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Role of guanidinium group in the insertion of L-arginine in DMPE and DMPC lipid interphases

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ABSTRACT

L-Arginine (Arg) is a positively charged amino acid constituent of peptides and proteins, participating in diverse mechanisms of protein–membrane interaction. The effect of Arg on phosphatidylcholine (PC) membranes has been previously related to water structure changes and to the presence of water defects in the hydrocarbon region. However, no information is available with regard to phosphatidylethanolamine (PE), another important component of lipid membranes. For this reason, the aim of this study is to determine the effect of Arg on DMPE membranes and partially methylated PEs in comparison to DMPC. The adsorption of the amino acid onto the lipid membranes was followed by determining the changes in the surface potential as a function of the bulk amino acid concentrations. The effects of Arg on the surface properties were also measured by changes in the surface pressure and the dipole potential. The onset of the transition temperature was measured with a fluorophore anchored at the membrane interphase. The results provide a new insight on amino acid—PE interactions, which can be ascribed to specific perturbations in the head group region induced by the guanidinium residue.

1. Introduction

The interaction of proteins with lipid membranes remains of central interest in biophysical research. Understanding a wide range of fundamental molecular mechanisms, such as the action of antibiotic peptides, the association of proteins involved in cell signaling and membrane fusion and also nongenomic action of some hormones is based on a molecular interpretation of the interaction of constitutive amino acids with the lipid matrix [1].

Several studies have considered the effect of the lipid composition on the adsorption, penetration and intercalation of functional and structural proteins and its consequences on the enzymatic activity and membrane structure [2–4]. Several homo- and hetero-synthetic peptides have been used as model systems to elucidate the effect of either naturally occurring or synthetic pore-forming peptides. A great emphasis was put on correlating the penetration of the different peptides into the membrane with the phase properties and the domain formation of lipid mixtures [5]. In general, the interaction of proteins with different types of membranes has been explained in terms of the insertion of some amino acids at different depths of the bilayer affecting the hydrocarbon core [6,7]. In this regard, some models postulate the partition of individual amino acids composing the protein of interest into different regions of the bilayer. Thus, thermodynamics of lipid–peptide side chain interactions becomes a critical step for clarifying the stabilization of amino acid side chains into lipid bilayers [2].

It has been suggested that flanking residues of transmembrane segments might influence the positioning of membrane proteins at the membrane interface [8]. This implies the presence of specific sites near the surface for defined amino acids. For instance, it was shown that the binding of polycationic peptides is mainly due to electrostatic interactions and that small peptides do not bind to membranes formed from electrically neutral lipids like PC [9]. However, this binding seems to be due not only to electrostatics [10]. In this regard, other studies suggest the participation of specific interactions of polycationic peptides with phosphocholine head groups [11]. The TAT (peptide rich in arginine) induces the formation of rodlike, presumably inverted micelles in DMPC, which may represent intermediates during the translocation. The molecular interactions responsible of this mechanism seem to involve the formation of complexes between the phosphate group and the arginine side chain [11]. Therefore, in order to understand the mechanism of peptides insertion, it appears of interest to have an insight on the effect of isolated amino acids on the lipid surface and phase properties of membranes of different lipid composition.

Among positively charged amino acids, L-arginine (Arg) is an important component of several peptides and proteins. This amino acid exposes a guanidinium group at the end of an apolar region in addition to the amine and carboxilic groups. There has been great interest in recent years concerning the protonation state of Arg residues in a lipid bilayer environment. This interest was partly triggered by the observation that several Arg residues on the S4 helix may come in contact with the hydrophobic region of the lipid

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membrane in a crystal structure of the potassium channel [12]. In addition, the interaction of the isolated amino acid has also received some attention. It was reported that isolated Arg is transported and accumulated in different types of cells, strongly suggesting the ability of this molecule to be transported through cells or plasma membrane vesicles [13–15]. In connection with these properties and functions, it should be mentioned that enhanced transport of L-arginine in smooth muscle cells [16] occurs when L-arginine is encapsulated in liposomes. This amino acid is associated with the generation of nitric oxide (NO) in living organisms and is involved in endothelial dysfunction associated with atherosclerosis, diabetes and other diseases [17,18].

Molecular dynamics studies, undertaken to achieve an understanding of the mechanism of partition, have shown that Arg may be either charged or uncharged at the center of PC membranes [19]. It has been suggested that this may be due to the formation of water defects connecting the side chains to bulk water [7]. Thus, the energetics of partitioning assumes that the process of Arg interaction with PCs involves the presence of water in the membrane structure. In consequence, the thermodynamics of this process is complex since burying a charge in the membrane involves protonation/deprotonation in bulk water and in the membrane. In turn, deprotonation of Arg may involve lipid membrane deformation and changes in the water structure due to the Arg charge electrostriction [19]. The polar headgroups of the lipids can stabilize the charged Arg residues in the membrane, causing the lipid membrane to deform and dehydrate locally [3]. This implies that the hydration properties of the lipid components of the membrane may regulate the amino acid partitioning. Therefore, the possible effects of Arg could be related to the hydration level of the membrane lipid components. If this is the case, effects of the amino acid could be different in membranes composed of lipids having different affinity for water, such as phosphatidylcholines and phosphatidyl ethanolamines.

The hydration rates of these lipid components are related to the fluctuations at the water–hydrocarbon interphase of the carbonyl groups and the exposure of the phosphate groups to the aqueous media [20]. This affects the compressibility and area per molecule, which appears important in the mechanism of Arg partition, as discussed above. In this particular, PEs may adopt different surface area and special arrangements, due to its molecular shape and to the strong lateral head group interactions due to the formation of H bonds [41].

In this regard, amino acids can be considered H-bonding compounds that may interact with membrane surface groups similarly to sugars and polyphenols, replacing water in the hydration sites [21–26]. Thus, in order to gain insight into the molecular interactions of Arg with membranes with different states of hydration, the effects on surface and dipole potentials have been investigated by means of surface pressure curves and fluorescence methods in DMPC and DMPE interfaces. In particular, the role of the hydration centers, carbonyl and phosphate groups in the two lipids, is of special interest since they are involved in the determination of the surface potentials, such as charge and dipole potential [27]. Constitutive groups of a lipid interface such as P=O and C=O groups and the water molecules polarized by them determine the dipole potential of lipid membranes [21,28]. For these reasons, we have investigated the effect of Arg on the zeta potential, dipole potential and surface pressure of monolayers and bilayers of different derivatives of saturated phosphatidylcholine (DMPC) and phosphatidyl ethanolamine (DMPE). Thus, changes in the zeta and dipole potential may be related to the binding of the amino acid to these groups.

Furthermore, the conformational changes and water content at the polar head groups may modulate local changes in the dielectric constant of the bilayer [29]. For this purpose, structural parameters at the lipid interphase and the hydrocarbon region were measured by fluorescence methodologies for different concentrations of Arg in PC and PE membranes.

2. Materials and methods

2.1. Chemicals

1,2-Dimyristoyl-sn-glycero- 3-phosphocholine (DMPC), 1, 2-di-*O*-tetradecyl-sn-glycero-3- phosphocholine (etherPC), 1,2- dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-di-*O*-tetradecyl-sn-glycero-3-phosphoethanolamine (etherPE), 1,2-dipalmitoyl-sn-glycero-3-phospho-ethanolamine *N*-monomethylated (mmDPPE) and *N*,*N*-dimethylated (dmDPPE) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used as received. The purity of lipids was checked by thin layer chromatography using a chloroform:methanol: water mixture as running solvent.

L-Arginine (Arg) was obtained from Sigma-Aldrich (Saint Louis, MO).

The fluorescence probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-dodecanoyl-2-dimetilaminonaftaleno (Laurdan) were obtained from Molecular Probes and used as received. Chloroform and KCl were analytical grade. Water was MilliQ quality.

The pHs of the solutions for zeta and dipole potentials were adjusted by titrating with HCl or with buffer carbonate when necessary.

2.2. Monolayer formation

Dipole potential and surface pressures were determined in lipid monolayers. Aliquots of chloroform solutions of the different lipids were spread on a clean surface of water, or on aqueous solutions containing Arg at the different concentrations tested. Data were collected when constant potential or pressures were reached and no changes were observed with further additions of lipids. In this saturation condition, the lipids in the monolayer are in equilibrium with lipids forming liposomes in the subphase. In this condition, a corresponding state between bilayer and monolayer is achieved since equilibrium is established by the transfer of lipid molecules to and from the monolayer and the outer monolayer of the vesicles [30,31]. Both experiments were performed at the same temperatures and conditions.

2.2.1. Determination of dipole potential in monolayers

The values of interfacial potential (V_{surf}) were determined through a high impedance circuit, by means of an ionizing electrode on the monolayer and a reference electrode in the aqueous subphase (KCl 1 mM) using the following expression:

$$V_{surf} = V_{Ag/AgCl} - V_{grd} = V_{solution} - V_{grd},$$

where $V_{Ag/AgCl}$ is the potential of the reference electrode and V_{grd} is the potential of the shield covering the ionizing electrode.

Temperature was set at the values indicated in each assay (mostly 18 and 28 °C) and measured with a calibrated thermocouple immersed in the subphase and maintained within ±0.5 °C.

The dipole potential of the monolayer (Ψ_D) was evaluated as

$$\Psi_D = V_{surf} - V_{lip} \quad (1)$$

where V_{surf} is the potential of the clean surface (without lipids) and V_{lip} is the potential after the monolayer was formed.

Different values of Ψ_D were obtained for the clean surface of the amino acid solution assayed and with a monolayer of lipids, in the conditions described below. These values are reported as a function of the amino acid concentration in the subphase solution [32].

2.3. Surface pressure measurements in monolayers

2.3.1. Area per lipid calculation

The formation of saturated monolayers of lipids, on the interface of solutions with and without amino acid, was monitored by measurements of the surface pressure of the different lipid monolayers in a

Kibron μtrough S equipment, at constant temperature (28 ± 0.5 °C) and area. The surface of an aqueous solution contained in a Teflon trough of fixed area was exhaustively cleaned. Then, a chloroform solution of the phospholipids was spread on the surface, until it reach a constant surface pressure for different Arg concentrations in the aqueous subphase. Results of surface pressure were expressed in mN/m.

In the conditions used (see monolayers formation), the measures are attained with lipids in the monolayer in equilibrium with lipids in the subphase. The lipid conformations are stabilized spontaneously according to the aqueous solution properties, without forcing the lipids by the application of any lateral pressure.

The saturation point of the monolayer, for each case, was determined considering the standard deviation of the results, at the plateau of the curve. Those points for which the difference with the mean point of saturation was higher than the standard deviation were not considered. With these criteria, areas per lipid were calculated with the first point of the saturation plateau of a curve of monolayer surface pressure vs. nmoles of lipid added to a constant area trough. Considering that each aliquot corresponds to 0.5 nmol, each determination is affected by an error in the area corresponding to ± 0.25 nmol. For this amount, the error expressed in area is ca. ± 4.7 and ± 3.58 $Å^2$ for PC and PE, respectively.

2.4. Surface pressure changes induced by L- arginine adsorption

Different aliquots of a chloroform solution of phospholipids were spread on the clean surface, to reach increasing surface pressures from 9 mN/m to that for monolayer saturation. In this range, the surface pressure–area isotherms of DMPC and DMPE show that the lipids are forming monolayers [31–34].

For each given initial surface pressure, a fixed volume of an Arg solution was injected in the subphase to reach a final concentration of 21.4 mM. At this concentration, Arg does not change the surface tension of the air–water interface without monolayers. In addition, it is the higher concentration that can be added to the subphase using the minimum volume in order to avoid a significant increase in the final volume of the trough. This value comes out from experiments in which the variation of the surface pressure was followed as a function of the amino acid concentration in the subphase. Surface pressure changes were followed during time up to reach a constant value.

The same procedure was followed for all monolayer compositions. Surface pressure and increases of surface pressure at constant surface area were automatically recorded. Spreading of the amino acid on a clean aqueous surface, as well as the injection of it into water, resulted in no changes in the surface pressure. Surface pressure values shown in the figures are the average of at least three measurements. The individual points were within 5% of the reported values.

2.5. Liposome preparation

Multilamellar liposomes were prepared in order to perform zeta potential and fluorescence measurements with Laurdan.

Multilamellar liposomes (MLVs) were prepared by dispersing the dry lipid films in water (fluorescence experiments) or KCl 1mM (zeta potential), at temperatures higher than that of the phase transition, for 60 min.

2.6. Zeta potential

The zeta potentials (ζ) of DMPC and DMPE liposomes were determined in a Zeta-Meter System 3.0 equipment, at 18 ± 2 °C. The voltage was fixed at 75 V.

The liposomes were prepared by dispersing a dry film in 1 mM KCl above the phase transition temperature of the corresponding lipid. The total lipid concentration in all cases was 52 μM.

Once prepared, liposomes were cooled down to 18 °C and incubated at that temperature with different Arg concentrations (5–100 mM) for 1 h.

Another batch of samples of DMPC liposomes were incubated at ca. 35 °C in the presence of the different Arg concentrations. Liposomes were then cooled to 18 °C, and the zeta potential was determined at that temperature.

A total of 20 measurements were carried out for each sample. Data reported are the average of the measurements done for each condition with, at least, three different batches of liposomes [35].

Multilamellar vesicles used in this study are not in a strained configuration, in contrast to the lipids in small sonicated vesicles. Since it is possible to see these large liposomes with a microscope, measurements are made on individual multilamellar vesicles, in contrast to electrode or dialysis measurements, which are made on an ensemble of vesicles and in consequence an absolute measure of the zero potential can be obtained.

2.7. Fluorescence measurements

Fluorescence measurements were carried out with a Perkin Elmer LS55, luminiscence spectrometer. MLVs were prepared as previously described, by addition of diphenyl hexatriene (DPH) or Laurdan to the chloroformic lipid solution, in a probe to lipid ratio of 1:300, in all cases. The temperature was controlled by an external system. The total lipid concentration, in all cases, was 0.1 mg/mL liposome suspension. L-Arg was added in a final concentration of 100 mM.

2.7.1. Steady-state anisotropy measurement with DPH

The excitation and emission λ were 350 and 452 nm, respectively.

2.7.2. Generalized polarization and anisotropy measurement with Laurdan

Emission intensity was acquired for several hundred seconds at 435 (I_{435}) and 500 (I_{500}) nm (excitation = 350 nm). Generalized polarization (GP) was calculated from the emission intensities according to Parasassi et al. [36].

The fluorescence intensity at 435 nm (350 nm excitation) was used to calculate the anisotropy. In all anisotropy values, a total of 10 measurements were carried out for each sample, at each temperature. Data reported are the average of measurements done for each condition with, at least, three different batches of liposomes. The individual points of the values shown in the figures were within 5% of the reported values.

3. Results

Arginine added to the external phase of gel DMPE liposomes at 18 °C adsorbs to the external lipid surface as denoted by the shift of the zeta potential to negative values (Fig. 1). Since the pH resulting from the dissolution of Arg in water is around 10 at all the concentrations tested, the zeta potential for the different Arg concentrations was compared with control samples of DMPC and DMPE liposomes at pH 10. In both cases, the zeta potential was not affected by the increase in pH from 7 to 10 in the absence of the amino acids. This is reasonable, since the pKa value of DMPE is 11.27 [38].

The presence of Arg displaced the zeta potential of DMPE in 20 mV toward negative values, but no effect was found when Lys was added in the same condition and pH (Table 1). The zeta potential shift to negative values cannot be ascribed to the deprotonation of the PE phosphates because at the higher ionization percentual, where PE is more anionic, the addition of Arg but not of Lys makes the zeta potential further negative. This is taken as a strong indication that the increase in the surface negative charge is due to amino acid adsorption on the external surface of the liposomes.

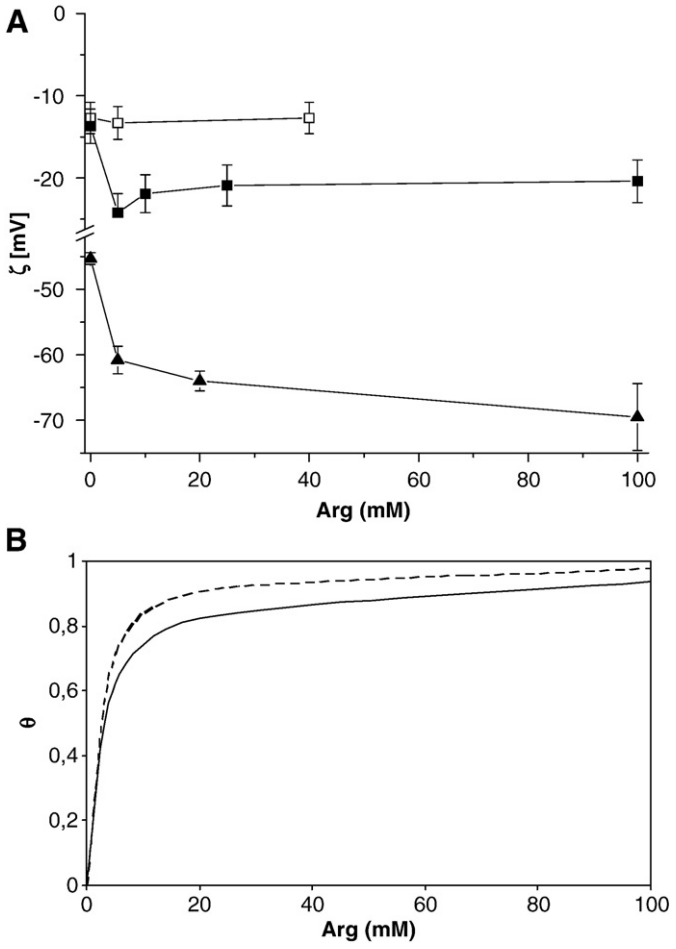

Fig. 1. (A) Binding of L-arginine on DMPC and DMPE liposomes, as measured by zeta potential changes at different Arg concentrations. Similar samples of liposomes prepared in water were dispersed in different concentrations of the amino acids and then incubated in the following conditions. (□) DMPC liposomes incubated and measured at 18 °C. (■) DMPC liposomes incubated at 30 °C and measured at 18 °C. (▲) DMPE liposomes incubated and measured at 18 °C. (B) Adsorption isotherm of Arg to DMPE liposomes in the gel state. The experimental data were fitted by Eq. (2) for $K = 2 \times 10^3\ M^{-1}$ and $n = 1$. The discontinuous line corresponds for a Langmuir isotherm ($n = 1$) considering the same affinity constant.

The degree of coverage (θ) of the external surface at each bulk Arg concentration can be calculated from the zeta potential measures, according to

$$\theta = \frac{\Delta\zeta}{\Delta\zeta max} = \frac{[Arg]^n}{K + [Arg]^n} \quad (2)$$

where K is the affinity constant and n is the heterogeneity parameter describing the width of energy distribution function, which is 1 for a Langmuir behavior [37]. From the fitting of the data according to Eq. (2) (Fig. 1B), $K = 0.2 \times 104\ M^{-1}$, and $n = 0.74$.

No adsorption of Arg was observed with DMPC liposomes in the gel state incubated with Arg in the same concentration range at 18 °C since the zeta potential remained unchanged and equal to the values of pure DMPC liposomes, within the experimental error. Thus, the shift to negative values observed with Arg in DMPE membranes seems to be due to specific interactions of the amino acid and this kind of lipid.

It should be noted that Arg can insert in DMPC membranes when the liposomes were incubated with different concentrations of Arg above the phase transition temperature (28 °C-fluid phase) and then cooled to 18 °C. The resulting small shift to negative values in comparison with the control suggests that the insertion of Arg is not limited to the external surface but instead it may penetrate into the liposome interior. This finding is similar to previous reports [5].

The different insertion of Arg in DMPE and DMPC membranes in the different phase states observed in liposomes is in agreement with studies on lipid monolayers in which the surface pressures were varied. At all pressures, changes are considerable more pronounced in DMPE that in DMPC.

At low surface pressures (Fig. 2A), the effect of Arg on DMPE is similar to that on DMPC at 28 °C, which is liquid expanded. However, at higher surface pressures, the effect of Arg on DMPE in comparison to DMPC is more significant. In addition, the greater surface pressure increase is attenuated when, at 18 °C, methylene groups are attached to the ethanolamine group. For a lipid chain length slightly longer (16:0), the results correlate well with the changes in the polar head group, congruent with the interpretation that the arginine-induced effects are promoted at the surface of gel phase membranes.

Expansion of the scale highlighted in Fig. 2B denotes that even at the higher surface pressure, Arg is able to insert in DMPEs, with a response several times greater than in DMPC, while it affects the surface pressure of DMPC negligibly at 18 and 28 °C within the experimental error (Fig. 2C).

The dependency of Arg insertion with respect to the surface pressure of DMPC and DMPE monolayer can be visualized from the plots of the surface pressure as a function of the initial surface pressure (Fig. 3). For monolayers held at constant area, the surface pressure increase is due to the insertion of molecules from the subphase into the lipid interface [30]. The injections of Arg to reach a final concentration of 21.4 mM into the subphase of DMPC and DMPE monolayers, at different surface pressures, give rise to curves of different slopes (see Materials and methods).

The increase in surface pressure decreases linearly with the initial surface pressure, extrapolating to a characteristic cutoff value that depends on the lipid phase state. However, Arg does not perturb (within the experimental error) monolayers of DMPC at 18 °C within the whole range of surface pressures, which is congruent with the absence of changes in the zeta potential of gel DMPC liposomes shown in Fig. 1.

The response of DMPE monolayers at 18 °C is similar to that corresponding to fluid DMPC monolayers at 28 °C. The curve for DMPE at 18 °C is quite parallel to that of DMPC at 28 °C being this last one shifted to lower values with little differences in the cutoff. The similar behavior of DMPE bilayers in the gel phase with those of DMPC at 28 °C indicates that Arg should be able to interact with PE affecting the organization of the interfacial region in the gel phase.

Table 1
Comparison of zeta potential and relative area per molecule increase with the onset of T_m and the relative change in surface pressure ($\Delta\pi/\pi_0$) on DMPC and DMPE monolayers at 18 °C in the presence of Arg and Lys.

Lipid	Zeta potential	% area increase	ΔT_m (°C) onset anisotropy Laurdan	$\Delta\pi/\pi_o$ at equilibrium, $\pi_o = 10$ mN/m
DMPC control	−13.7 ± 2.1	–	–	–
DMPC – arg	−13.3 ± 2.0	11	0	0.1
DMPE control	−45.3 ± 0.9	–	–	–
DMPE – arg	−70.0 ± 3.2	8.2	−10	0.5
DMPE – lys	−47.5 ± 3.3	ND	0	ND

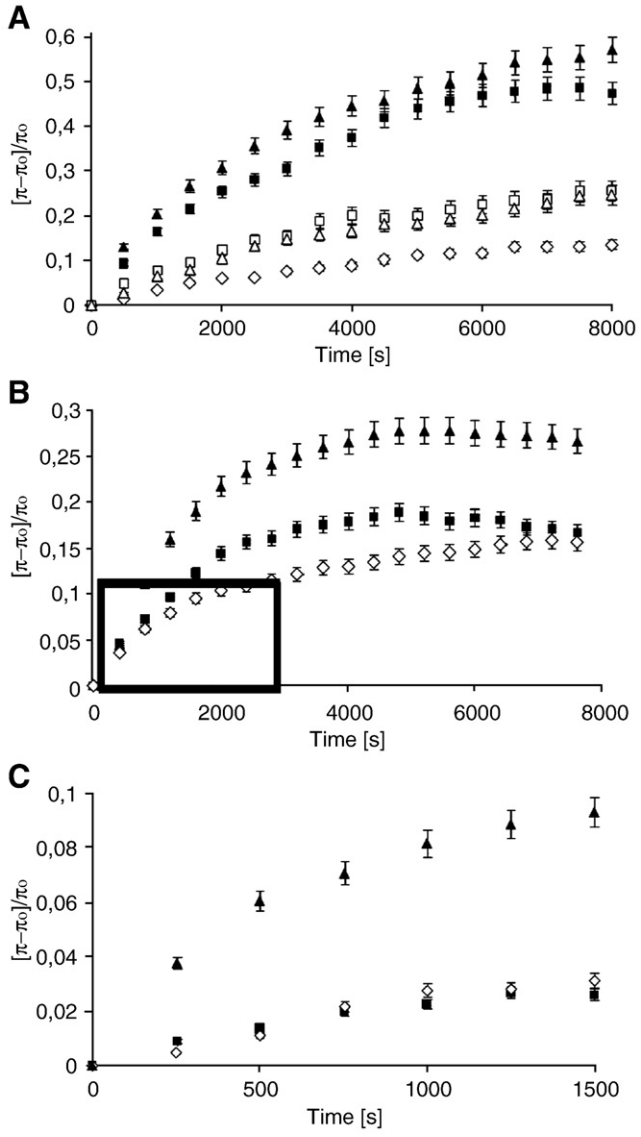

Fig. 2. Relative increase in the surface pressure ($\pi - \pi_0/\pi_0$) of gel DMPE (▲), gel DMPC (◊), fluid DMPC (■), gel mm-DPPE (△) and gel dm-DPPE (□) monolayers with 21.4 mM Arg at 18 and 28 °C as a function of time. (A) $\pi_0 = 10$ mN/m, (B) $\pi_0 = 30$ mN/m, (C) $\pi_0 = 42$ mN/m.

Comparison of the rate of surface pressure changes induced by the injection of 21.4 mM Arg into the subphase of PC and PE monolayers and the mono and dimethyl PCs-monolayers, suggests again the importance of the head group methylation of the ethanolamine group in the Arg interaction. This becomes more apparent when the effect of Arg on DMPC and DMPE membrane interfaces was followed by changes in fluorescence anisotropy measurements using Laurdan (Fig. 4). It is observed that Arg decreases at about 10 °C, the onset of the transition in DMPE in comparison with a control that does not contain the amino acid. In contrast, no effect is observed in DMPC membranes for a similar Arg concentration. In addition, similar values of anisotropy were obtained with both lipids above the phase transition temperature. For comparison, measurements were carried out with Gly (without side chain) and Lys (without guanidium group). Neither Gly nor Lys affected the transition onset (data not shown). These findings suggest that the arginine-induced perturbation is preferentially ascribed to the presence of the guanidinium group in Arg.

The displacements of the GP values for DMPC and DMPE, reflecting changes in hydration, show some differences between DMPC and DMPE (Fig. 5). In the case of DMPC, neither the gel nor the fluid phase was affected, although a small shift to lower temperatures ca. 1.7 °C is observed. In the case of DMPE, the GP values of the gel and fluid phases are decreased. A similar effect was observed when the anisotropy of the lipid membrane is measured in the hydrocarbon core with DPH (Fig. 6).

Alternatively, stabilization of Arg in monolayers of the ester and ether forms of DMPC and DMPE resulted in a lower dipole potential, in comparison to water, but with a similar decrease in all the lipids (around 100 mV for Arg 100 mM in the aqueous subphase) (Fig. 7). The absence of carbonyl groups in the alkyl derivatives (etPC and etPE) did not modify appreciably the decrease in comparison to the acyl analogous PCs and PEs. The

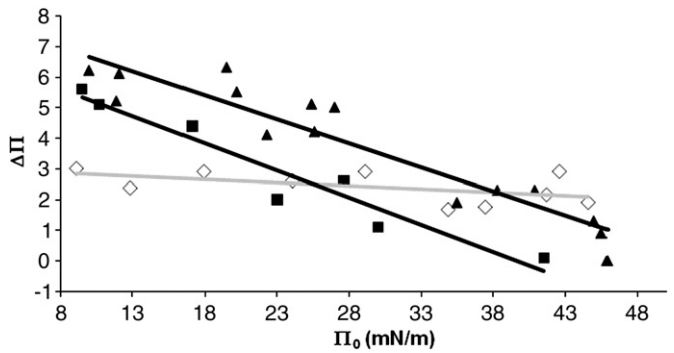

Fig. 3. Effect of Arg 21.4 mM on the surface pressure of monolayers (Δπ) of DMPE at 18 °C (▲), DMPC at 18 °C (◊) and DMPC at 28 °C (■) at different initial pressures.

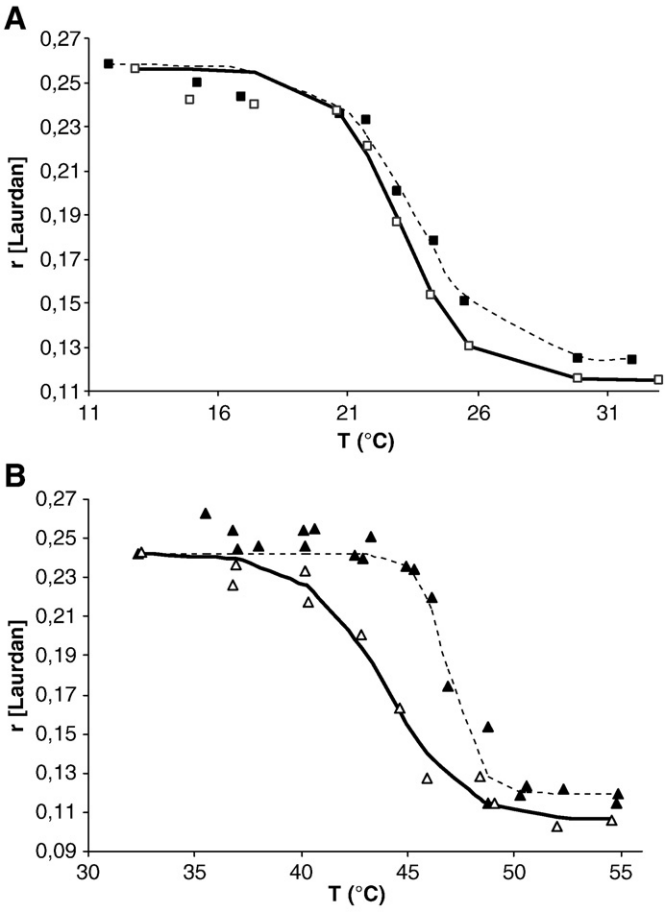

Fig. 4. Effect of L-arginine on the anisotropy measured with Laurdan, in (A) DMPC and (B) DMPE. (■) DMPC, (□) DMPC-Arg 100 mM, (▲) DMPE, (Δ) DMPE-Arg 100 mM.

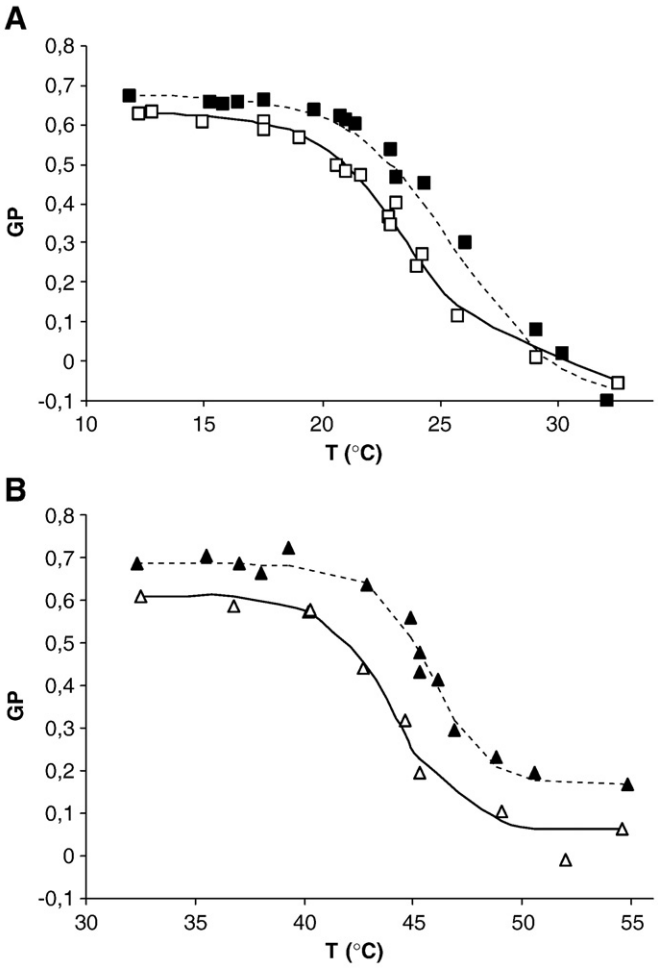

Fig. 5. Effect of L-arginine on the GP values of Laurdan, in (A) DMPC and (B) DMPE. (■) DMPC, (□) DMPC-Arg 100 mM, (▲) DMPE, (Δ) DMPE-Arg 100 mM.

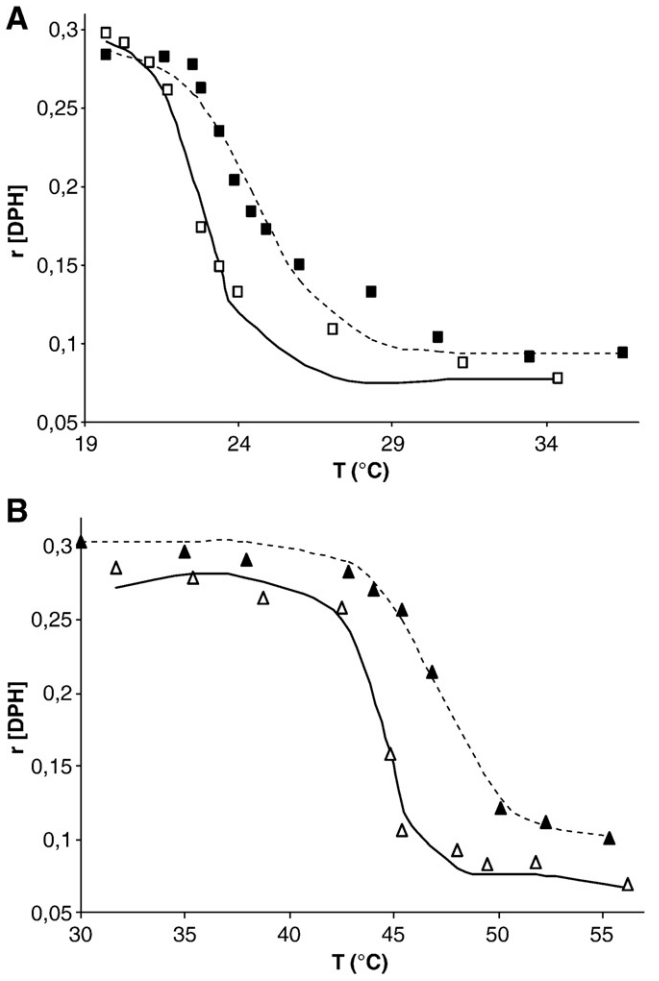

Fig. 6. Effect of L-arginine on the anisotropy measured with DPH, in (A) DMPC and (B) DMPE. (■) DMPC, (□) DMPC-Arg 100 mM, (▲)DMPE, (Δ) DMPE-Arg 100 mM.

decrease observed in the dipole potential of DMPC and DMPE is congruent with the observation of an increase in the area per lipid in the presence of Arg. Table 1 summarizes the relative changes in zeta potential, area and the decrease on the onset of the phase transition measured with Laurdan, for DMPC and DMPE in the gel state and the methyl PEs.

4. Discussion

The present results show striking differences in the interaction of Arg with DMPC and DMPE. Evidences for the penetration of Arg into phosphatidylcholine membranes and its encapsulation in the interior of liposomes have been reported elsewhere [5]. Changes in the zeta-potential were attributed to incorporation of a portion of Arg in the interior of the liposome, suggesting that most of the amino acid molecules can be located in the Gouy–Chapman ionic mobile layer of the outer bilayer. Possible mechanisms for this insertion include either passive diffusion through the bilayer (which is higher at the main lipid phase transition), or by a flip-flop mediated transport.

Data shown in Fig. 1 indicate that there is no effect of Arg on the zeta potential of DMPC liposomes at 18 °C. Interestingly, Arg adsorbs on DMPE membrane in the gel state. DMPE liposomes at 18 °C adsorb Arg following a non-Langmuir isotherm, with an affinity binding constant $K = 2 \times 10^5\ M^{-1}$ and $n = 0.74$, [37]. This means that the adsorption takes place in nonindependent sites, suggesting surface rearrangements. In addition, the affinity constant is in the same order as that reported for Arg on dodecyl hydrogen phosphate [39].

The control experiments varying the pH solution demonstrate that the zeta potential is not affected by the pH increase. Even at pH 10, at which the PE may be considered as an anionic lipid, the addition of Arg shifts the zeta potential by 20 mV to more negative values. This effect is absent when Lys is added to PE in the same conditions. In another set of measurements, Gly did not change the surface potential

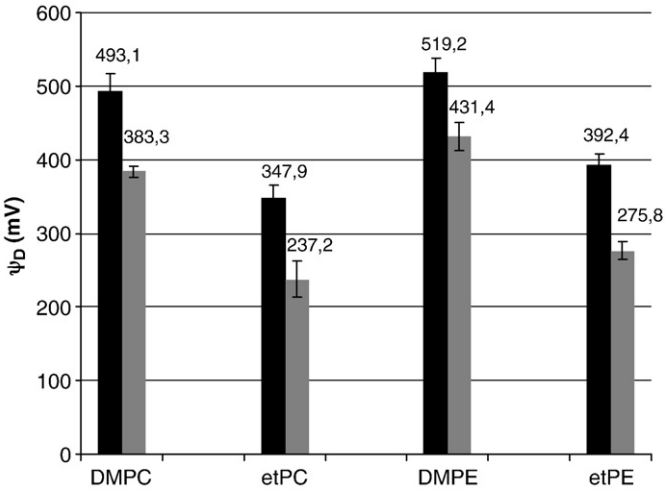

Fig. 7. Effect of Arg 100 Mm on the dipole potential of DMPC, etPC, DMPE and etPE at 28 °C. (■) Pure lipids, ■ lipid + Arg.

of PE liposomes (data not shown). These results indicate that the particular different residue on Arg, the guanidinium group, determines the insertion.

The same conclusion can be derived from the studies on monolayers (Figs. 2 and 3). At similar initial surface pressures, the relative increase of surface pressure is higher for DMPE than for DMPC suggesting that the insertion of Arg is favored. In addition, methyl substituted PEs (mm-DPPE and dm-DPPE) show an intermediate response, indicating that the insertion is disfavored when the amine is blocked by bulky methyl groups. In spite of the increase in the acyl chain, the effects in membranes in the gel state correlate well with the changes in the head group, promoting evidence that the Arg main site is located at the interphase.

This result is unexpected since it is known that the packing of PEs is higher than those of PCs due to the formation of H bonds with the neighbor molecules leading to a lower hydration degree and a much lower area per lipid in PEs [40].

The kinetics of Arg insertion can be described (Fig. 2) by

$$d\pi / dt = RT / A(dn / dt) = RT(d\Gamma / dt)$$

where A is the interface area and n is the total number of molecules at the interface (lipids plus amino acid). The changes in surface pressure as a function of time are a direct measure of the insertion in the interface given by the increase of the surface excess (Γ) of Arg molecules at constant area and constant lipids in the monolayer [30].

It is clear that the kinetics of insertion decreases abruptly for DMPC in the condensed state and when DMPC is compressed from 10 to 42 mN/m.

The kinetics of insertion in DMPE also decreases with the lateral pressure but is always higher than that of DMPC in the same conditions.

On the other hand, the effects on membrane surface properties, surface potential and onset of the phase transition are significant only if the guanidinium group is present since no effect has been observed in the presence of Lys. These data suggest that the interaction of Arg with lipids is favored by the presence of nonmethylated NH_2 groups in the lipids and guanidine N_3C group in the arginine. Moreover, the shift in the zeta potential of DMPE liposomes induced by Lys is not significant within the experimental error as compared to that induced by Arg in membranes (Table 1). Thus, the increase in the negative surface charge induced by Arg is an additional evidence that guanidine group is responsible for its mechanism of insertion.

It is possible that the higher negative surface potential of DMPE in comparison with DMPC would favor the interaction with the positive group of Arg (Table 1). This hypothesis is consistent with the zeta potential values, -39.15 ± 2.3 mV and -29.9 ± 3.2 mV for the mono and dimethyl-DPPEs, respectively, for which the effect of Arg on the surface pressure was shown to be intermediate between those of DMPC and DMPE (Fig. 2).

Pure DMPE liposomes' potential is higher at pH 10 than that for DMPC. This condition could be responsible for a higher electrostatic interaction with the positive end of the amino acid, stabilizing the molecule, which would lead to the exposure of the negative portion (carboxyl groups) to the aqueous media, explaining the larger negative charge of Arg-DMPE liposomes.

Thus, considering the results obtained with Lys, for which no changes in the zeta potential were observed, the increase in the negative surface charge can be driven by the presence of the guanidinium group.

Judging from the dipole potential measures (Fig. 7), the final orientation of Arg is similar in DMPC and DMPE membranes. In addition, Arg dipole should oppose the PO dipoles, since Arg also decreases the dipole potential in the same magnitude in phospholipids without carbonyls.

As the dipole potential decrease and the effect on the hydrocarbon core is similar in DMPC and DMPE (Fig. 6), the substantial difference of the effect of Arg in these lipids is more likely related to the mechanism of insertion to achieve the final equilibrium position of the dipoles.

In DMPC, the insertion is possible only if the membrane goes through phase transition. In DMPE, the insertion takes place in the gel phase. This disruption at the interfacial level, as shown by the Laurdan experiments, seems to be related to the presence of the guanidinium group since the comparison with Lys gives such dissimilar results. The particular action of Arg on DMPE may be caused by the higher negative surface charges of DMPE in comparison to DMPC membranes. The electrostatic interaction would promote the insertion of the positive moieties explaining the onset of the transition 10 °C lower than the control in DMPE and the negative shift of the surface potential.

One possibility to achieve this stabilization is that the guanidinium group would be oriented into the membrane. It is well known that the guanidinium group interacts with the phosphates of the lipid membrane through a possible hydrogen bond [41]. In addition, there is evidence that the H bonds with the imine group are thermodynamically favored with respect to H bonds with the amine ones [42]. Thus, driven by electrostatic interaction, guanidine moieties would compete for the H-bonds between the phosphate and amine groups of the PEs at the surface.

The significantly higher change in Δπ observed for PE than for PC is consistent with the onset decrease of the phase transition. The disorder introduced by Arg in PE membranes is congruent with the higher rate of penetration and the magnitude of the change in the surface pressure for similar initial surface pressures in PCs (Fig. 2).

It has been suggested that when Arg is added from the outside to PC bilayers, the hydrophobic side is buried, exposing a negative portion to the aqueous phase. The energetic of partitioning resulting from molecular dynamics simulations postulates that the charged Arg molecule can be placed at the center of the PC membranes by the rapid formation of a water defect rather than simple partitioning between water and a hydrophobic phase of PCs [6,7]. The expectation that Arg can adopt the protonated state despite the low dielectric nature of the bulk lipid membrane means that the molecule should be stabilized by other interactions.

Partitioning of polar and charged residues into the hydrocarbon core may be accompanied by water penetration. This would explain the effects of the gel and fluid state measured with DPH in DMPE liposomes.

It is likely that this mechanism can take place also in PEs. The evidences presented here allow one to conclude that the increase in the negative surface charge of DMPE liposomes is due to the presence of the guanidinium group and that this interaction is attenuated by the methylation of the ethanolamine group. In addition, the presence of carbonyl groups in the lipids seems not to be relevant for this insertion.

Summarizing, Arg interacts preferentially with DMPE monolayers and bilayers in comparison to DMPC. This interaction appears as a direct consequence of the presence of a guanidinium group—in the amino acid molecule—and of the ethanolamine group—in the lipid molecule. This conclusion is derived from the observation that the methylation of the ethanolamine group decreases the response and that Lys did not cause any perturbations at the interface. As a result, the polarity of the interphase is increased as well as the negative charge surface density. The presence of interfacial carbonyls is not involved in this interaction. The stability of the complex Arg-PE might be higher due to the formation of strong bonds between the imine and the phosphates groups, stabilized by the resonance of the CN bond in the guanidinium group.

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