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Stability and fusion of lipid vesicles containing headgroup-modified analogues of phosphatidylethanolamine

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We have used lipid mixing, contents mixing and contents-leakage assays to characterize the divalent cation-mediated interactions of vesicles composed of various headgroup-modified analogues of phosphatidylethanolamine, PE (N- and C-2-alkylated derivatives, and analogues with increased separations of the phosphoryl and amino groups) together with a low mole percentage of phosphatidylserine (PS). Vesicles containing different structural analogues of PE exhibit marked differences, both in the threshold divalent cation concentrations that are required to initiate vesicle–vesicle interactions and in the rates of contents mixing and leakage observed at suprathresholds divalent cation concentrations. The efficiencies of divalent cation-promoted contents leakage, and to a slightly lesser extent those of contents mixing, for PS/PE (analogue) vesicles show a marked inverse correlation with the lamellar-to-hexagonal II transition temperature (T_H) of the PE (analogue) component. However, the destabilization kinetics for such vesicles show no abrupt changes over the temperature range around the equilibrium T_H value measured for the vesicle lipids. Vesicles combining PS with different PE analogues exhibit divalent cation thresholds for aggregation that are not correlated with the T_H values of the PE (analogue) components but appear instead to be correlated with the equilibrium interbilayer separations measured in multilamellar dispersions of these species. We have identified headgroup-modified analogues of PE that can be used to prepare vesicles that fuse more rapidly under a given set of conditions, or that show a better ratio of fusion-to-contents-leakage rates, than do PE-containing vesicles. These results may be useful both for understanding better the bases for the high fusion-supporting ability of PE and for the preparation of lipid vesicles 'tailored' for particular practical applications.

Introduction

Of the neutral phospholipids commonly found in the membranes of higher organisms, PE is exceptional in its tendency to favor the destabilization and fusion of bilayer lipid membranes. This property of PE has been exploited in the construction of various types of PE-rich lipid vesicle, including immunoliposomes [1–3], pH-sensitive liposomes [4–13] and cationic liposomes [14,15], which have been used to deliver hydrophilic bioactive molecules to animal cells.

The relatively weak hydration of the PE headgroup [16] and the ability of unsaturated PEs to form non-lamellar structures under near-physiological conditions [17–19] have been suggested to be important physical determinants of the strong fusion-supporting activity of this lipid [16–26]. The relative importance of these (and possibly additional) factors in determining the overall fusion-promoting ability of PE remains to be fully assessed. The specific structural features of the PE headgroup that determine the fusion-supporting properties of this lipid have also so far not been definitively assigned. Among factors of possible importance in this regard are the relatively small size of the PE headgroup, the high hydrogen-bonding capacity of the amino group,

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Abbreviations: ANTS, 8-aminonaphthalene-1,3,5-trisulfonic acid, disodium salt; DPX, *N,N′-p*-xylylenebis(pyridinium bromide); DEPB, dielaidoylphosphatidyl-4-amino-1-butanol; DEPE, dielaidoylphosphatidylethanolamine; DEPP; dielaidoylphosphatidyl-3-amino-1-propanol; DEPS, dielaidoylphosphatidylserine; DOPB, dioleoylphosphatidyl-4-amino-1-butanol; DOPP, dioleoylphosphatidyl-3-amino-1-propanol; DOPE, dioleoylphosphatidylethanolamine; DOPS, dioleoylphosphatidylserine; LUV, large unilamellar vesicle(s); *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; OAP, *N*-oleoyl-2-aminopalmitic acid; PE, phosphatidylethanolamine; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPP, 1-palmitoyl-2-oleoylphosphatidyl-3-amino-1-propanol; PS, phosphatidylserine; *N*-Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; Tes 2[{2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid, sodium salt; T_H, lamellar-to-hexagonal II transition temperature.

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and an optimal spacing of the amino and phosphoryl groups that may facilitate the formation of cooperative networks of hydrogen bonds among neighboring lipid molecules [27].

We have recently characterized the physical properties of a variety of PE analogues with modified headgroup structures [28,29]. The results of these studies have helped to define better the structural bases for some of the distinctive physical properties of PE, including its relatively weak surface hydration and its propensity to form nonlamellar structures. In the present study, we have examined the fusion-supporting abilities of a variety of PEs and headgroup-modified PE analogues by measuring the cation-regulated interactions of LUVs that combine these species with low mole fractions of anionic lipids. The results of these experiments help to define better the physical and structural features of PE that constitute the primary determinants of its strong fusion-supporting abilities. Through this work, we have identified certain PE analogues that can be used to prepare lipid vesicles that show higher 'fusion competence' than do vesicles containing the corresponding PE. These analogues of PE may be useful in the construction of liposomes that offer enhanced rates of fusion and/or destabilization under particular conditions for specific practical applications

Materials and Methods

Materials

Derivatives of PE in which the ethanolamine moiety is replaced by 3-amino-1-propanol, 4-amino-1-butanol, DL-2-amino-1-propanol, DL-2-amino-1-butanol, *N*-methylethanolamine or *N*-ethylethanolamine were prepared from the corresponding phosphatidylcholines (obtained from Avanti Polar Lipids, Birmingham, AL) by phospholipase-D-mediated transphosphatidylation as described previously [30,31]. Phosphatidylserines, phosphatidyl 2-amino-2-methyl-1-propanols and phosphatidyl 2-amino-3-methyl-1-butanols were synthesized from the corresponding phosphatidic acids as described elsewhere [28,29,32]. N-Palmitoyl-2-aminopalmitic acid was synthesized as described previously [11]. *N*-NBD-PE was obtained from Avanti Polar Lipids, and *N*-Rho-PE was synthesized from transphosphatidylated egg PE as described elsewhere [33].

ANTS and DPX were obtained from Molecular Probes (Eugene, OR) and used as received. Diethyl ether used in the preparation of reverse-phase evaporation vesicles was twice washed with distilled water and then redistilled before use. All other solvents used in lipid preparations were redistilled before use. All inorganic chemicals and buffers were of at least reagent grade.

Methods

LUVs were prepared by reverse-phase evaporation and filtration through 0.1 μm pore-size Nucleopore membranes as described by Wilschut et al. [34]. Vesicle encapsulated volumes were determined by measuring trapping of either carboxyfluorescein [34] or ANTS/DPX [5]. Lipid mixing between vesicles was assayed by the procedure of Struck et al. [33], using vesicles labeled with 1 mol% NBD-PE and 0.35 mol% *N*-Rho-PE together with a 9-fold excess of unlabeled vesicles. Mixing and leakage of vesicle contents was assayed by the procedures of Ellens et al. [5,9], using vesicles loaded in the former case with either 90 mM DPX or 50 mM ANTS, and in the latter case with 45 mM DPX plus 25 mM ANTS. All solutions used in vesicle preparations contained 0.1 mM EDTA plus 2 mM histidine and 2 mM Tes (pH 7.4) and were adjusted with NaCl to an osmolarity equivalent to that of 150 mM NaCl/2 mM Tes/2 mM histidine/0.1 mM EDTA (pH 7.4). Vesicle phospholipid concentrations were assayed by the procedure of Lowry and Tinsley [35], with the modification that samples were digested for at least 4h.

Lamellar-to-hexagonal II phase transition temperatures of PE (analogue)/PS mixtures were determined using a Microcal MC-1 differential scanning calorimeter as described previously [32]. Samples containing 10 μmol of phospholipid were dispersed in 0.8 ml of 140 mM NaCl/20 mM Tes (pH 7.4), then mixed with calcium or magnesium chloride to a final concentration of 20 mM and incubated for 2 h at 30°C (for POPE-containing samples) or 0°C (for DOPE-containing samples) and finally incubated overnight at 0°C. Sample thermograms were recorded at a heating rate of 24 C°/h.

Results

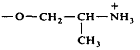
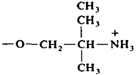
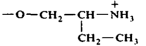
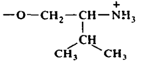
Fusion-supporting ability of head-group modified DEPE analogues

In Table I are shown the structures of the alkanolamine moieties of the headgroup-modified analogues of PE that were examined in this study. All of these PE analogues could be used to prepare reverse-phase evaporation vesicles when combined with 25 mol% PS. After filtration through 0.1 μm Nucleopore filters, the trapped volumes of such vesicles, measured by their retention of carboxyfluorescein, were very similar (typically 2.5–3 μl/μmol lipid), regardless of from which PE analogue they were prepared. All of the experiments described in this paper were carried out using vesicles prepared in this manner.

In Fig. 1 are shown time-courses of calcium-induced lipid mixing, contents mixing and leakage, measured as described in Materials and Methods, for LUVs prepared from DEPE, or one of three headgroup-modified

TABLE I

Structures of the alkanolamine groups of the PE analogues examined

Abbreviation	Alkanolamine group
PE	$-O-CH_2-CH_2-\overset{+}{N}H_3$
PP	$-O-CH_2-CH_2-CH_2-\overset{+}{N}H_3$
PB	$-O-CH_2-CH_2-CH_2-CH_2-\overset{+}{N}H_3$
N-Methyl PE	$-O-CH_2-CH_2-\overset{+}{N}H_2-CH_3$
N-Ethyl PE	$-O-CH_2-CH_2-\overset{+}{N}H_2-CH_2-CH_3$
C-2-Methyl PE	 $-O-CH_2-\underset{CH_3}{CH}-\overset{+}{N}H_3$
C-2-Dimethyl PE	 $-O-CH_2-\overset{CH_3}{\underset{CH_3}{C}}H-\overset{+}{N}H_3$
C-2-Ethyl PE	 $-O-CH_2-\underset{CH_2-CH_3}{CH}-\overset{+}{N}H_3$
C-2-Isopropyl PE	 $-O-CH_2-\underset{CH(CH_3)_2}{CH}-\overset{+}{N}H_3$

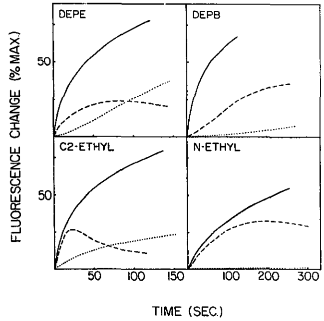


Fig. 1. Time-courses of lipid mixing (———), contents mixing (— — —) and contents leakage (- - - - - -), measured by fluorescence assays as described in Materials and Methods, when vesicles combining 25 mol% DEPS with DEPE, DEPB, dielaidoylphosphatidyl 2-amino-1-butanol (C-2-ethyl) or dielaidoylphosphatidyl-*N*-ethylethanolamine (*N*-ethyl), were exposed at zero time to calcium at a concentration equal to roughly twice the threshold concentration. The curves shown were recorded at 4, 5, 7.5 and 30 mM calcium, respectively, for the vesicles containing DEPE, DEPB, C-2-ethyl DEPE and *N*-ethyl DEPE. All assays were performed at 45 °C, using a total lipid concentration of 30 μM. For the lipid-mixing assay, a 1:9 ratio of labeled to unlabeled vesicles was used, while for the contents-mixing assay, a 1:4 ratio of ANTS-containing to DPX-containing vesicles was used. Other experimental details were as described in Materials and Methods.

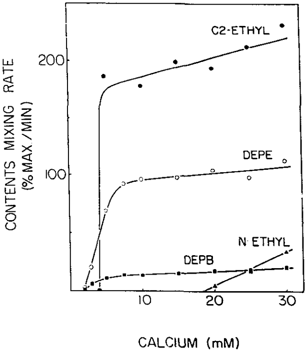


Fig. 2. Initial rates of contents mixing, measured by the ANTS/DPX assay, when vesicles combining 25 mol% DEPS with DEPE (○), DEPB (■), dielaidoylphosphatidyl 2-amino-1-butanol (●) or dielaidoylphosphatidyl *N*-ethyl ethanolamine (▲) were mixed at zero time with calcium chloride at the indicated concentrations. Details of the contents-mixing assay were as described in the legend to Fig. 1.

analogues, together with 25 mol% DEPS. For each of these types of vesicle, the initial rate of lipid mixing is substantially faster than that of contents mixing or of contents leakage *. The effective reaction order for the lipid-mixing process (determined by varying the vesicle concentration) was nearly 2.0 for all of these preparations of vesicles at either high or low concentrations of divalent cations (results not shown), indicating that the process is rate-limited by vesicle aggregation [36]. By contrast, the effective reaction orders for the mixing and leakage of vesicle contents were in most cases substantially lower, indicating that these processes were not strictly aggregation rate-limited.

In Fig. 2, the initial rates of contents mixing measured for the four vesicle preparations described above are shown as a function of the added calcium con-

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* Under our experimental conditions, a single round of interactions between simple pairs of vesicles would produce roughly 45% of the maximum possible fluorescence change in the lipid-mixing assay, roughly 80% of the maximum in the contents-mixing assay and 100% of the maximum in the contents-leakage assay. To avoid excessive (and, to some extent, model-dependent) corrections to the primary data presented in this paper, the initial rates presented in the figures and tables have not been corrected for the above effect. However, this factor should be taken into account in comparing the results of the different types of assay in Tables II and III, particularly in comparing measured rates of lipid mixing to those measured for mixing and leakage of aqueous contents.

centration. Each type of vesicle preparation exhibits a characteristic threshold concentration of calcium, only above which is contents mixing observed. Essentially identical threshold concentrations of calcium were required to produce detectable lipid mixing, contents mixing or contents leakage for vesicles of a given composition (data not shown). For simplicity, in the remainder of this paper we will refer to this quantity simply as the 'calcium threshold' for a given type of vesicle. Since the calcium threshold varies considerably for vesicles with different compositions, it is inappropriate to compare the interactions of different types of vesicle at a single fixed concentration of calcium. Instead, as a more uniform measure of the 'fusion competence' of different types of vesicle, we define a quantity termed the 'efficiency' of contents mixing, which corresponds to the rate of contents mixing at a calcium concentration equal to twice the threshold [32]. Analogous quantities are defined in the same manner for the processes of lipid mixing and contents leakage.

In Table II we compare the divalent-cation thresholds and the efficiencies measured at 42° C for the calcium- and magnesium-induced interactions (lipid mixing, contents mixing and leakage) of LUVs prepared from various DEPE analogues together with DEPS (75:25 mol/mol). The lipid-mixing efficiencies measured for such vesicles generally vary less dramatically with the headgroup structure of the PE analogue component than do the efficiencies of contents mixing and leakage. This result probably reflects the fact that lipid mixing between such vesicles is largely aggregation-limited under our experimental conditions; it was not technically feasible in most cases to overcome this limitation by working at much higher vesicle concentrations [36]. It appears that the rate constant for vesicle aggregation in these systems is in general a less sensitive function of the vesicle composition than are the rate constants for subsequent fusion and destabilization of the vesicles.

As is illustrated in Table II, replacement of the PE component in DEPE/DEPS vesicles by a headgroup-modified analogue can strongly alter both the efficiencies of divalent cation-promoted vesicle fusion and destabilization and the divalent-cation thresholds measured for these processes. Different types of modifications of the PE headgroup produce distinct patterns of effects on these quantities. Vesicles containing *N*-alkyl derivatives of DEPE, for example, exhibit much higher divalent-cation thresholds, and show generally lower efficiencies of fusion, than do similar vesicles containing DEPE. Interestingly, while vesicles containing *N*-ethyl DEPE exhibit much higher divalent-cation threshold than do vesicles containing *N*-methyl DEPE, the efficiencies of contents mixing measured for DEPS/*N*-ethyl DEPE vesicles are comparable to or greater than those measured for DEPS/*N*-methyl DEPE vesicles. This result illustrates the general finding that the efficiencies measured for the fusion (or destabilization) of vesicles combining PS with different analogues of PE show no clear and systematic relationship to the divalent-cation thresholds measured for these vesicles (see Tables I and II and Fig. 2).

In contrast to the behavior of DEPS/*N*-alkyl DEPE vesicles, vesicles that combine DEPS with C-2-alkylated derivatives of DEPE show substantially higher efficien-

TABLE II

Threshold divalent-cation concentrations and efficiencies measured for the calcium- and magnesium-promoted interactions of DEPS/DEPE (Analogue) vesicles

Neutral lipid component	Calcium threshold (mM)	Efficiency of calcium-promoted			Magnesium threshold (mM)	Efficiency of magnesium-promoted			T_H (° C) [b]
		lipid mixing [a]	contents mixing [a]	leakage [a]		lipid mixing [a]	contents mixing [a]	leakage [a]	
DEPE	1.7	107	32.6	3.2	3.0	76	14.1	1.7	63.5
DEPP	2.0	143	15.1	0.9	3.5	93	6.5	0.46	88.5
DEPB	2.0	152	9.3	0.45	4.2	89	6.4	0.14	> 95
N-Methyl DEPE	7.0	160	15.6	1.45	11.0	118	5.3	0.24	> 95
N-Ethyl DEPE	18.2	54.1	52.1	1.35	23	28.7	5.2	0.06	> 95
C-2-Methyl DEPE	4.0	267	47.7	24	7.3	179	18.9	5.2	57.5
C-2-Dimethyl DEPE	5.0	181	145	78	9.5	158	70	39	55.5
C-2-Ethyl DEPE	4.8	175	181	23	9.5	180	242	10	< 47.8 [c]
C-2-Isopropyl DEPE	4.0	122	122	73	7.0	200	162	67	< 62.0 [c]
Dipalmitelaidoyl PE	1.9	154	10.6	1.75	3.0	143	8.1	0.45	92.5

[a] All rates were determined at 42° C and are presented as a percentage of the maximum possible fluorescence change per minute. Other assay conditions were as described in the legend to Fig. 1.

[b] From Refs. 29 and 39.

[c] Values listed are for a direct transition of a dehydrated gel phase to the hexagonal II phase. Since the liquid–crystalline phase is metastable with respect to the dehydrated gel phase at all temperatures below this transition, a direct transition from the liquid–crystalline lamellar phase to the hexagonal II phase could not be observed for these species. However, the temperature of this transition (T_H as defined for the other species listed in this table), if it could be observed, would have to lie below the value listed in the table.

TABLE III

Threshold divalent-cation concentrations and efficiencies measured for the calcium- and magnesium-promoted interactions of vesicles combining DOPS with cis-unsaturated PE analogues

Neutral lipid component	Calcium threshold (mM)	Efficiency of calcium-promoted			Magnesium threshold (mM)	Efficiency of magnesium-promoted			T_H(°C) [b]
		lipid mixing [a]	contents mixing	leakage [a]		lipid mixing [a]	contents mixing [a]	leakage [a]	
DOPE	1.8	70	43	40	3.0	39	13.8	43	8
DOPP	2.0	87	15.7	9.2	3.5	90	10.0	7.4	35
DOPB	2.0	115	17.6	7.4	3.5	131	12.7	2.9	80
N-Methyl DOPE	9.0	43	35	24.5	13	21	8.3	5.6	70
N-Ethyl DOPE	17.0	193	66.4	31	22	139	11.9	3.4	71.5
C-2-Methyl DOPE	5.0	218	n.d. [c]	107	9	128	n.d. [c]	130	< 5
C-2-Dimethyl DOPE	7.5	325	n.d. [c]	322	10	174	n.d. [c]	221	< 5
C-2-Ethyl DOPE	9.5	134	69.2	96	11	106	38.9	197	< 5
Dipalmitoleoyl PE	1.8	– [d]	– [d]	17.0	3.0	– [d]	– [d]	4.5	43.5
POPE	1.8	85	5.3	1.33	3.0	41.1	4.3	0.65	69.0
POPP	1.8	49	– [d]	0.22	3.0	32.4	– [d]	0.15	> 95
N-Methyl POPE	10	27.5	– [d]	0.35	13.5	14.8	– [d]	0.24	> 95
C-2-Methyl POPE	4.5	154	– [d]	4.5	7.0	68.4	– [d]	1.15	62.3

[a] Initial rates were determined at 25°C and are presented as percentages of the maximum possible fluorescence change per minute. Other reaction conditions were as described in the legend to Fig. 1.

[b] From Refs. 29 and 39 and unpublished calorimetric observations.

[c] n.d. The maximum fluorescence change for these samples was too small, and the initial rate of the fluorescence signal change was too rapid, to permit accurate quantitation of the rate of contents mixing.

[d] Not determined.

cies of divalent-cation-mediated fusion and leakage than do similar vesicles containing DEPE. This effect is more pronounced for vesicles that contain DEPE analogues with more extensive modifications at C-2 of the ethanolamine group (see Table II). In general, replacement of the PE component in DEPE/DEPS vesicles by C-2-alkylated PE leads to a greater enhancement in the efficiency of divalent-cation-induced contents leakage than in the efficiency of contents mixing. Interestingly, however, vesicles containing the C-2-ethyl derivative of DEPE exhibit a large enhancement in fusion (contents mixing) efficiency when compared to DEPE/DEPS vesicles, without a greatly disproportionate increase in their rates of contents leakage in the presence of either calcium or magnesium. Vesicles composed of DEPS plus C-2-alkylated derivatives of DEPE, even though they show higher efficiencies of fusion than do DEPS/DEPE vesicles, nonetheless require substantially higher threshold concentrations of divalent cations to elicit vesicle fusion than do DEPS/DEPE vesicles.

Modification of the DEPE headgroup by addition of extra methylene residues between the phosphoryl and amino groups produces a different pattern of changes in the fusion-supporting ability of this lipid. Vesicles combining DEPS with DEPP or DEPB exhibit divalent-cation thresholds comparable to those measured for DEPE/DEPS vesicles. Moreover, the efficiencies of divalent-cation-mediated lipid mixing are somewhat higher for vesicles containing DEPP or DEPB than for vesicles containing DEPE itself. However, the efficiencies of contents mixing and contents leakage measured for such vesicles decline progressively as the DEPE component is replaced successively by its 3-amino-1-propanol and 4-amino-1-butanol analogues.

It can be concluded from the data in Table II that, while the fusion-supporting ability of a given DEPE analogue is clearly affected by the structure of the polar headgroup, the bases for these effects are relatively complex and do not rest on such simple features of the headgroup structure as the size of the polar moiety. Some aspects of the fusion-supporting abilities of various DEPE analogues can be more simply correlated with their lamellar-to-hexagonal II phase transition temperatures, T_H, which are listed in the final column of Table II. Specifically, the efficiencies of calcium- and magnesium-promoted contents mixing and contents leakage for different types of DEPE(analogue)/DEPS vesicles show a strong inverse correlation with T_H. By contrast, the efficiencies of lipid mixing measured for such vesicles show only a weak correlation, and the divalent-cation thresholds show no clear correlation, with the T_H value of the neutral lipid component.

Effects of acyl chain composition

Previous studies [17–19,21,22,25,37,38] have shown that the polymorphic phase behavior and, to some extent, the fusion-supporting properties of a given type of PE depend significantly on its acyl chain composition. To generalize the results described above concerning the effects of headgroup structure on the fusion-supporting abilities of DEPE analogues, we therefore examined also the divalent-cation-mediated interactions of vesicles that contained PE or PE analogues with other types of acyl chain. In Table III we summarize the results of a series of measurements of the divalent-cation-mediated interactions of vesicles that combine various *cis*-unsaturated PEs, or their headgroup-modified analogues, with DOPS. The divalent-cation thresholds measured for such vesicles are generally quite comparable to those measured for similar vesicles containing the corresponding dielaidoyl lipids (see Table II). By contrast, the efficiencies of divalent-cation-induced contents mixing and leakage for such vesicles depend strongly on their acyl chain composition. Moreover, the relative rates of lipid mixing, contents mixing and leakage measured for vesicles containing PE or a particular PE analogue also depend strongly on the lipid fatty acyl composition. For example, vesicles containing analogues of DOPE typically show much greater rates of divalent-cation-promoted contents leakage, relative to their rates of lipid and contents mixing, than do vesicles containing the corresponding analogues of DEPE or POPE.

While the fusion-supporting ability of a PE (or PE analogue) clearly varies with its fatty acyl composition, a given structural modification to the headgroup of a 'parent' PE modifies its fusion-supporting properties in a generally consistent manner regardless of the fatty acyl composition of the parent species. As can be seen from Table III, *N*-alkylations, or the addition of extra methylene residues, to the ethanolamine moiety of DOPE affect the fusion-supporting ability of this species in a manner very similar to that observed when DEPE is similarly modified (Table II). C-2-alkylated derivatives of DOPE, like the corresponding derivatives of DEPE, give vesicles that show considerably greater efficiencies of leakage in the presence of divalent cations than do similar vesicles containing the parent PE. Apparently because of this fact, vesicles combining DOPS with C-2-methyl or -dimethyl DOPE showed only a very limited extent of contents mixing under our experimental conditions, precluding accurate determination of contents-mixing rates for these vesicles. However, vesicles prepared from DOPS plus C-2-ethyl DOPE showed substantial contents mixing under our assay conditions, with an efficiency significantly greater than that observed for DOPS/DOPE vesicles. At least qualitatively, therefore, the effects of C-2-alkylations on the fusion-supporting ability of DOPE are comparable to those observed when DEPE is similarly modified.

As was observed for the DEPE analogues described above, the efficiencies of divalent-cation-induced contents mixing and leakage for the vesicle preparations listed in Table III show a strong inverse correlation with T_H of the neutral lipid component (indicated in the last column). This correlation, while highly significant, is not perfect: vesicles containing *N*-ethyl DOPE, for example, show a significantly higher efficiency of contents mixing than do vesicles containing *N*-methyl DOPE, even though the two *N*-alkyl PE species show very similar T_H values by calorimetry [29].

A particularly interesting observation regarding the effects of acyl chain composition on the 'fusion competence' of PE-containing vesicles concerns the effect of the PE acyl chain length. As shown in Table III, vesicles composed of dipalmitoleoyl PE plus DOPS (75:25 mol/mol) show significantly lower efficiencies of divalent-cation-induced destabilization (contents leakage) than do similar vesicles containing DOPE. A similar result is obtained with vesicles containing the corresponding *trans*-unsaturated species (Table II): vesicles containing dipalmitelaidoyl PE exhibit significantly lower efficiencies of divalent-cation-promoted contents mixing and leakage, but higher efficiencies of lipid mixing, than do vesicles containing DEPE. These results can be correlated with the fact that T_H values for the 9-*cis*- and 9-*trans*-hexadecenoyl PEs are significantly higher than those for the corresponding octadecenoyl PEs [17,29,39]. This result contrasts with the frequent observation that isolated vesicles composed of shorter-chain lipids are more prone to disruption than are vesicles composed of longer-chain lipids [40,41].

Effects of temperature

As noted above, the efficiencies of destabilization of PE(analogue)/PS vesicles in the presence of divalent cations show a strong inverse correlation, at a fixed temperature, with T_H of the neutral phospholipid component. To explore the basis for this correlation in more detail, we examined as a function of temperature the rates of calcium- and magnesium-induced destabilization for three preparations of vesicles (DOPE/DOPS, DOPP/DOPS and POPE/DOPS), whose constituent lipids exhibit lamellar-to-hexagonal II transitions in bulk dispersions at quite different temperatures.

In Fig. 3 are plotted the rates of destabilization (contents leakage) measured for DOPE/DOPS, DOPP/DOPS or POPE/DOPS vesicles at various temperatures in the presence of 10 mM magnesium, a concentration well above the threshold for all three types of vesicle. For clarity, the data in Fig. 3 are presented as Arrhenius plots (of ln(leakage rate) vs. $1/T$), although the overall process of vesicle leakage is of course more complex than the single-step reactions for which such plots are rigorously appropriate [5,36]. The Arrhenius plots obtained for POPE/DOPS and DOPP/DOPS vesicles are linear over the full temperature range 5–60 °C, while the curve obtained for DOPE/DOPS vesicles almost levels off above 35 °C. The apparent activation energies measured for the destabilization of all three vesicle preparations are very similar at lower temperatures (18, 20 and 22 $\text{kcal} \cdot \text{mol}^{-1}$, respectively, for DOPE/DOPS,

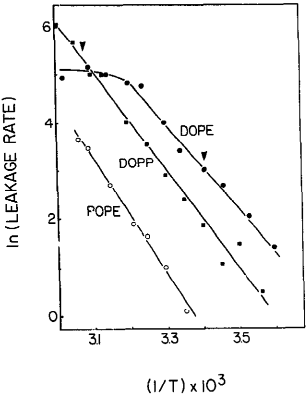

Fig. 3. Temperature-dependence of the initial rates of contents leakage, measured by the ANTS/DPX assay, when vesicles composed of 25 : 75 DOPS/DOPE (●), 25 : 75 DOPS/dioleoylphosphatidyl-3-amino-1-propanol (■) or 25 : 75 DOPS/POPE (○) were mixed with 10 mM magnesium. Samples were incubated at a lipid concentration of 30 μM; the temperature in the cuvette was recorded after each run. Arrows indicate the T_H values measured for these lipid mixtures in the presence of magnesium; the T_H measured for POPE/DOPS dispersions under these conditions falls above the temperature range shown in this figure.

DOPP/DOPS and POPE/DOPS vesicles). For none of these three vesicle preparations does the rate of contents leakage show any sharp change in its temperature-dependence at or near the equilibrium T_H value, which was measured by calorimetry for the same lipid mixture in the presence of magnesium (arrows). Parallel measurements of the leakage of contents from these preparations of vesicles in the presence of 20 mM magnesium or 10 mM calcium (data not shown) led to the same conclusion.

Proton-induced destabilization of vesicles containing PE analogues and a protonatable amphiphile

In all of the above experiments, the abilities of different PE analogues to support bilayer destabilization and fusion were evaluated through measurements of the interactions of PE(analogue)/PS vesicles in the presence of divalent cations. To test our conclusions from these experiments, we also examined the proton-induced destabilization of vesicles that combined POPE, or two headgroup-modified analogues, with OAP, a protonatable amphiphile that converts from an anionic to a neutral form at weakly acidic pH [11]. In Fig. 4 are shown the initial rates of contents leakage measured when these different types of vesicles, prepared at pH 7.8, were abruptly exposed to citrate buffers with the indicated pH values. It can be seen that vesicles containing *N*-methyl POPE require a higher threshold concentration of protons to elicit vesicle destabilization than do vesicles containing POPE, and that the rate of contents leakage for the former vesicles is considerably slower than that for the latter, even at proton concentrations well above the threshold (note the 10-fold difference in scales for these two curves in Fig. 4). Vesicles containing C-2-methyl POPE require a threshold proton concentration for destabilization that is intermediate between those measured for POPE- and for *N*-methyl POPE-containing vesicles. However, the rate of contents leakage from vesicles containing the C-2-methyl species is considerably higher, at proton concentrations well above the threshold, than that measured for the other two types of vesicle. The relative thresholds and efficiencies measured for the proton-induced destabilization of these three types of vesicle thus follow exactly the same pattern that we observe if we compare the analogous quantities for the divalent-cation-promoted destabilization of vesicles that com-

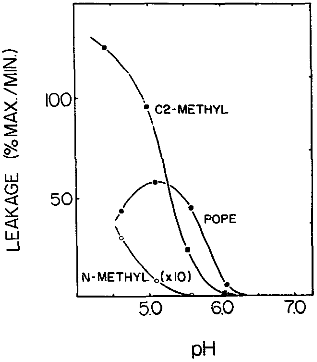

Fig. 4. Initial rates of contents leakage, measured by the ANTS/DPX assay, when vesicles composed of 25 mol% *N*-oleoyl-2-aminopalmitic acid together with POPE (●), C-2-methyl POPE (■) or *N*-methyl POPE (○) were abruptly exposed to a weakly acidic pH at zero time. Note that the curve plotted for vesicles containing *N*-methyl POPE has been scaled up by a factor of 10 for clarity. Samples of vesicles prepared at pH 7.8 and suspended in 150 mM NaCl/1 mM Tes/0.1 mM EDTA (pH 7.8) were mixed at zero time with concentrated citrate buffer solutions to give the final pH values shown. Measurements were carried out at 25° C using a total lipid concentration of 30 μM (phospholipid concentration of 22.5 μM).

bine POPE, C-2-methyl POPE or *N*-methyl POPE with DOPS (see Table III).

Discussion

A number of previous studies [15,31–34,37,38] have reported that lipid vesicles that are rich in PE show a much higher fusion competence than do similar vesicles containing N-methylated derivatives of PE, including phosphatidylcholine. We have observed previously [31] that even a single N-methylation of PE sharply reduces the ability of this lipid to support the calcium-mediated interactions of vesicles in which it is combined with PS. However, previous studies have not clearly established whether the reduced fusion-supporting abilities of N-methylated PEs can be attributed to their larger headgroup volumes, to their diminished hydrogen-bond donating capacity, or to perturbation of some still more specific feature of the PE headgroup structure.

The results obtained in the present study indicate that two specific structural features of the PE headgroup, namely the absence of substituents on the amino group and an apparently optimal separation of the phosphoryl and amino groups, are the primary determinants of the high fusion-supporting ability of this lipid. If we compare the fusion-supporting abilities of PE analogues in which both the phosphoryl-to-amino group separation and the number of *N*-alkyl substituents are held constant, we can further observe that the fusion-supporting ability of a PE analogue increases as we increase the extent of alkyl substitution of the ethanolamine group. This result suggests a general contribution of the headgroup 'hydrophobic bulk' to the fusion-supporting ability of the PE analogues studied here, in addition to the more specific effects described above. The small size of the PE headgroup per se thus does not appear to be an important factor in determining the fusion-promoting ability of this lipid.

Since the fusion-supporting ability of a given PE derivative shows a rather complex dependence on the detailed structure of the headgroup, it is appropriate to ask whether this functional property can be correlated more directly with some physical property or properties of the PE analogues examined here. As discussed above, information about T_H for a given PE analogue provides a reasonably useful basis on which to predict the ability of that lipid species to support vesicle fusion and destabilization. This correlation is strongest when we compare the fusion-supporting abilities of a series of PEs or PE analogues that differ either in their headgroup structures or in their acyl compositions while the other structural feature is held constant. Taking as a reference a specific diacyl PE, we find that modifications of either the ethanolamine group or the acyl chains that increase or decrease T_H typically diminish or enhance, respectively, the ability of the modified derivative to support contents mixing and destabilization of lipid vesicles.

The rates of divalent-cation-induced destabilization (contents leakage) measured for DOPE/DOPS, DOPP/DOPS and POPE/DOPS vesicles showed no abrupt changes in their temperature-dependence at or near the T_H values measured for the vesicle lipids at equilibrium. This result contrasts with the findings of Ellens et al. [21] and of Bentz et al. [22], who reported that the rate of proton-triggered destabilization of vesicles composed of pure PEs or *N*-methyl PEs showed a pronounced change in its temperature-dependence in the region of T_H. Our present findings may seem surprising in view of these results and our observation that the T_H value for a given PE (analogue) is an important determinant of its ability to support vesicle fusion and destabilization. However, the fusion and destabilization of lipid vesicles are kinetic rather than equilibrium phenomena, which presumably occur through local fluctuations in the organization of vesicle bilayers. Such fluctuations may occur at temperatures well below those at which the system adopts a nonlamellar organization at equilibrium, and they should evolve much more gradually as a function of temperature than would a true nonlamellar phase [21–26]. It would appear that the structural and physical factors that favor the ability of a given lipid or lipid mixture to form nonlamellar phases at equilibrium also promote its ability to exhibit local fluctuations from a lamellar geometry, such as those implicated in the fusion and contact-dependent destabilization of lipid vesicles, even at temperatures well below the equilibrium T_H [24,26].

While the T_H values for different PEs and PE analogues appear to be significant determinants of the abilities of these species to support lipid vesicle fusion and destabilization, other physical properties may also play a role in determining the 'fusion competence' of lipid vesicles that contain these compounds. For example, vesicles containing *N*-monoalkylated PEs show significantly higher efficiencies of divalent-cation-mediated fusion and leakage than one would predict simply on the basis of their T_H values. This result may reflect the fact that these species, in contrast to the PE analogues with unsubstituted amino groups, readily form localized nonlamellar structures at temperatures as much as 50 °C below those at which they form the hexagonal II phase itself [22,29,31,42]. Ellens et al. [21,22] and Siegel [23,24,26] have suggested that the ability of a lipid vesicle to undergo fusion, as opposed to collapse and leakage of contents, may rest on its ability to form localized nonbilayer structures that do not propagate to cause destabilization of the vesicle as a whole. In the light of this suggestion, it is also noteworthy that vesicles containing N-alkylated PEs show higher efficiencies of contents mixing, relative to contents leakage, than do vesicles containing PE itself. By contrast, vesicles containing C-2-alkylated derivatives of PE, which form the hexagonal II phase per se more readily than does PE [29], show lower ratios of contents mixing to contents leakage efficiencies than do vesicles containing the corresponding PE.

While the efficiencies of divalent-cation-mediated fusion of vesicles containing PE analogues can be correlated with the abilities of these analogues to form nonlamellar structures, no similar correlation can be drawn between the latter property and the divalent-cation thresholds measured for such vesicles. The calcium (or magnesium) thresholds observed for one representative series of PS/PE(analogue) vesicles, for example, vary with the structure of the neutral lipid component in the order PE < phosphatidyl-3-amino-1-propanol ≈ phosphatidyl 4-amino-1-butanol ≪ phosphatidyl-2-amino-1-propanol ≪ *N*-methyl PE. The threshold proton concentrations required to induce destabilization of vesicles combining OAP with POPE, *N*-methyl POPE or C-2-methyl POPE also fall in the above sequence. This sequence of threshold values cannot be clearly correlated with the efficiencies of fusion (or leakage of contents) for these preparations of vesicles, or with the T_H values of their PE (analogue) components. It is moreover highly unlikely that the above sequence can be related to variations in the affinity of PS for divalent cations in vesicles containing different types of PE analogue; PS has been shown to bind calcium (or magnesium) with essentially equal affinity when it is combined with neutral phospholipids as different as PE and PC [43,44].

Another physical property of various PE analogues may be more reliably correlated with the divalent-cation thresholds that are measured for vesicles that combine these analogues with PS. We have recently found that fully hydrated multibilayers, formed from a series of PE analogues with the fatty acyl composition of egg PC, show lamellar spacings that vary with the structure of the lipid headgroup in a sequence identical to that given above for the divalent-cation thresholds of vesicles that combine different PE analogues with PS (Silvius, J. and Rand, R.P., unpublished results). This correlation may be more than purely coincidental. The equilibrium separation of two bilayers composed of a given PE analogue, in the presence of excess water, will depend on the balance of Van der Waals' attractions and steric and 'hydration' repulsions between the two lipid surfaces [16]. These same forces will be of key importance in determining how completely the surface charge must be titrated in order to permit aggregation of two vesicles that combine a given PE analogue with a given proportion of charged lipids [16,44,45]. We are currently pursuing further experiments to explore the extent and the basis of this correlation in more detail.

The results presented in this paper demonstrate that different types of modification to the PE headgroup can produce different patterns of effects on the ability of this species to promote the fusion and destabilization of lipid vesicles. As noted previously, the efficiencies of fusion of vesicles containing various PEs and PE analogues (as monitored by contents-mixing assays), as well as the relative efficiency of fusion vis-à-vis leakage of contents for these vesicles, are strongly affected by the structures of both the lipid acyl chains and the polar headgroup. By contrast, the threshold ionic conditions that determine the range of pH, divalent-cation concentrations, etc., in which vesicles containing a PE analogue will be stable or unstable depend mainly on the structure of the analogue headgroup. These properties of vesicles containing different PE analogues appear to be predictable, to a useful level of approximation, from the knowledge of a few basic and measurable physical properties of these compounds (e.g., their T_H values and their equilibrium bilayer separations in multilamellar dispersions). These findings should be useful in the design of lipid vesicles, such as those currently under investigation as carriers of bioactive molecules to animal cells [1–14], that show optimal stability under one range of conditions and yet become highly prone to efficient fusion and/or destabilization under another.

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