of lysis was indicated by the lack of release of entrapped radiolabeled citrate during PA import. It is possible that the reduction in pH_i arises in part due to leakage of internal Na^+ ions, which would allow the inward movement of protons.

As indicated under Materials and Methods, assuming that only the neutral (protonated) form of PA is transported, a plot of $\ln \{[[PA(t)]_0 - [PA(eq)]_0]/[PA(0)]_0\}$ vs time should yield a straight line with slope k, where the half-time ($t_{1/2}$) for transbilayer movement of the PA is given by $t_{1/2} = 0.693\ k^{-1}$. As shown in Figure 4B, a satisfactory linear fit employing the data of Figure 4A could be achieved employing k and $[PA(eq)]_0$ as variables. This analysis results in a rate constant of 1.67×10^{-1} min^{-1}, corresponding to a half-time for transbilayer movement ($t_{1/2}$) of DOPA of 4.1 min at 45 °C.

Influence of pH and Temperature on PA Transport. The kinetic analysis of PA transport employed here assumes that

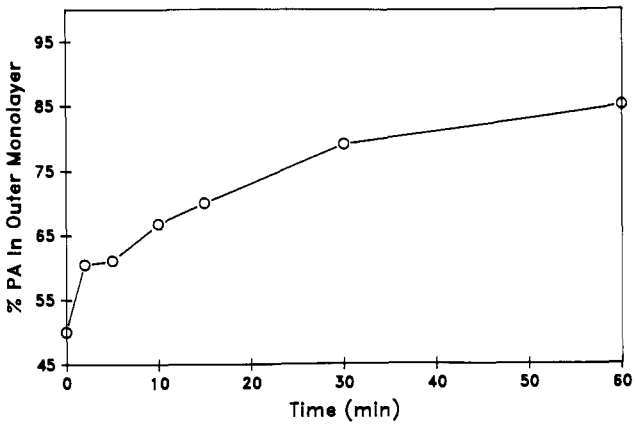

FIGURE 6: Transport of DOPA to the outer monolayer of DOPC/DOPA (97.5:2.5) LUVs in response to a transbilayer pH gradient (interior acidic). Vesicles were prepared in 300 mM citrate (pH 4.0), and the external buffer was exchanged for 150 mM Na_2SO_4/1 mM citrate (pH 4.0). At zero time, outward DOPA transport was initiated by addition of 100 mM EPPS buffer (pH 9.0). Transport was quenched after incubation for the indicated times at 40 °C by addition of 100 mM ammonium acetate/100 mM citrate buffer (pH 6.0) precooled to 0 °C. DOPA asymmetry was assayed employing the TNS protocol.

the neutral (protonated) form of PA is the permeating species. Indeed, it is generally accepted that weak acids and bases permeate through membranes in the neutral form [see, for example, Gutnecht and Walter (1981b)]. However, it has been reported that weak bases can cross lipid bilayers in the charged form (McLaughlin, 1975) and that fatty acids can act as proton ionophores (Gutnecht & Walter, 1981a) which implies they can move across the membrane in both the charged and uncharged form. As previously shown for PG (Redelmeier et al., 1990), determination of the rate constant k as a function of exterior pH provides a method for unambiguously determining whether the neutral form is the primary permeating species, as k should vary linearly with the exterior proton concentration via the relation $k = [H^+]_0 K/K_a$ if this is the case. As shown in Figure 5A, the rate of PA transport was found to be strongly dependent on the exterior pH, increasing by nearly an order of magnitude for every unit pH_0 is lowered. A plot of log k vs log $[H^+]_0$ (Figure 5C) reveals a straight line with a slope 0.9 ± 0.05, strongly indicating that PA is permeating the membrane in the neutral (protonated) form.

The temperature also strongly influenced the rate of DOPA transport. An analysis of transport rates over the range 25–45 °C revealed that the transport rate increased nearly 5-fold for every 10 °C increase in temperature (results not shown). An Arrhenius plot of the rate constants indicated an activation energy for DOPA transport of 28 kcal/mol or 117 kJ/mol.

PA Asymmetry in LUVs with an Acidic Interior. The results to this point demonstrate transport of DOPA from the outer monolayer to the inner monolayer of LUVs with a basic interior. Conversely, it would be expected that in LUVs with an acidic interior, PA should move from the inner to the outer monolayer. This behavior is illustrated in Figure 6 where it is shown that the percentage of DOPA in the outer monolayer increases from 50% of the total DOPA to more than 85% over a 1-h time course for DOPC/DOPA (95:5, mol/mol) LUVs with an initial interior pH of 4.0 and an exterior pH of 9.0.

As indicated above, the TNS assay for asymmetry should be generally applicable to determine the transbilayer distributions of a variety of acidic lipids in addition to PA and PG. We have therefore employed this assay to determine possible ΔpH-induced asymmetry in DOPC systems containing 5 mol % bovine liver PI, DOPS, and 3 mol % bovine heart cardiolipin.

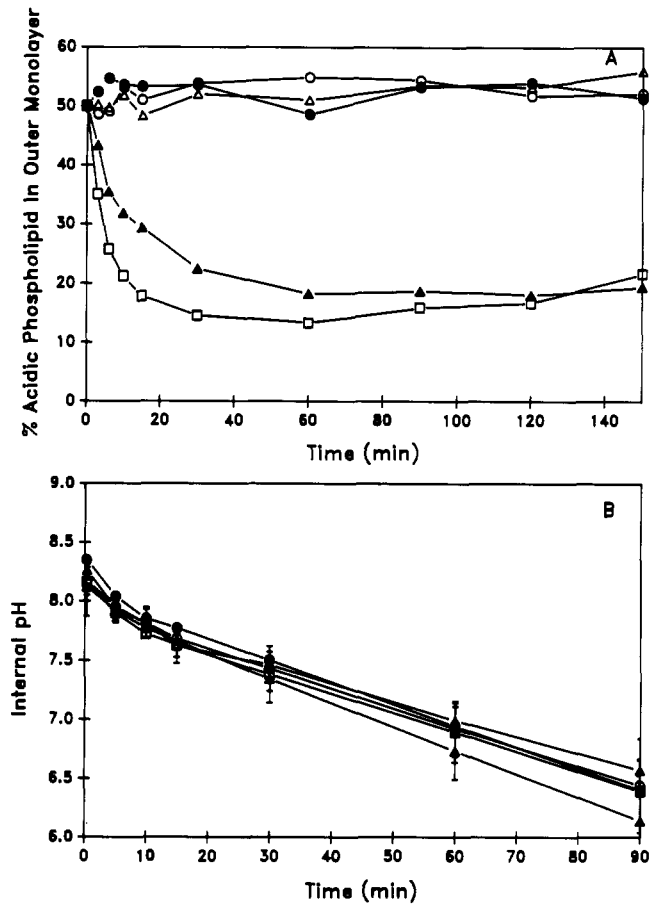

FIGURE 7: (A) Transbilayer distributions of acidic phospholipids following incubation at 45 °C in the presence of a transmembrane pH gradient (pH_i = 9.0, pH_0 = 4.0). The LUVs were prepared, pH gradients were established, and the amount of acidic lipid in the outer monolayer was assayed as indicated under Materials and Methods. The lipid compositions corresponding to the various symbols are as follows: (□) DOPC/DOPA (95:5, mol/mol); (▲) DOPC/EPG (95:5, mol/mol); (●) DOPC/bovine brain PI (95:5, mol/mol); (△) DOPC/DOPS (97:3, mol/mol); (○) DOPC/bovine heart cardiolipin (97:3, mol/mol). (B) Residual pH gradients detected employing pyranine as a probe of internal pH. Vesicles were prepared as described earlier except that 1 mM pyranine was added to the internal buffer. The internal pH of the vesicles was monitored (see Materials and Methods) by measuring the fluorescence of samples using excitation wavelengths of 405 and 463 nm and an emission wavelength of 511 nm.

As shown in Figure 7A, no ΔpH-induced asymmetry could be detected at 45 °C for these phospholipids under conditions similar to those for which DOPA and egg PG exhibit considerable transbilayer movement. This is in agreement with preliminary observations described elsewhere (Hope et al., 1989). As shown in Figure 7B, this inability to induce asymmetry for PI, PS, and cardiolipin does not arise from depletion of the transmembrane pH gradient, as ΔpH values in excess of 2 units are maintained over the experimental time course.

Discussion

The results of this investigation establish TNS as a useful probe for determining transbilayer distributions of acidic lipids in LUV systems and provide new information on the mechanism and kinetics of the transbilayer movement of PA. With regard to the TNS assay, the obvious advantages are convenience and generality. Tedious syntheses to achieve ^{13}C-labeled or spin-labeled varieties of acidic phospholipids are avoided, the behavior of the acidic phospholipid itself (rather than a labeled variety) is detected, and the assay is relatively

rapid. A potential disadvantage of the TNS assay is that the asymmetry to be assayed must be relatively stable. This is clearly not a problem for the DOPA asymmetries assayed here—the half-time for transbilayer movement of DOPA at 20 °C and pH 6.0 can be estimated from the results of this study as 12.6 days (see below). Such stable asymmetries are not available for other lipids which are weak acids, such as fatty acids, which exhibit much faster transbilayer diffusion rates (Hope & Cullis, 1987).

With regard to the mechanism of DOPA transport in response to transmembrane pH gradients, the results of this investigation strongly support a first-order process involving permeation of the neutral (protonated) form. The linear dependence of the rate constant on the exterior proton concentration $[H]_0$ provides particularly compelling evidence in this respect. This behavior is consistent with that previously documented for EPG and DOPG (Redelmeier et al., 1990) and the generally accepted view that weak acids permeate through membranes in the neutral form (Gutnecht & Walter, 1981b). Within this formalism, the rate constant k can be written as $k = [H^+]_0 K/K_a$ where K is the rate constant for transbilayer movement of the neutral form and K_a is the weak acid dissociation constant. Given that $k = 1.67 \times 10^{-1}$ min^{-1} at 45 °C for DOPA in LUVs with an exterior pH of 4.0, we obtain $K = (1.67 \times 10^3)K_a$ min^{-1}, corresponding to $K \approx 1.67$ min^{-1} ($t_{1/2} = 25$ s), assuming a K_a for DOPA of 10^{-3} (Tocanne & Teissie, 1990). This is somewhat smaller than, but comparable to, the rate constant for the neutral form of PG under similar conditions ($K = 6$ min^{-1}; Redelmeier et al., 1990). A more precise comparison is difficult to achieve given the variability in K_a values reported for PG and PA (Tocanne & Teissie, 1990).

The high activation energy (28 kcal/mol) observed for DOPA transport is similar to that observed for EPG (31 kcal/mol) and likely reflects requirements related to dehydration of the phospholipid headgroup, as discussed elsewhere for PG (Redelmeier et al., 1990). In this regard, the similarity between PA and PG activation energies clearly establishes the (protonated) phosphate group as the dominant impediment to transbilayer diffusion. It should also be noted that the combination of a high activation energy and the linear dependence on the proton concentration imparts an exquisite sensitivity of the rate constant for transbilayer movement of DOPA (and PG) to the experimental temperature and pH. Given $k = 1.67 \times 10^{-1}$ min^{-1} at 45 °C, pH 4.0, a generalized rate constant for DOPA can be written as

$$k(T,\mathrm{pH}) = 1.67 \times 10^{3-\mathrm{pH}} \exp[44.3(1 - 318/T)] \text{ min}^{-1}$$

where T is temperature (in degrees kelvin). Thus, at pH 4 and 60 °C, the rate constant can be calculated to be 1.23 min^{-1} ($t_{1/2} = 34$ s), whereas at pH 7.0 and 20 °C $k = 3.8 \times 10^{-6}$ min^{-1} ($t_{1/2} = 127$ days). This obviously allows the preparation of LUVs exhibiting stable asymmetric transmembrane lipid distributions following a brief incubation at low pH and/or high temperature to induce the asymmetry. Aside from the fact that this provides convenient conditions for assaying asymmetry as indicated above, such systems are of potential utility in their own right. Two areas of interest concern the influence of lipid asymmetry on membrane fusion processes and the influence of lipid asymmetry on the transbilayer movement of other lipids.

The inability to induce asymmetry for other acidic phospholipids (bovine brain PI, bovine heart cardiolipin, and DOPS) is of interest. In the case of DOPS, this can be attributed to the zwitterionic nature of the headgroup, bestowing a net (positive) charge to the headgroup even when both acidic functions are protonated. The lack of response of PI and cardiolipin is surprising, and likely reflects the influence of the bulky polar inositol group and/or low phosphate pK_a values.

In summary, this investigation establishes TNS as a useful probe of asymmetric transbilayer distributions of acidic phospholipids in LUV systems. Application of this assay to monitor the ΔpH-dependent transport of DOPA in LUV systems indicates that DOPA traverses the membrane in the neutral form which exhibits transbilayer redistribution times which can be on the order of seconds. Finally, the sensitivity of the rate constant for transport to pH and temperature allows the generation of systems with relatively stable asymmetric distributions of PA.

Registry No. DOPA, 14268-17-8; DOPG, 62700-69-0.

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