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Association and release of prostaglandin E_1 from liposomes

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Abstract

PGE1–lipid interactions were studied in several liposome systems. Data from both circular dichroic (CD) measurements and differential scanning calorimetry (DSC) indicated that PGE1 in the protonated form seeks the less polar environment of the lipid bilayer. CD measurements made on PGE1 in solution showed that the wavelength of maximum absorbance red shifted approximately 8 nm with decreasing solvent polarity. The CD spectrum of liposomal PGE1 prepared in pH 4.5 but not pH 7.2 buffer was also red shifted. There was no red shift in the CD spectrum of PGE1 detected at pH 4.5 in the absence of phospholipid. DSC measurements on DSPC bilayers prepared with 5 mol% PGE1 at pH 4.5 but not pH 7.2 revealed an almost complete loss of the pre-transition as well as broadening of the main phase transition. The amount of ^{3}H-PGE1 initially associated with EPC, POPC or DSPC liposomes was determined using size exclusion filters and centrifugation. This amount was found to be dependent on the pH of the buffer (pH 4.5 $\gg$ pH 7.2) and fluidity of the bilayer (EPC = POPC > DSPC), but independent of the lamellarity of the liposome. In all cases, addition of cholesterol reduced the amount of PGE1 associated with the liposome. The time-dependent release of PGE1 from the liposomes was determined by rapidly diluting the sample 100-fold into pH 7.2 buffer. Lipid saturation was a key factor influencing this release. Gel-phase liposomes of DSPC showed a rapid initial release ($t_{1/2} < 2$ min) of PGE1, corresponding to the amount in the outer monolayer, followed by a very slow, almost negligible release of the remaining PGE1. A rapid initial release also occurred in fluid-phase membranes, followed by a more gradual release of the remaining PGE1 over several hours. This release rate could be slowed by increasing the lamellarity of these liposomes, or adding cholesterol to decrease the fluidity of the membrane. © 1997 Elsevier Science B.V.

Keywords: Prostaglandin E_1; Phospholipid; Vesicle; Drug delivery; Inflammation

1. Introduction

Prostaglandins are a family of cyclic, oxygenated fatty acids that exert diverse and potent effects on cell function in many organ systems. In particular, prostaglandins E_1 and E_2 have a well documented role in the series of events that encompass the inflammatory cascade [1,2]. Since both pro- and anti-in-

Abbreviations: EPC, egg phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-GA, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[glutaryl]; DOPE-GA, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[glutaryl]; PGE1, prostaglandin E_1; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MLV, multilamellar vesicle; LUV, large unilamellar vesicle; SPLV, stable plurilamellar vesicle

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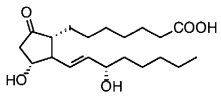
flammatory actions [3–11] have been attributed to these molecules, it is likely that some therapeutic uses could be expanded but these would undoubtedly depend on the timing, dose and targeting of prostaglandins to their sites of action.

A major problem, however, exists regarding some clinical uses of prostaglandin E_1 (PGE1):



When administered intravenously, this molecule is rapidly metabolized during circulation through the lungs, which results in an in vivo half-life of approximately 1 min [12]. In order to circumvent these difficulties, considerable efforts have been made in designing delivery vehicles for PGE1 that might prolong its in vivo half-life and deliver the molecule to its site of action [13–18]. One area of research has been in the design of lipid emulsions to improve the chemical stability and retention of PGE1 [14,16]. Such formulations are reported to exhibit increased biological activity and reduced side effects as compared to the free drug in the treatment of peripheral vascular diseases and diabetic neuropathy [19–21].

Here we describe the physical characteristics of various liposomal formulations as potential PGE1 delivery systems. This investigation has been fuelled by encouraging preclinical [22–26] and clinical [27,28] results using liposomal PGE1 in the treatment of a number of inflammatory diseases. The formulation used in these studies consists of extruded (0.1 μm) liposomes prepared from EPC and PGE1 in a 200:1 molar ratio. At physiologic pH, the PGE1 rapidly dissociates from these vesicles. This combination of liposomes with PGE1 has been shown to toggle the activity of PGE1 from pro- to anti-inflammatory in animal models [22,23] and has also shown significant effect in clinical trials [27,28]. Potentially, the preparation of liposomal PGE1 with increased drug retention could provide access to the treatment of other disease indications.

In this report we describe the association and release characteristics of PGE1 in various liposomal formulations. Factors such as pH, dilution, lipid composition and vesicle lamellarity were investigated.

2. Materials and methods

2.1. Chemicals

EPC, POPC, DSPC, DOPE-GA, DSPE-GA and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). [5,6(n)-^{3}H]PGE1 was obtained from Amersham Life Science (Arlington Heights, IL) and PGE1 from Chinoin Pharm. and Chemical Works (Budapest, Hungary). HPLC grade solvents, methylene chloride, chloroform and methanol were purchased from Baxter (McGaw Park, IL) and sterile 0.9% saline from Abbott Laboratories (North Chicago, IL). Acetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI) and Hepes from Sigma Chemical Co. (St. Louis, MO). All lipids, chemicals and solvents were used without further purification.

2.2. Liposome preparation

Stable plurilamellar vesicles (SPLVs) of EPC, POPC or DSPC and PGE1 were prepared by a variation of the method of Gruner et al. [29]. Briefly, the phospholipid and PGE1 were mixed in organic solvent ($CHCl_3$, CH_2Cl_2, ethanol) and 3% of the total buffer volume (50 mM sodium acetate, pH 4.5, 0.9% saline) was added. The solvent was evaporated under a continuous stream of nitrogen until a wet lipid paste formed. This paste was then hydrated with buffer to the desired volume. Typical concentrations were 10 mg/ml phospholipid and 25 μg/ml PGE1. The SPLVs were then extruded under pressure 10 times through 14 μm polycarbonate filters using a Lipex extruder (Lipex Biomembranes Corp., Vancouver, BC). For DSPC SPLVs, the hydration and extrusion steps were performed in 10 mM sodium acetate, pH 4.5, 0.9% saline at 70°C. Samples were radioactively labeled by adding small quantities of ^{3}H-PGE1 (0.4 μCi/ml) in ethanol to the organic phase before evaporation. For samples containing either cholesterol (10, 30 or 40 mol%), DOPE-GA (10 mol%) or DSPE-GA (10 mol%), these lipids (in $CHCl_3$) were added to the organic solvent phase and the POPC or DSPC molar concentration was reduced appropriately. All liposome preparations used in the release assays had a molar ratio of total lipid to PGE1 of 200:1. To determine the apparent partition coefficient

of liposomal and aqueous PGE1 at pH 4.5, samples were made at 0.5–8 mol% PGE1 by holding the PGE1 concentration constant at 25 μg/ml and decreasing the lipid concentration.

Unilamellar liposomes of DSPC were made by combining phospholipid and PGE1 in organic solvent then drying by rotary evaporation. Multilamellar vesicles were formed from this film by adding buffer (10 mM sodium acetate, 0.9% saline, pH 4.5) and incubating at 70°C for 30 min, vortexing periodically. Typical concentrations were 4 mg/ml phospholipid and 10 μg/ml PGE1. After 10 freeze/thaw cycles, the sample was extruded 10 times through two-stacked 0.1 μm polycarbonate filters at 70°C. Unilamellar liposomes of EPC/PGE1 were made in a similar way, except the lipid film was hydrated in 50 mM sodium acetate, and all steps were carried out at room temperature. Vesicle size was confirmed by quasi-elastic light scattering using a Nicomp Model 270 submicron particle sizer (Nicomp Instruments, Inc., Goleta, CA).

2.3. Circular dichroic measurements of PGE1

Circular dichroic measurements of PGE1 were made using a JASCO J-710 Spectropolarimeter (Japan Spectroscopic Co., Tokyo). Spectra were obtained between 250 nm and 350 nm in order to monitor the $n \rightarrow \pi^*$ transition of the carbonyl group of PGE1. For these experiments, liposomes composed of EPC (2 mg/ml) and PGE1 (50 μg/ml) were prepared via extrusion through 50 nm polycarbonate filters. Small extruded liposomes were used for these experiments in order to minimize light scattering. Samples were prepared in 50 mM sodium acetate, 0.9% saline (pH 4.5), as well as in 10 mM Hepes, 0.9% saline (pH 7.2). CD measurements were made at room temperature using a 10 mm path length quartz cell and a scanning speed of 50 nm/min. A minimum of eight scans was collected for each sample. Appropriate blanks were subtracted before reporting molar circular-dichroic absorbance (cm^2/mmol).

2.4. Differential scanning calorimetry

Phase transition temperatures of multilamellar vesicles were determined using a differential scanning calorimeter (Microcal MC-2 with DA-2 data acquisition software, Northampton, MA). Multilamellar vesicles were prepared by dispersing a thin film of DSPC (100 mg) in the absence and presence of PGE1 (2.5 mg) in 25 ml of 10 mM acetate buffer (150 mM NaCl, pH 4.5). The lipid was hydrated at 65°C for 0.5 h. Their melting behavior was measured using that same buffer solution as a reference. Heating and cooling scans were recorded from 10°C to 65°C at a rate of 20°C/h. Samples were equilibrated at the starting temperature for at least 1 h prior to each scan.

2.5. NMR spectroscopy

Measurements were completed on a Bruker AC300 spectrometer operating at 121.5 MHz. In a typical experiment, the ^{31}P signals were collected using a 90° pulse of 15 μs for the total 3000 transients. The gated proton decoupler was used to suppress phosphorus–proton couplings with exponential line broadening of 50 Hz applied to all FIDs. EPC (50 mg/ml)/PGE1 (125 μg/ml) SPLVs were made as described above. Samples were then extruded 10 times through a 14 μm or 0.8 μm polycarbonate filter. In addition, one sample was frozen and thawed 10 times then extruded through a 0.1 μm filter. The lamellarity of the liposomes was determine by titrating the relaxation agent Mn^{2+} (as $MnCl_2$) into each sample [30], and recording spectra at each titration point. The integrated area of the EPC peak was determined relative to an external triphenyl phosphite standard. The percent of total lipid exposed to the bulk aqueous phase was calculated from the ratio of EPC area at saturating Mn^{2+} to EPC area at 0 mM Mn^{2+}.

2.6. Measurement of initial association and release of PGE1 from liposomes

The amount of PGE1 initially incorporated into the liposomes was determined using ^{3}H-PGE1. Liposomes and associated PGE1 were separated from bulk buffer and unassociated PGE1 by centrifugation using Centricon-30 microconcentrators (Amicon, Beverly, MA) in a fixed-angle rotor at 3000–5000 $\times g$ for 15–60 min at 23°C. Aliquots of the starting liposome preparation and the lipid-free filtrate were counted on a LS6800 liquid scintillation counter (Be-

ckman Instruments, Irvine, CA). The percentage of PGE1 associated with the liposome was calculated from the radioactivities in the filtrate and the starting sample.

The time-dependent release of PGE1 from liposomes after dilution was determined by rapidly mixing 1 ml of the tritiated sample with 100 ml of 10 mM Hepes, 0.9% saline, pH 7.2. PGE1 is monomeric under neutral (0–0.4 mM, the highest concentration is a saturated solution) as well as basic (0–45 mM) conditions [31]. All experiments were performed at room temperature, except those using DPPC MLVs at 55°C, as noted. Aliquots were removed at various time points ranging from 3 min to 20 h. The liposome-associated and non-associated PGE1 were separated by centrifugation in a fixed-angle rotor at 5000–8000 $\times g$ for 1–2 min using either Centricon-30 or Microcon-100 microconcentrators (Amicon, Beverly, MA). No difference was found between the two types of concentrators or speed of centrifugation. The microconcentrators were also tested using radiolabeled liposomes to ensure that the filtrate did not contain PGE1 that was still liposome-associated. The percentage of PGE1 that remained associated with the liposomes was calculated as described above for initial incorporation. Control experiments were also performed in order to ensure that the PGE1 release profiles were indicative of the kinetics of PGE1 release, not PGE1 equilibration. A 50-fold and a 250-fold dilution were substituted for the 100-fold dilution. Under both of these conditions, the same release profiles were obtained.

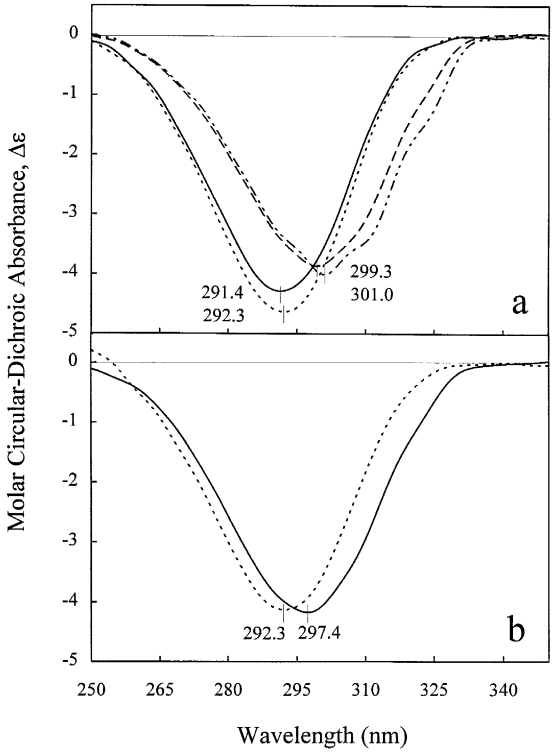


Fig. 1. Circular dichroic spectra of 'free' and liposomal PGE1. (a) Circular dichroic spectra of PGE1 (50 μg/ml) dissolved in ethanol (---), hexane (– · · –) and aqueous buffers at pH 4.5 (————) and pH 7.2 (· · ·). The vibrational fine structure evident in the CD spectrum of PGE1 dissolved in hexane has been observed for other carbonyl compounds in hydrocarbon solvents [32]. (b) Circular dichroic spectra of liposomal PGE1 in aqueous buffers at pH 4.5 (————) and pH 7.2 (· · ·). In both cases, the LUV dispersion was prepared by extrusion through 50 nm polycarbonate filters and consisted of EPC (2 mg/ml) and PGE1 (50 μg/ml).

3. Results and discussion

3.1. PGE1 associates with lipid membranes at pH 4.5

Circular dichroic (CD) measurements of optically active carbonyl compounds, such as PGE1, are known to be sensitive to solvent environment [32]. Specifically, the $n \rightarrow \pi^*$ transition of the carbonyl group is red shifted with decreasing solvent polarity. The absence of hydrogen bonding in nonpolar solvents raises the energy level of the n orbital, thus decreasing the $n \rightarrow \pi^*$ transition energy. We hypothesized that this property could be used as an indicator of liposomal PGE1 environment (surface vs. intra-membrane association). CD spectra of free and liposomal PGE1 were obtained between 250 nm and 350 nm, as shown in Fig. 1. As expected, the wavelength of maximum absorbance of free PGE1 red shifted approximately 8 nm with decreasing solvent polarity. The magnitude of this shift is typical of that seen with other carbonyl compounds [32]. The wavelength of maximum absorbance of free PGE1 in aqueous buffer remained unchanged at pH 4.5 or pH 7.2. In contrast, the CD spectra of liposomal PGE1 (Fig. 1b), was dependent on the pH of the buffer used to prepare the sample. The spectrum in pH 7.2 buffer was very similar to that of free PGE1 in buffer at pH 7.2 or pH 4.5. In this case, very little (9%) of the

PGE1 was liposome associated (detected using tritiated PGE1). At pH 4.5 however, where approximately 80% of the PGE1 was found to be associated with the liposome, the spectrum was red shifted. This data indicates that, at pH 4.5, PGE1 locates within the lipid bilayer. In these fluid-phase membranes the bulk of the PGE1 molecule likely resides primarily in the head group region with the hydrophobic C_{20} end penetrating to approximately the C_2–C_5 region of the bilayer. The pK_a of PGE1 in water is reported to be 4.8, as determined by methanol–water titrations [33]. Thus, at pH 7.2 PGE1 exists predominantly as the carboxylate anion. Significant liposome association is only achieved at lower pH where a larger percentage of PGE1 is protonated and incorporation into the lipid membrane of the less water-soluble species becomes thermodynamically favorable.

Further evidence of PGE1 membrane association was provided from differential scanning calorimetry experiments gathered from multilamellar vesicles composed of DSPC (4 mg/ml) prepared in 10 mM sodium acetate (pH 4.5) in 0.9% saline (Fig. 2). PGE1 decreased the main phase transition temperature of DSPC by 0.5°C and increased the peak width at half-height by five-fold. The pre-transition was almost completely obliterated. This is typical of changes that can occur when a perturbing molecule is present within the bilayer [34–37]. When we performed the same experiments using liposomes prepared in pH 7.2 Hepes buffer (data not shown), the main phase transition of DSPC was unaffected but

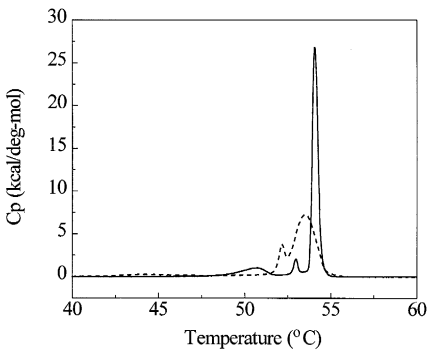


Fig. 2. High-sensitivity excess heat capacity profiles of DSPC bilayers in the absence (————) and presence (---) of PGE1. MLVs were prepared in 10 mM acetate buffer (150 mM NaCl, pH 4.5). The sub-main phase transition evident in both DSC traces is caused by a salt effect [55,56].

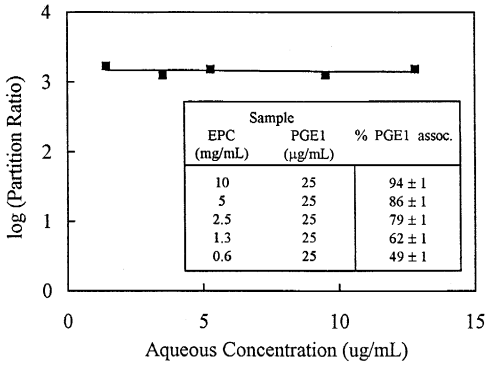


Fig. 3. PGE1 partitions between aqueous and organic (lipid) phases. Plot of the log of the partition ratio as a function of PGE1 concentration in the aqueous phase. EPC/PGE1 SPLVs in 50 mM sodium acetate in 0.9% saline (pH 4.5) were prepared such that the lipid concentration varied from 10 to 0.6 mg/ml while the PGE1 concentration was held constant at 25 μg/ml. Each preparation was labeled with tritiated PGE1 in order to determine the extent of liposome association (as described in Section 2). This data is shown in the inset chart. The apparent partition coefficient, K_{app}, was determined for each preparation using the concentration of PGE1 found in the aqueous and lipid phases.

the pre-transition was lowered slightly (1°C). Using tritiated preparations, we found that in these cases only approximately 10% of the PGE1 was liposome-associated. This low level of association was either insufficient to cause observable endotherm broadening or the deprotonated molecule bound only to the interfacial regions of the membrane causing no disruption of acyl-chain packing.

The partitioning behavior of PGE1 between immiscible organic (lipid) and aqueous phases is shown in Fig. 3 for EPC/PGE1 SPLVs in 50 mM sodium acetate in 0.9% saline (pH 4.5). Partition coefficients measured for PGE1 in several liposomal preparations are tabulated in Table 1. In each case the lipid concentration varied from 10 to 0.6 mg/ml while the

Table 1
PGE1 apparent partition coefficients in several liposomal formulations

Liposome formulation	K_{app}
DSPC-MLV	7.4×10^2
DSPC/CHOL(90:10)-MLV	3.1×10^2
EPC-SPLV	1.5×10^3
EPC-MLV	2.4×10^3
EPC/CHOL(90:10)-MLV	1.6×10^3

PGE1 concentration was held constant at 25 μg/ml. Each preparation was labeled with tritiated PGE1 in order to determine the extent of liposome association. The apparent partition coefficient:

$$K_{app} = [PGE1]_{lipid}/[PGE1]_{aq}$$

was determined for each preparation using the concentration of PGE1 found in the aqueous and lipid phases. These calculations were made using values of 0.983 ml/g and 0.952 ml/g for the specific volume of EPC [38] and DSPC [39], respectively, and a value of unity for the activity coefficient.

The apparent partition coefficients measured for fluid-phase bilayers composed of EPC or EPC/cholesterol agreed well with the octanol–water partition coefficient of PGE1 measured at pH 4.5 by Avdeef et al. [33]. A significant decrease (3–8-fold) in PGE1 partitioning was observed in DSPC or DSPC/cholesterol gel-phase bilayers. This decrease is a likely consequence of the increased surface density of the bilayer chains, which leads to solute exclusion [40,41].

3.2. The association of PGE1 with liposomes prepared in pH 4.5 buffer is dependent upon the lipid composition

As predicted by the apparent partition coefficients measured for PGE1 in fluid- and gel-phase membranes, the degree of association of PGE1 with liposomes prepared in pH 4.5 buffer was found to depend upon lipid composition (Table 2). PGE1 was more highly incorporated into fluid unsaturated (EPC or POPC) than gel-phase saturated (DSPC) bilayers. It is not surprising that fluid-phase lipid membranes with a higher degree of alkyl-chain disorder accommodate the functionalized cyclopentane ring of PGE1 more effectively than tightly packed and highly ordered gel-phase membranes.

Cholesterol reduced PGE1 association only slightly in fluid-phase bilayers but quite significantly in gel-phase bilayers (note the 2-fold decrease in K_{app} for DSPC/cholesterol bilayers as compared to DSPC bilayers in Table 1). We suspect that the fused-ring structure of cholesterol in combination with gel-phase lipid is highly dimensionally uncomplimentary to that of PGE1. The fluidizing effect that cholesterol has on gel-phase lipid [42] was overshadowed by these geometric constraints.

Table 2
Percentage of PGE1 initially associated with various liposome formulations

Liposome formulation [a]	PGE1 associated (%)
DSPC-LUV [b]	68 ± 1
DSPC-SPLV [c]	84 ± 1
DSPC/DSPE-GA (90:10)-LUV [b]	72 ± 1
DSPC/CHOL (90:10)-LUV [b]	45 ± 1
DSPC/CHOL (60:40)-LUV [b]	7 ± 2
EPC-LUV [c]	94 ± 3
EPC-SPLV [c,d]	94 ± 1
EPC-SPLV [c,e]	94 ± 1
POPC-SPLV [c]	95 ± 2
POPC/DOPE-GA (90:10)-SPLV [c]	93 ± 3
POPC/CHOL (90:10)-SPLV [c]	93 ± 2
POPC/CHOL (70:30)-SPLV [c]	78 ± 3

[a] Formulations at total lipid:PGE1 mol ratio of 200:1.
[b] 10 μg/ml PGE1.
[c] 25 μg/ml PGE1.
[d] EPC SPLV with 10% of total phospholipid located in the outer leaflet.
[e] EPC SPLV with 20% of total phospholipid located in the outer leaflet.

The effect of negative surface charge on PGE1 incorporation was investigated by the addition of the phospholipid derivatives DSPE-GA or DOPE-GA into liposomes prepared from saturated and unsaturated lipids, respectively. These preparations were of interest to us from an experimental as well as a practical point of view because these lipid derivatives are known to increase the circulation lifetime of liposomes in vivo [43,44]. As shown in Table 2, the addition of 10 mol% of these negatively charged lipid derivatives had no effect on the degree of PGE1 incorporation.

Association was also independent of liposome lamellarity, as shown by the identical incorporation of PGE1 into EPC LUVs and SPLVs. A slightly higher PGE1 association is reported for DSPC SPLVs than LUVs because the SPLVs were prepared at a higher concentration.

3.3. The dissociation of PGE1 from liposomes at physiologic pH is dependent upon liposome lamellarity and lipid composition

The release of PGE1 from liposomes prepared in either 10 or 50 mM sodium acetate and 0.9% saline

(pH 4.5) was measured using a method that relies upon a 100-fold dilution of the sample into a pH 7.2 Hepes buffer. These conditions were chosen in order to mimic the pH change and rapid sample dilution that would occur in vivo. Results from these experiments are shown in Fig. 4. We studied the release of PGE1 from EPC LUVs that were prepared by extrusion through 0.1 μm filters. Quasi-elastic light scattering measurements revealed them to have a mean diameter of approximately 91 nm. The integration of ^{31}P-NMR spectra in the absence and presence of Mn^{2+} revealed approximately 43% of the total phospholipid was located in the outer leaflet. Release of PGE1 from these single lamellar liposomes was rapid and essentially complete within 3 min, which was the first convenient measurement point. Upon dilution into pH 7.2 buffer, the PGE1 putatively located in the outer leaflet was rapidly deprotonated, becoming much more soluble in the aqueous phase. PGE1 located in the inner leaflet was also rapidly released from the vesicle, which indicates that in this situation there was virtually no barrier to PGE1 'flip-flop' across the membrane.

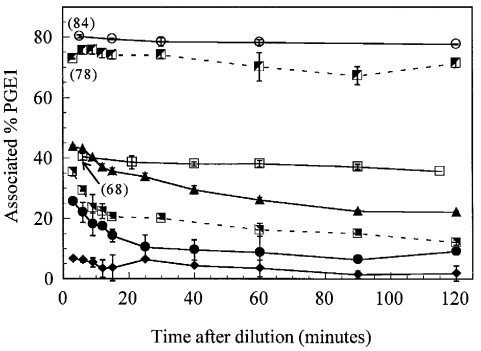


Fig. 4. Dissociation of PGE1 from liposomes at physiologic pH depends upon liposome lamellarity and lipid composition. PGE1 release profiles from DSPC SPLVs (○) and LUVs (□), DPPC MLVs (rt) (◩) and 55°C (◥), EPC SPLVs with 10% (▲) and 20% (●) of total phospholipid located in the outer leaflet, EPC LUVs with 43% (♦) of total phospholipid located in the outer leaflet. Lamellarity calculations are based on the integration of ^{31}P-NMR spectra in the absence and presence of Mn^{2+}. In all cases, 1 ml of the preparation was rapidly diluted with 100 ml of 10 mM Hepes in 0.9% saline (pH 7.2). Aliquots were removed at various time points (3 min to 20 h) and the liposome-associated and non-associated PGE1 were separated by centrifugation using microconcentrators. Data is plotted as the percentage of PGE1 remaining associated with the liposome as a function of time. The graphs are not normalized; 100% retention is equal to the total amount of PGE1 initially present (liposome associated and non-associated) in each preparation. In each case, the percentage of PGE1 initially associated with the liposome was 94%, except where noted in parentheses.

Similar rapid release of PGE1 from 200 nm lipid microspheres (Lipo-PGE1) has been reported [45]. Lipo-PGE1 is a 10% oil in water (pH 4.5–6.0) emulsion prepared from soyabean oil, EPC and other components. The release of PGE1 from these microspheres was determined by ultrafiltration in a similar manner to the method we report here and was found to be over 90%. Conflicting results using a dialysis method to measure PGE1 release from Lipo-PGE1 have also been reported [13,46]. In this case, Lipo-PGE1 was enclosed in cellulose tubing and immersed into pH 7.4 buffer (10-fold increase in volume) at 20°C. In these studies only 50% of the PGE1 was released after 2 h but the rate-limiting step was probably diffusion through the cellulose membrane *not* release of PGE1 from the lipid microspheres. We have found that equilibration of free PGE1 across dialysis membranes requires extended time frames ($t > 60$ min).

PGE1 release kinetics from DSPC LUVs were in marked contrast to the release profile from EPC LUVs (Fig. 4). LUVs prepared from DSPC released only approximately 50% of the initially associated PGE1. After this rapid release phase the remaining fraction of PGE1 (ca. 48% of initially associated PGE1) remained associated for > 5.5 h. Presumably, PGE1 exposed to the pH 7.2 buffer in the outer leaflet was rapidly deprotonated and released from the membrane. PGE1 located in the interior leaflet was rigidly embedded in the tightly packed gel-phase lipid and transbilayer movement ('flip-flop') across the membrane is essentially abolished.

The release kinetics of PGE1 from multilamellar fluid- and gel-phase membranes are also shown in Fig. 4. Release profiles of PGE1 from EPC SPLVs with 10% or 20% of total phospholipid located in the outer leaflet (data from ^{31}P-NMR) showed a rapid initial release of PGE1, followed by a more gradual release over a period of several hours. (DSPC SPLVs showed long-term retention of the bulk of the initially associated PGE1). Clearly, liposome lamellarity plays a significant role in the retention of PGE1. For multilamellar vesicles, the majority of PGE1 is located within internal lipid membranes. Release of the

drug requires crossing multiple aqueous compartments and lipid bilayers, which greatly attenuates its release from fluid-phase systems. For gel-phase systems the fraction of PGE1 located in internal lamellae is apparently unable to gradually migrate through successive bilayers, only material in the outer leaflet is released. This conclusion is supported by data collected on the release of PGE1 from DPPC MLVs at room temperature and 55°C (Fig. 4). Below the phase transition these liposomes showed long-term retention of the bulk of the initially associated PGE1, similar to the DSPC SPLVs. However, above the phase transition a rapid release phase is followed by a gradual release over a period of several hours, similar to fluid-phase EPC multilamellar membranes. These results strongly support our conclusion that the rate of transbilayer movement of PGE1 is greatly influenced by the physical state of the membrane.

The effect of lipid composition on PGE1 dissociation was further investigated for POPC and DSPC liposomes that incorporated cholesterol or negative surface charge. The addition of increasing amounts of cholesterol to POPC SPLVs decreased the PGE1 release rate, particularly the initial rapid release phase, from these systems (Fig. 5a). The effect of cholesterol on lipid bilayers has been extensively studied [47–53]. In a membrane above its phase transition, cholesterol reduces the freedom of the lipid acyl chains. This results in bilayers that are more tightly packed and less fluid, more like a membrane composed of lipids that are in the gel phase. Dissociation of PGE1 from this type of membrane is more difficult, as previously determined for gel-phase DSPC bilayers.

On the other hand, addition of increasing amounts of cholesterol to DSPC LUVs had a profound effect on the ability of PGE1 to be incorporated into these membranes (Table 2) but no effect on release (Fig. 5b). The release profiles were identical, after considering the amount of PGE1 initially associated in each liposome preparation. In all cases approximately 50% of the PGE1 was rapidly released and the remaining fraction had long-term retention. Of course, in DSPC/cholesterol (60/40) LUVs, virtually no PGE1 was initially associated with this membrane.

Incorporation of DOPE-GA or DSPE-GA into liposomes had only a slight effect on the rate of PGE1 release (data not shown). Both POPC SPLVs, prepared with 10 mol% DOPE-GA, and DSPC LUVs, prepared with 10 mol% DSPE-GA, showed a slight increase in the initial rate of release of PGE1 from the membrane as compared to POPC SPLVs or DSPC LUVs prepared without these lipid derivatives. In both cases, approximately 35% of the initially associated PGE1 remained with the liposome at the first time point measured (3 min). After this rapid initial release the data were identical to the underivatized liposomes in both cases. This small increase in the initial rate of PGE1 release was likely due to charge repulsion or the change in liposome morphology produced by the incorporation of these molecules [54].

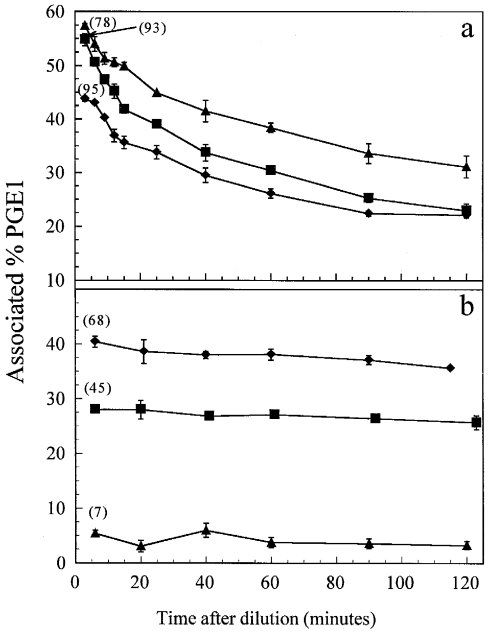


Fig. 5. Effect of cholesterol on PGE1 release. (a) PGE1 release profiles from POPC SPLVs prepared with 0% (♦), 10% (■), and 30% (▲) cholesterol. (b) PGE1 release profiles from DSPC LUVs prepared with 0% (♦), 10% (■), and 40% (▲) cholesterol. Experimental conditions are the same as described in Fig. 4. The value in parentheses is the percentage of PGE1 initially associated with the liposome.

4. Conclusions

We have shown that protonated PGE1 associates with lipid membranes and the degree of this association is highly compositionally dependent. The disso-

ciation of PGE1 from such systems was measured using rapid dilution at physiologic pH and could be related to the degree of lipid saturation, lamellarity and negative surface charge in a systematic fashion. Fluid-phase membranes accommodated more PGE1 than gel-phase membranes. Addition of cholesterol reduced the ability of both fluid- and gel-phase membranes to accommodate this molecule. At physiological pH values PGE1 embedded in gel-phase membranes showed greatly reduced release kinetics compared to PGE1 embedded in fluid-phase membranes.

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