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Incorporation of *N*-acylethanolamine phospholipids into egg phosphatidylcholine vesicles: characterization and permeability properties of the binary systems

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We have studied the effect of the *N*-acylphosphatidylethanolamine (*N*-acylPE) on the permeability properties of liposomes composed primarily of egg phosphatidylcholine using a fluorescent anionic dye, carboxyfluorescein, as model solute. Leakage from liposomes decreased and vesicle size increased with increasing *N*-acylPE content. In addition, measurement of the trapped aqueous space, using the same dye marker, showed a correlation between trapped volume and vesicle size determined by dynamic light scattering. Permeability parameters were calculated according to the pseudo-first-order analysis. It appears that *N*-acylPE stabilizes liposomes at least in part through its ability to impart surface negative charge, in accord with the results obtained with potassium chloride as encapsulated solute. These results agreed well with osmotic response of anionic lipid vesicles. Cholesterol stabilizes *N*-acylPE liposomes in a proportional manner to the molar fraction of the effector.

Introduction

Recent studies have shown the presence of *N*-acylethanolamine phospholipids in mammalian cells which are undergoing degenerative changes, such as the granular cells of the epidermis [1], degenerative BHK cells [2], canine myocardial infarct [3] and post-decapitative cerebral ischemia of the immature rat [4]. However, these lipids were also detected in normal vertebrate tissues, such as bovine erythrocyte stroma [5] and the central nervous system of fresh water pike (*Esox lucius*) and carp (*Cyprinus carpio*) [6] and they are normal constituents of higher plants [7] and microorganisms [8–10].

The biosynthesis of *N*-acylphosphatidylethanolamines (*N*-acylPEs) is important because they are catabolized to free *N*-acylethanolamines, compounds of known pharmacological and biological activity, which are found to protect mitochondrial membranes against induced increases in permeability and Ca^{+2} efflux [11–13]. The *N*-acylation reaction, followed by the catabolic sequence, could thus constitute an inducible metabolic pathway of some significance under pathological conditions.

The physical properties of *N*-acylethanolamine phospholipids have recently been studied by Newman et al. [14], by Akoka et al. [15] and by Lafrance et al. [16], who examined the phase behaviour of their aqueous dispersions by differential scanning calorimetry, phosphorus magnetic resonance spectroscopy and FT-IR. They reported that *N*-acylPEs are bilayer lipids, which form closed structures in excess of water, as also revealed by freeze-fracture electron microscopy.

Regarding all the different properties and functions of biological membranes, they are barriers which selectively control the transfer of solutes in both directions. The permeability properties of a membrane depend on the physical state of its lipid constituents, which determines the organization of the bilayer. Natural membranes contain a high percentage of phosphatidylethanolamine (PE), an H_{II}-forming lipid which destabilizes the bilayer and affects its barrier functions. Conversion of PEs into their *N*-acyl derivatives, catalyzed

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Abbreviations: *N*-acylPE, *N*-acylphosphatidylethanolamine; Chol, cholesterol; PC, phosphatidylcholine; CF, 5(6)-carboxyfluorescein; SUV, small unilamellar vesicles; TLC, thin-layer chromatography; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

in vivo by a Ca^{+2}-dependent membrane-bound enzyme [17,18] would allow the formation of lamellar structures and the *N*-acylation reaction might thus have an important stabilizing effect on the bilayer with regard to its properties as a barrier.

The physicochemical and functional properties of phospholipid bilayers have been studied extensively because of their use as model membrane systems. However, there is relatively little information about the phase behaviour of acidic phospholipids and even less about the permeability properties of pure or multicomponent systems containing acidic phospholipids. Because of this and the possible physiological significance of *N*-acylethanolamine phospholipids, we have studied the influence of their presence on the permeability properties of phosphatidylcholine (PC) liposomes, as model membrane systems. The effect of cholesterol on the leakage of a fluorescent marker incorporated into vesicles has also been studied.

Reddy et al. [17] demonstrated that the biosynthesis of *N*-acylethanolamine phospholipids in dog heart is effected by a transacylation reaction which predominantly utilizes the acyl groups at the *sn*-1 position of ethanolamine and choline phospholipids. For this reason and in order to obtain the species that would be synthesized in physiological conditions, we have synthesized an *N*-acylPE with a saturated third chain and a fatty-acid composition which maintains the same relation between the acyl groups 16:0 and 18:0 as the corresponding PC from which it was prepared.

Materials and Methods

Synthesis of N-acylPE. Egg phosphatidylethanolamine was prepared from egg lecithin (EPC) (Merck) by transphosphatidylation reaction with phospholipase D (Boehringer-Mannheim) [19]. Lecithin was first purified by active carbon treatment, aluminium oxide column chromatography and preparative thin-layer chromatography (TLC) with chloroform/methanol/water (65:25:4 (v/v)) as eluent. *N*-acylPE was synthesized by condensing a mixture of palmitoyl and stearoyl chlorides (Merck Schuchardt), in the appropriate molar ratio, with the purified eggPE [20]. The product was purified by silicic-acid column chromatography and preparative TLC with chloroform/methanol/ammonium hydroxide (40:10:1 (v/v)) as eluent. All phospholipids were identified by proton-NMR and IR spectroscopy (amide-I and amide-II bands of *N*-acylPE were seen at 1660 and 1540 cm^{-1}, respectively (results not shown)) and assayed for purity by TLC with G-25 silica gel plates (Macherey-Nagel) and two solvent systems (chloroform/methanol/ammonium hydroxide/water, 60:35:2:2 (v/v) and chloroform/methanol/water, 65:25:4 (v/v)). Fatty-acid composition was also determined by gas-liquid chromatography of the methyl esters. Lipid concentrations were determined by phosphorus analysis [21].

Preparation of vesicles. Small unilamellar vesicles (SUV) were prepared by sonication of a phospholipid suspension following standard procedures [22]. Phospholipids were mixed in the desired molar ratio (total lipid concentration was 0.5 mg/ml) and the dried lipid film was hydrated by vortexing for 10 min with 20 mM Tris-HCl buffer (pH 7.4). The suspension was then sonicated for 20 min (with intervening periods of 30 s every 90 s) in a Braun Labsonic 2000 sonifier, equipped with a titanium probe (9 mm tip diameter), in contact with a refrigerant bath at a temperature above T_m, under a continuous nitrogen stream. Undispersed lipids and titanium particles were removed by centrifugation and the liposome suspension was sealed under nitrogen and left for 1 h at 40°C to facilitate annealing processes [23]. Lipid recovery was usually greater than 95%, as judged by phosphate measurements, and it was verified that lipids did not suffer degradation due to the sonication process.

Permeability experiments. The permeability of small unilamellar liposomes was first examined by the fluorescence technique described by Weinstein et al. [24], using 5(6)-carboxyfluorescein. The dye, obtained from Eastman-Kodak, was purified by Sephadex LH-20 column chromatography and acid precipitation (pH 4.5) as described previously [25]. Liposomes were prepared as described above, but in the presence of a 50 mM carboxyfluorescein Tris-buffered solution. Non-encapsulated dye was removed by Sephadex G-50 column chromatography (20 × 1 cm); a 0.5-ml aliquot of vesicle suspension was applied to the column and eluted with 20 mM Tris-HCl buffer (pH 7.4), containing 100 mM NaCl. The composition of liposomes eluted in the void volume was determined by TLC and phosphorus analysis of spots. The data obtained showed that liposome composition was the same as that of the film used for its preparation. Carboxyfluorescein release from SUV was monitored on a Kontron SFM25 spectrofluorimeter, using excitation and emission wavelengths of 492 and 520 nm, respectively. The cuvettes were maintained at the assay temperature by a circulating water bath. Small aliquots of the vesicle suspension (200 μl) were added to each cuvette in a total volume of 3 ml and the fluorescence was measured for 2–4 h. Total carboxyfluorescein fluorescence was determined by adding 150 μl of a 10% (v/v) Triton X-100 (Merck) solution.

Potassium efflux from unilamellar vesicles was also assessed. Vesicles (0.5 mg lipid/ml) were prepared as for CF release experiments but in 150 mM KCl buffered at pH 7.4 with 10 mM Tris-HCl, containing 0.1 mM EDTA. Extraliposomal K^+ was removed by Sephadex G-50 column chromatography (20 × 1 cm) eluted with 150 mM Tris-buffered choline chloride. The release of

K^+ was measured potentiometrically [26] with a combination of a K^+-selective glass electrode (Ingold, type 15 221 3000) and a reference electrode (Ingold, type 373–90-WTE-ISE-S7), both connected to a microp H 2002 Crison pH-meter. After measuring the K^+ leak for 20 min, cholate was added to liberate the residual trapped K^+.

Determination of entrapped volume. The volume of the aqueous space of PC dispersions containing increasing amounts of *N*-acylPE (in μl μmol^{-1} lipid) was found by measuring fluorescence at 520 nm of a 100 μl-aliquot of the liposomal suspension free of non-encapsulated carboxyfluorescein eluted from the Sephadex G-50 column for permeability experiments. The sample was diluted in 2 ml of 20 mM Tris-HCl buffer in the presence of a 10% Triton X-100 solution, to destroy any permeability barrier [27]. The carboxyfluorescein concentration was determinated by comparison with a standard curve. Lipid concentration was quantified by phosphorus analysis.

Particle size analysis. Vesicle size was determined by dynamic light scattering using a PCS41 optics unit (Malvern Autosizer IIC) and a 5 mW He-Ne laser (Spectra Physics) at an excitation wavelength of 633 nm. Data were collected with a Malvern 7032N 72 data channel correlator and the mean hydrodynamic diameter was calculated from a cumulant analysis of the intensity autocorrelation function.

Results

Fatty-acid content of phospholipids

The fatty-acid composition of PC, PE and *N*-acylPE was determined by gas-liquid chromatography of the methyl esters (Table I). Palmitic was the predominant acyl group in all species and the 16:0 and 18:0 fatty-acid content of *N*-acylPE was that which would correspond to the transacylation of *sn*-1 acyl groups from PC to PE demonstrated in vivo [17].

Characterization of liposomes

The behaviour of liposomes is determined by factors such as physical size, chemical composition, and the purity of starting materials and quantity of entrapped solutes. Knowledge of these parameters is necessary for evaluating the results of experiments using phospholipid vesicles.

TABLE I

The fatty-acid composition of lipids used

16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid.

Lipid	Fatty acids (% total mass)				
	16:0	18:0	18:1	18:2	20:4
Egg-yolk PC	37.61	11.06	31.62	16.38	3.34
Transphosphatidylated egg-yolk PE	37.87	10.12	31.49	17.48	3.04
N-acylPE	50.61	15.80	19.24	10.92	2.17

TABLE II

Characteristic parameters of N-acylphosphatidylethanolamine-containing liposomes

Data are mean ± S.D., $n = 3$.

Lipid composition (molar ratio)	D_H [a] (nm)	Trapped volume [b] (μl/μmol lipid)
PC	67.7 ± 8.9	0.41 ± 0.02
N-acylPE/PC (1:3)	70.8 ± 9.4	0.49 ± 0.14
N-acylPE/PC (1:1)	80.4 ± 11.3	0.75 ± 0.12
N-acylPE/PC (2:1)	97.5 ± 16.5	1.12 ± 0.12
N-acylPE/PC (3:1)	103.2 ± 16.7	1.23 ± 0.20
N-acylPE	159.1 ± 29.8	1.69 ± 0.28
N-acylPE/Chol (2:1)	178.2 ± 32.2	2.08 ± 0.06
N-acylPE/Chol (1:1)	169.6 ± 28.5	1.86 ± 0.19

[a] Calculated from dynamic light scattering data.
[b] Determined by carboxyfluorescein dye retention.

Liposomes of different composition were analyzed for size and size distribution by quasi-elastic light scattering. Lipids were hydrated with a 50 mM carboxyfluorescein-buffered solution, as for permeability experiments, and vesicles were prepared by sonication, as described in Materials and Methods. The results are summarized in Table II. The mean hydrodynamic diameter of eggPC vesicles derived from a cumulant analysis of the intensity autocorrelation function increased with increasing *N*-acylPE content of vesicles, results related to the higher molecular area of tricatenary phosphilipid. Further, the presence of cholesterol increases the size of *N*-acylPE vesicles. For all dispersions studied the polydispersity coefficient was of the order of 0.30, which denotes some degree of heterogeneity.

The aqueous space volume of mixed *N*-acylPE/PC or *N*-acylPE/Chol liposomes was measured using a carboxyfluorescein dye method. The data obtained for the different systems (Table II) show an increase in the volume entrapped when increasing the *N*-acylPE molar fraction of the binary systems, which correlates well with the greater size for *N*-acylPE and *N*-acylPE/PC liposomes compared with PC vesicles, as observed by photon correlation spectroscopy. However, when the *N*-acylPE content of mixed vesicles was higher the increase in size was not correlated with the increase in trapped volume, in agreement with a possible reduced solute concentration related to the negative surface charge due to the incorporation of *N*-acylPE.

When vesicles contained cholesterol in two different molar ratios, there was also an increase in the volume captured, according to the measured sizes of the corresponding liposomes.

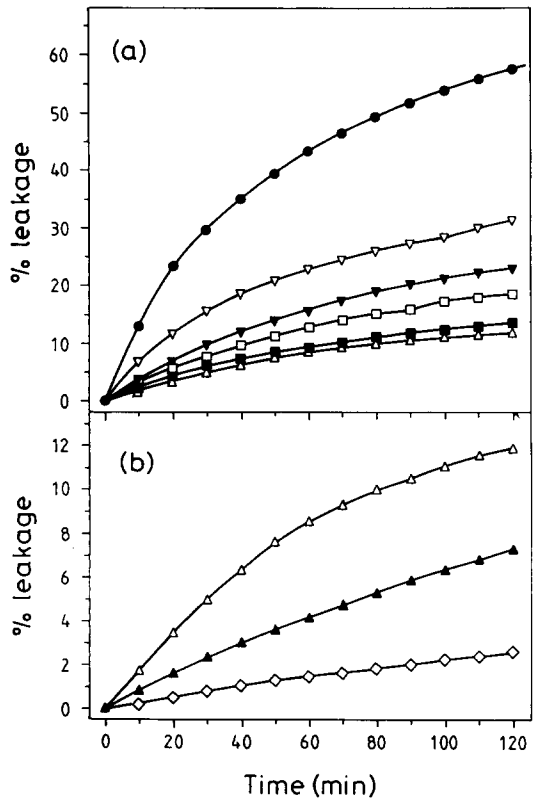

Fig. 1. Time curves of carboxyfluorescein efflux from mixed PC/*N*-acylPE (a) and *N*-acylPE/Chol (b) liposomes. Efflux was measured during 2-h periods and is expressed as the percentage of initial trapped solute lost over a given time. The results illustrated are the means of individual experiments performed in triplicate. (a) PC (●); *N*-acylPE/PC 1:3 (▽); *N*-acylPE/PC 1:1 (▼); *N*-acylPE/PC 2:1 (□); *N*-acylPE/PC 3:1 (■) and *N*-acylPE (△). (b) *N*-acylPE (△); *N*-acylPE/Chol 2:1 (▲) and *N*-acylPE/Chol 1:1 (◇).

Permeability experiments

Fig. 1 illustrates the time course for carboxyfluorescein release from mixed *N*-acylPE/PC and *N*-acylPE/Chol liposomes. The effluxes are expressed as the percentage of initial entrapped dye lost versus time. The initial entrapped dye or initial latency is the initial fluorescence self-quenching of carboxyfluorescein which is related to the concentration into vesicles and is defined by $E_0 = 100(1 - (F_0/F_\infty))$, where F_∞ and F_0 correspond to the fluorescence values obtained by adding Triton and that found at time zero, respectively.

The data corresponding to *N*-acylPE/PC vesicles revealed a decrease in carboxyfluorescein release when the liposomal content of *N*-acylPE was raised, which agrees with the greater molecular order both at the hydrophobic and at the head group levels (data not shown) and with their higher vesicular size. The results obtained when Chol was incorporated to vesicles show a decrease in the permeability of the bilayer, which are the consequence of its rigidifying effect.

The effect of the medium osmolarity on the behaviour of *N*-acylPE vesicles was also shown by changes

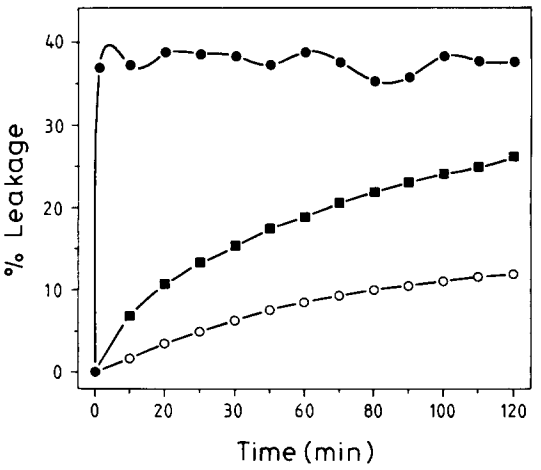

Fig. 2. Influence of the medium osmolarity on carboxyfluorescein release from *N*-acylPE unilamellar vesicles. The experimental conditions are the same that in Fig. 1 but the outer medium was 20 mM Tris-HCl buffer (pH 7.4) with 0.1 mM EDTA and 100 mM NaCl (○), 100 mM NaCl and 364 mM sorbitol (equivalent osmolarity to 200 mM NaCl) (■) and without NaCl (●).

in their permeability properties. The results are plotted in Fig. 2. When the osmolarity of the outer medium was higher than that of the vesicular interior there was an increase in carboxyfluorescein release, whereas when the outer medium was hypotonic, there was a sudden leakage of the entrapped dye. In this case, 35% of the marker was lost in 5 min, but no subsequent release occurred. This was confirmed by a second chromatography of the free non-encapsulated carboxyfluorescein liposomes through Sephadex G-50. The effect of osmotic pressure was also studied as a function of the phospholipid composition of the bilayer. Fig. 3 shows that there was a decrease in osmotic swelling-induced permeability when the PC molar content of vesicles increased.

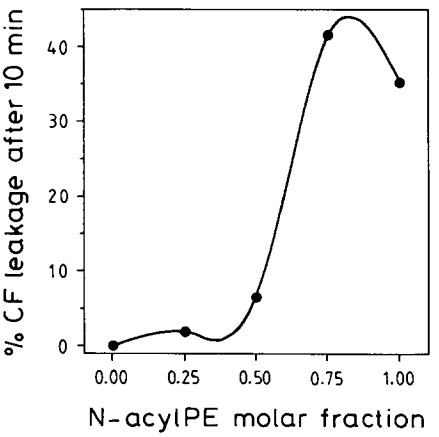

Fig. 3. Permeability of mixed *N*-acylPE/PC liposomes as function of *N*-acylPE molar fraction. Leakage was measured in osmotic gradient condition (higher inside) across the bilayer. 200 μl of liposomes encapsulating 50 mM CF were diluted at time zero with 2.8 ml of 20 mM Tris-HCl buffer (pH 7.4) with 0.1 mM EDTA.

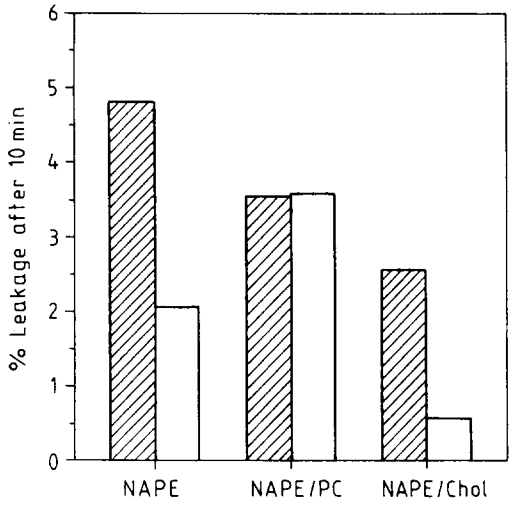

Fig. 4. Comparison of the permeabilities to K^+ and carboxyfluorescein in different liposomes. Vesicles of *N*-acylPE, *N*-acylPE/PC 1:1 and *N*-acylPE/Chol 1:1 were prepared as described in the text in either buffer containing the solute. Permeability was expressed by the mean release of three independent experiments of entrapped solute after 10 min. Potassium leakage is depicted by shaded columns, carboxyfluorescein leakage by clear columns.

Allen et al. [27] reported the influence of the negative surface density charge on calcein release from SUV and observed a decrease of this related with the electrostatic repulsion between the fluorescent marker and the bilayer. Consequently, we also determined the permeability to K^+ of *N*-acylPE-containing bilayers, in order to establish whether the negative charge of the *N*-acyl derivative determines the slower release of the aqueous marker. Results are given in Fig. 4, which shows comparative data from K^+ and CF effluxes. The most significant differences with regard to the release of the encapsulated material during a short time correspond to *N*-acylPE and *N*-acylPE/Chol systems, that is, those with the greatest negative charge.

Kinetic analysis

Usually, the kinetic release of a fluorophore can be expressed as a first-order process [28,29], according to the equation

$$f_t = 100(1 - \exp(-k_{rel} \cdot t)) \tag{1}$$

where $f_t = 100((F(t) - F_0)/(F_\infty - F_0))$ is the percentage of leakage, being $F(t)$, the fluorescence found at time t, F_∞ and F_0 as before and $\lim f_t = 100$. The constant rate is $k_{rel} = PA_i/V_i$, where V_i is the effective inner volume and A_i is the inner surface of the vesicle.

In our case, the semilogarithmic plots of percentage of leakage dye versus time were non-linear in all cases, except for PC liposomes, indicating that the leakage process is not a single exponential one and that a direct estimation of $t_{1/2}$ values from efflux curves was not possible (results not shown).

We apply the expression used to interpret ion-flux experiments [30]

$$J = \frac{\ln Q}{Q-1} P(C_e - QC_i) \tag{2}$$

where

$$Q = \exp(-ZF\psi_i/RT) \tag{3}$$

with C_e and C_i being the concentrations of probe outside and inside of liposome, respectively, and ψ_i the surface potential in the inner monolayer. The other parameters have the usual meaning. This model assumes that the electric field is constant within the membrane and the membrane potential, ψ_M, is linear. The agreed outer surface potential is equal to zero ($\psi_e = 0$).

On the other hand, in a preparation of n liposomes with an effective inner volume V_i (the volume is constant in isosmotic conditions) and an inner surface A_i, the variation of the total number of mols of an encapsulated substance, n_i, is

$$\frac{dn_i}{dt} = \frac{d(C_i V_i n)}{dt} = V_i n \frac{dC_i}{dt} = JA_i n \tag{4}$$

Replacing Eqn. 2 in Eqn. 4 gives

$$\frac{dC_i}{dt} = \frac{PA_i}{V_i} \frac{\ln Q}{(Q-1)} (C_e - QC_i) \tag{5}$$

However, the internal volume of liposomes is so low that the release of their contents will not bring about a significant increase in the extraliposomal concentration of the substance, then $C_e \lll QC_i$ and Eqn. 5 becomes

$$\frac{dC_i}{dt} = -\frac{PA_i}{V_i} \frac{Q \ln Q}{(Q-1)} C_i \tag{6}$$

Thus, Eqn. 6 can be integrated between C_i^0 to C_i and $t = 0$ to $t = t$, obtaining

$$C_i = C_i^0 \exp(-k'_{rel} t) \tag{7}$$

where

$$k'_{rel} = \frac{PA_i}{V_i} \frac{Q \ln Q}{(Q-1)} = k_{rel} \frac{Q \ln Q}{(Q-1)} \tag{8}$$

In charged bilayers, the distribution of ions at the interface has been described by the Debye-Hückel model, which generally assumes a Boltzmann distribution of ions as follows [31]

$$C_i^0 = c_{i0}^0 \cdot \exp(-Z\psi_i/kT) = C_{i0}^0 Q \tag{9}$$

TABLE III

Permeability parameters of N-acylPE / PC and N-acylPE / Chol binary systems

Data are mean ± S.D., $n = 3$.

Lipid composition	Initial (%)	f_{eq} (%)	$k_{rel} \times 10^{-3}$ (min^{-1})
PC	95.2 ± 0.7	100	10.8 ± 1.2
N-acylPE/PC 1:3	90.7 ± 0.9	42.7 ± 1.2	7.0 ± 0.9
N-acylPE/PC 1:1	81.0 ± 0.4	30.0 ± 2.0	4.2 ± 0.4
N-acylPE/PC 2:1	89.4 ± 1.2	23.0 ± 1.0	3.2 ± 0.1
N-acylPE/PC 3:1	89.0 ± 0.9	15.8 ± 0.6	2.5 ± 0.3
N-acylPE	80.5 ± 1.8	14.5 ± 0.7	2.1 ± 0.3
N-acylPE/Chol 2:1	92.1 ± 1.1	14.4 ± 1.0	0.9 ± 0.1
N-acylPE/Chol 1:1	94.0 ± 0.6	5.6 ± 0.6	0.3 ± 0.05

where ψ_i is the surface potential, C_i^0 and C_{i0}^0 are the inner interface and the bulk concentration of ions, respectively, and the other parameters have the usual meaning. Then Eqn. 7 turns into

$$C_i = C_{i0}^0 Q \exp(-k'_{rel} t) \quad (10)$$

The inner fluorophore concentration, C_i, at time t is proportional to $(F_\infty - F(t))$ and the bulk concentration, C_{i0}^0, to F_∞. Eqn. 10 expressed as % of retention (E) is

$$E = 100Q \exp(-k'_{rel} t) \quad (11)$$

and expressed as % of release with respect to initial retention

$$f_t = 100Q(1 - \exp(-k'_{rel} t)) \quad (12)$$

where $f_t = (100Q - E)$ and the $\lim f_t = 100Q$.

The kinetic data can be fitted to a pseudo-first-order equation (Eqn. 12) by a non-linear regression program (Enzfitter), using time as the dependent variable (results not shown).

In our case, since the phospholipid has a negative charge, the negative potential repels anions (i.e., carboxyfluorescein) from the inner lipid-water interface. Then, the surface potential controls the ability of ions to penetrate into the membrane to a position suitable for ion migration in the interstitial spaces between acyl chains. Thus, there is a net exit of ions provided the free energy, available in the concentration gradient, is sufficient to allow the ions to move against the electrical potential gradient. If not, the ions are in equilibrium [30]. That is to say, the driving force on an ion is zero when the total electrochemical free energy is zero.

It has been demonstrated in non-charged bilayers [32] that the electrostatic forces control the penetration by carboxyfluorescein, which at an initial stage releases as a univalent anion, whereas afterwards the release is regulated by counterion flux.

In this context, we define the leakage in the equilibrium, $f_{eq} = 100Q$, as the release of the dye at electrochemical equilibrium.

The permeability parameters obtained according to this model are given in Table III. Numerical data show a slow release of the fluorescent dye entrapped into PC/*N*-acylPE vesicles as their *N*-acylPE content increases, as can also be seen in Fig. 1.

Discussion

The object of this study was to ascertain the effect of the presence of *N*-acyl derivatives of PE on the permeability properties of phospholipid bilayers, in order to evaluate the possible physiological role of *N*-acylation reactions of membrane phospholipids. To this end, *N*-acylPEs were incorporated in increasing amounts to liposomal bilayers and the formation of closed vesicles and their characteristic parameters were established.

The photon correlation spectroscopic data show that PC/*N*-acylPE vesicles grow with increasing *N*-acylPE content. This result might be related with the fact that when phospholipids have the same acyl chain moiety there is a correlation between effective head-group size and vesicle diameter [33]. *N*-acylPE, which bears a net negative charge, may have a larger effective cross-sectional area than PC because of the electrostatic repulsion at the bilayer surface. Therefore, the incorporation of *N*-acylPE molecules into sonicated PC liposomes could favour a reduced bilayer curvature and a consequent increase in vesicle size. The importance of both lipid composition and the presence of anionic phospholipids for vesicle size has already been established. Hammond et al. [34] reported that the radii of mixed PC/PI vesicles increase with PI content, and Masserini et al. [35] showed that the presence of ganglioside GM1 decreases the hydrodynamic radius of PC sonicated vesicles. The variation of liposomal diameter

with the molar content of *N*-acylPE was non-linear, which indicated a second-order dependence between these variables. This result demonstrates the unequivalence of PC and *N*-acylPE and confirms the larger effective head-group size of the latter. Another remarkable finding is that the *N*-acylPE vesicles are greater than 100 nm. To our knowledge, this is the first report of vesicles with a diameter of over 100 nm being obtained by sonication method.

The presence of Chol also increases the size of *N*-acylPE liposomes in an *N*-acylPE concentration-dependent way, as for PC/*N*-acylPE binary systems. The effect of cholesterol in increasing the size of sonicated vesicles has been studied previously for PC [36], PC/PG [37], DOPA [38] and more complex liposome formulations [27] and has been related with the increased phospholipid headgroup separation due to cholesterol incorporation at levels $> 30\%$ [39,40]. The greater diameter of vesicles with 33% of cholesterol compared with those with a greater content agrees with the results obtained for eggPC liposomes by McIntosh et al. [41], and might be explained bearing in mind that a concentration of cholesterol as high as 50% might reduces the steric hindrances at the head-group level with a resulting decrease in the surface charge density, which would lead to a decrease in vesicle radius.

The increase in vesicle size was accompanied by differences in the trapped volumes of SUV containing both *N*-acylPE and cholesterol, which were 4–5-fold larger than that corresponding to PC vesicles. The variation in both parameters did not correlate with the linear dependence between the trapped volume and diameter (Table II). It could therefore be assumed that an increase in surface charge density may result in reduced solute concentrations of the trapped buffer, as has been observed in SUV of PS [42]. On the other hand, the initial latency values determined (between 80–95% of fluorescence self-quenching of carboxyfluorescein) indicate an intraliposomal carboxyfluorescein concentration equal or greater than 35 mM [43], which allows us to perform kinetic studies of permeability.

Both the vesicle size and the negative charge of the *N*-acylPE molecule might determine the permeability properties of the liposomal bilayer to entrapped carboxyfluorescein. Allen et al. [27] have shown that the stabilizing effect of trisialoganglioside (G_T) in eggPC liposomes of different size is not strictly related to size changes, but seems to be dependent on the negative charge of the sialic acid moiety at the surface.

Permeability parameters were calculated according to a pseudo-first-order analysis, except for PC vesicles in which case it was a first-order analysis, as indicated in the Results section. The use of charged solutes for permeability measurements allows us to study, among other aspects, the physiological consequences of the synthesis of *N*-acyl derivatives of membrane phospholipids, the presence of which may modify the bilayer surface charge, on the membrane ionic transport. Incorporation of *N*-acylPE into PC bilayers leads to a marked variation of kinetic data for carboxyfluorescein release from mixed PC/*N*-acylPE vesicles (Table III), so phospholipid bilayers are less permeable to entrapped dye when the *N*-acylPE molar fraction increases. The slow release of carboxyfluorescein when the *N*-acylPE content of vesicles increases could be the result of three different factors: the increased size of liposomes, the high molecular order both at the head group and at the hydrocarbon region levels and the presence of a negative charge in the bilayer. The variation of f_{eq} (Fig. 5) seems to indicate an asymmetric distribution of *N*-acylPE, the preference of which for the inner monolayer would account for the invariability of this kinetic parameter at an *N*-acylPE content of over 75% and for its sudden initial change. Moreover, if we assume a symmetrical distribution of phospholipids and that the factor which determines f_{eq} (parameter Q) is the surface charge density-dependent potential barrier which increases with the molar fraction of *N*-acylPE, then $\ln(f_{eq})$ vs. $x_{N\text{-acylPE}}$ would show a linear dependence. That is, we assume the linear dependence between surface potential and surface charge density, valid for surface charge density of 0.02 C/m^2 or less and ionic strength of 0.1 M or more [31]. This fact is confirmed in Fig. 5 (inset), which suggests that the surface charge density is the main factor to control f_{eq}. The variation of k_{rel} vs. $x_{N\text{-acylPE}}$ showed the same dependence (Fig. 5). The variation of k_{rel} =

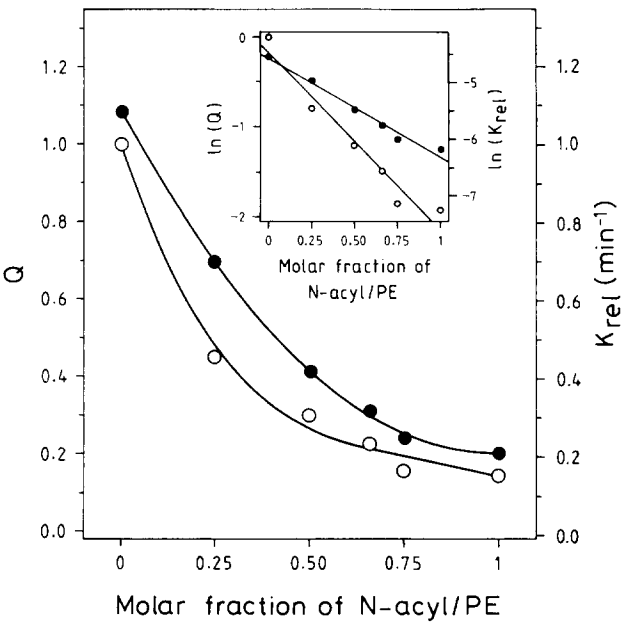

Fig. 5. Kinetic of carboxyfluorescein leakage of *N*-acylPE/PC vesicles as a function of *N*-acylPE molar fraction. The inset shows a semilogarithmic plot versus *N*-acylPE content. The f_{eq} (○) and k_{rel} (●) were calculated as described in the text.

PA_i/V_i [29] is proportional to the permeability coefficient and reciprocal to vesicular radius (r_i). The dependence of k_{rel} upon $1/r_i$ is not linear, which means that the decrease of permeability observed in vesicles containing *N*-acylPE does not depend exclusively on its radius. On the other hand, a decrease in $P = f(K,l,D)$ could be related with an increase in the order parameter at the hydrophobic and head-group levels when the *N*-acylPE molar fraction increases, as shown by the results obtained by fluorescence polarization. However, its variation with the molar fraction of *N*-acylPE shows exponential behaviour (Fig. 5, inset) and this dependence again suggests the importance of the surface charge for the leakage of carboxyfluorescein. This last point is clearly shown when the K^+ releases are analyzed. The monocomponent system constituted by *N*-acylPE shows greater permeability than the *N*-acylPE/PC 1/1 system, in contrast to the carboxyfluorescein release. This finding is probably related to an increase in the outside-inside cation concentration gradient due to the increment of K^+ concentration in the inner monolayer interface, which gives rise to the efflux of cation.

The response of a bilayer to osmotic stress yields information on its elasticity and curvature, both related with the lipid molecular area in the membrane, and which allows us to study the intermolecular forces and to establish the role of the lipid structure on them. Several studies have shown that osmotic stress increases the fluidity of a bilayer, which results in a rise in its permeability [44]. Haines et al. showed that the surface charge density determines the mechanical properties of vesicles constituted by acidic phospholipids [45]. When this type of vesicle reaches its elastic limit under osmotic stress, it bursts, releases the encapsulated solution and returns to the unstressed state of the bilayer. Similar behaviour is shown by *N*-acylPE vesicles under osmotic stress (hypotonic outside). The inhibition of the subsequent carboxyfluorescein release is due to the decrease in the concentration gradient obtained, which cannot overcome the electrostatic potential barrier.

An increase in the charge density of the bilayer restricts its expansion (that is the increase in surface area per lipid molecule) during osmotic stress [45]. In this way, a decrease in the permeability induced by the osmotic stress can thus be observed on decreasing the molar fraction of *N*-acylPE (Fig. 3), which is also related with the lower osmometric character associated with smaller vesicles.

The presence of Chol decreases the permeability of the systems constituted by *N*-acylPE in a proportional rate to the molar fraction of the effector (both CF and K^+) (Fig. 4). The stabilizing effect of Chol is related with its rigidifying effect on the bilayer (data not shown), since it has been demonstrated that the cholesterol interaction with anionic phospholipids results in a slight surface charge density decrease [37].

Our results indicate that the ionic permeability mechanisms in the lipid bilayer could be related to the surface charge density. In this way, the *N*-acylation of ethanolamine phospholipids establishes a mechanism by which to modify this permeability, as the molecular order in the bilayer increases because of the presence of a third saturated fatty-acid chain or because of the modification of the surface charge density. It has been shown that *N*-acylethanolamines, the biological degradation product of *N*-acylPE, can prevent swelling and Ca^{2+} release in mitochondrial membranes of myocardial cells from ischemic injury and the *N*-acylethanolamine generation as a defense mechanism aimed at minimizing irreversible injury and infarct size [46]. In this regard, further work is needed to examine the effect of the presence of *N*-acylPE in Ca^{2+} permeability.

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