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The surface organization of diacylglycerol pyrophosphate and its interaction with phosphatidic acid at the air–water interface

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ABSTRACT

Diacylglycerol pyrophosphate (DGPP), a phosphorylated form of phosphatidic acid (PA), gained attention recently due to its role as signaling lipid. However, little is known about its surface organization and potential impact on membrane-mediated function. In this work we investigated the interfacial behavior of Langmuir monolayers formed with pure DGPP and of its mixtures with PA. We found that changes of the subphase pH affect the surface behavior of DGPP. At pH 8, DGPP forms liquid expanded monolayers with a compressibility modulus of about 60 mN m^{-1} at collapse. On acidic solutions, the compressibility modulus increases to 90 mN m^{-1} and the average molecular area is smaller. At pH 8, DGPP and its precursor PA form thermodynamically favored topographically homogeneous non-ideal mixtures. The interaction among these lipids leads to a non-ideal diminution of the mean molecular area and consequently, to an increase of the compressibility modulus, with variations of the surface electrostatics. The favorable interaction of PA and DGPP, leading to changes of the film packing suggest that DGPP may act as a structural signal transducer in membrane-mediated cellular processes.

1. Introduction

Diacylglycerol pyrophosphate (DGPP) is a novel phospholipid found in biological membranes, with a relatively simple chemical structure within the glycerophospholipid family (Wissing and Behrbohm, 1993a). DGPP is synthesized from phosphatidic acid (PA) and ATP via the reaction catalyzed by phosphatidate kinase (PAK) and dephosphorylated to PA by the enzyme DGPP phosphatase (Wissing and Behrbohm, 1993b). Both enzymes were identified in several plants, bacteria and eukaryotic microbes (Wissing and Behrbohm, 1993b; Wu et al., 1996; Dillon et al., 1996; Marchesini et al., 1998). However, in mammalian cells such phospholipid has not been identified to date (van Schooten et al., 2006). The average concentration of DGPP in cell membranes is usually very low (Wissing and Behrbohm, 1993a) but recent evidence suggests that DGPP may act as a novel second messenger with important roles in diverse cellular processes related to drought and osmotic stress or salinity (van Schooten et al., 2006 and reference therein). Since its formation is transient and it is always associated with variations in the amount of PA, the DGPP formation may also be a way of attenuating PA levels (Munnik et al., 1996; Racagni et al., 2008; Villasuso et al., 2003).

DGPP is an anionic phospholipid with a pyrophosphate group attached to diacylglycerol. It has been suggested that, depending on the pH, the pyrophosphate moiety of DGPP could display 2 or 3 negative charges making it a highly polar molecule (Zalejski et al., 2005). Although it has not been demonstrated *in vivo*, it has also been suggested that the pyrophosphate group may play an important role for electrostatic interactions between DGPP and proteins as well as bivalent cations like Zn^{2+} and Ca^{2+} (Han et al., 2001; Zalejski et al., 2006).

The exact mechanism of DGPP action in these diverse processes is not yet clear, although two possibilities are likely. DGPP may function through the activation/recruitment of effector proteins by direct interaction and/or by a modulation of membrane properties such as packing, curvature and electrostatics (Zalejski et al., 2005). Hence, it is of great importance to first understand the interfacial packing and electrostatic behavior of this bioactive lipid as well as the interaction of this molecule with its precursor.

PA, the lipid precursor of DGPP, is the glycerophospholipid with the simplest chemical structure in biological membranes; its behavior is crucial for cell survival since it is a phospholipid intermediate used for the synthesis of phospholipids and triacylglycerols and plays a role in cell signaling (Athenstaedt and Daum, 1999).

Abbreviations: BAM, Brewster angle microscopy; DAG, diacylglycerol; DGPP, diacylglycerol pyrophosphate; FM, fluorescence microscopy; PA, phosphatidic acid; PLD, phospholipase D; PLs, phospholipids.

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It has been recently shown that the specificity of PA–protein interaction is likely related to the ionization properties of the phosphomonoester head group and a model for such mechanism has been proposed on the basis of the ionization properties (Kooijman et al., 2007; Kooijman and Burger, 2009). Even though the overall molar concentration of either PA or DGPP is low, during signaling process the local concentration may transiently reach high levels and this fact could affect local membrane properties at different levels. Little is known about the interaction of PA with DGPP and if these lipids can molecularly mix, with or without interactions that may modify their individual properties. In this study, we provide evidence on the molecular packing, in-plane elasticity, and surface electrostatic of films of pure DGPP, and on the variation of those properties that occur as a consequence of intermolecular interactions between DGPP and PA in mixed monolayers at the air–water.

2. Experimental procedures

Dioleoylglycerol pyrophosphate (DGPP), 1-palmitoyl-2-oleoyl-*sn*-glycerol 3 phosphate (PA) and the lipophilic fluorescent probe l-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl)-ammonium salt were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The lipids were dissolved in chloroform–methanol (2:1, v/v) to a final concentration of 10 nmol μl^{-1}. For all the experiments, the subphase was 150 mM NaCl, 5 mM EDTA, pH 8 or 5. The pH was stable during the time of the experiment. The subphase was prepared with ultra-pure water obtained from a Millipore purification system (18.2 MΩ). Solvents were of the highest available purity from Merck (Darmstadt, Germany).

Langmuir monolayers of the individual lipids and their binary mixtures were spread from premixed solutions in the desired proportion to an initial average molecular area of about 2 nm^2/molecule. Surface pressure and surface potential *vs* molecular area isotherms were obtained at 24 ± 1 °C in a Teflon trough of a Langmuir film balance (Monofilmmeter, Mayer Feintechnik, Germany). Temperature was maintained within ±1 °C with a refrigerated Haake F3C thermocirculator and air-conditioning the room temperature.

Surface pressure was measured with a platinized-Pt Wilhelmy plate. The surface potential measurements were carried out with a high impedance millivoltmeter connected to a surface ionizing ^{241}Am electrode positioned 5 mm above the monolayer surface and to a reference Ag/AgCl/Cl^{-1} (3 M) electrode tip in the aqueous subphase.

Absence of surface-active impurities in the subphase and in the spreading solvents was routinely controlled as described elsewhere (Maggio, 2004). At least triplicate monolayer isotherms were obtained and averaged at a compression rate of 0.45–0.60 nm^2 $molecule^{-1}$ min^{-1}; for each mixture, it was ascertained that reducing the compression speed produced no change in the isotherms and that recompression after 5 min equilibration of the expanded isotherm at surface pressures below 2 mN m^{-1} led to no significant changes of the limiting mean molecular areas which rules out film loss or kinetically-limited artefacts.

Reproducibility was within a maximum S.E.M. of ±1 mN m^{-1} for surface pressure, ±30 mV for surface potential, and always below ±0.03 nm^2 for molecular areas; S.E.M. was calculated from the average of at least triplicate monolayer isotherms. The monolayers of the pure components and of all mixed films were stable and reproducible by recompression.

The monolayer compressibility modulus (κ), also known as in-plane elasticity, was calculated for each mixture as $\kappa = -A_m(\partial\pi/\partial A_m)_T$ and the results were compared with those of ideal mixtures calculated from $\kappa_{ideal} = k_1k_2(x_1A_1 + x_2A_2)/A_1x_1k_2 + A_2x_2k_1$ as explained in Brown and Brockman (2007). In this equation k_1 and k_2 correspond to DGPP and PA compressibility modulus respectively; x_1 and x_2 correspond to DGPP and PA mole fraction; A_1 and A_2 correspond to DGPP and PA molecular area. Interactions and molecular miscibility were ascertained from deviations of the ideal behavior of the mean molecular area, of the resultant perpendicular molecular dipole moment and of the compressibility modulus. Besides, the partial molecular area of both lipids in the mixture was calculated from the average molecular area *vs* the mole fraction in the mixture using the methods of intercept, as previously described (Maggio, 2004). The resultant perpendicular molecular dipole moment ($\mu_\perp$) was calculated considering the capacitor model (Gaines, 1966) after subtracting the double layer potential (Ψ_0) to the measured surface potential, as $\mu_\perp = (\Delta VA/37.7) - \psi_0$.

The double layer potential was calculated using the Gouy–Chapman model which, for an ionized surfactant monolayer, reads: $\Psi_0 = (2RT/F)\sinh^{-1}(22.9q/A_mC^{1/2}T^{1/2})$ (Davies and Rideal, 1963). In this equation, q is the average charge per molecule (in elemental units), A_m the molecular area (in nm^2), T the absolute temperature, C the molar ion concentration, F the Faraday constant and R the ideal gas constant.

For a partially ionized surfactant monolayer, the value of q is the average of the charge of each i-species (z_i) weighted by their fraction of the total concentration of acid species α_i, $q = \sum_i \alpha_i z_i$.

The distribution of species at the interface depends on the local pH. Since the proton ions are positively charged species, the proton concentration will vary in the interfacial region according to a Boltzmann distribution (Davies and Rideal, 1963), $[H^+]_s = [H^+]_b \exp(-\Psi_0 F/RT)$.

All these considerations lead to the following equation, which can be solved numerically, knowing the pK_as and the involved chemical species:

$$[H^+]_s = [H^+]_b \exp\left(-\frac{2RT}{F}\sinh^{-1}\left(\frac{22.9\left(\sum_i \alpha_i z_i\right)}{A_m C^{1/2}T^{1/2}}\right)\right)$$

For example, in the case of pure DGPP monolayers with a bulk pH of 8 the more abundant species at the interface would be the three- and the two-negatively charged species and the involved pK_a value is pK_{a3} = 6.7. In that case, $q = -2 \times (1 - \alpha_{3-}) - 3\alpha_{3-}$, where α_{3-} is the fraction of the three-negatively charged species ($\alpha_{3-} = [DGPP^{3-}]/[DGPP]_{total} = Ka3/(Ka3 + [H^+]_s)$).

In order to asses thermodynamically favorable or unfavorable interactions, the excess free energy of mixing was calculated as the difference between the area under the experimental and the ideal surface pressure–molecular area isotherms, integrated between 2 and 35 mN m^{-1} ($\Delta G = \int_{2mN/m}^{35mN/m}(A_{mix} - x_{PA}A_{PA} - x_{DGPP}A_{DGPP})\,d\pi$); these conditions reduce errors at high surface pressures derived from variations of compressibility due to proximity to the monolayer collapse and those at low surface pressures arising from the rather variable gaseous region of the isotherms below 1 mN m^{-1} that can be markedly dependent on technical artefacts during spreading. Thus, the values discussed herein for this parameter include only the liquid expanded and/or condensed state of coherent films before entering the collapse region and leave out the contribution from the gaseous states of the isotherms.

The films were observed by fluorescence microscopy (FM) or Brewster angle microscopy (BAM) while simultaneously registering the surface pressure *vs* molecular area isotherms. An automated Langmuir film balance (microthrough, Kibron, Helsinki, Finland or Nima Technology Ltd., Coventry, England, model 102M), with a Pt Wilhelmy plate for surface pressure determination, was placed on the stage of the fluorescence or the Brewster angle microscope.

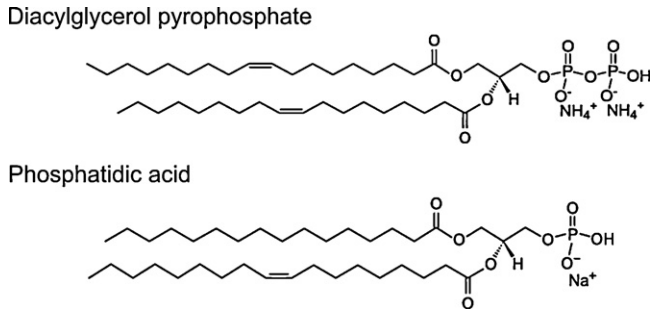

Fig. 1. Chemical structure of diacylglycerol pyrophosphate and phosphatidic acid.

The fluorescence microscope employed was a Zeiss Axiovert 200 (Carl Zeiss, Oberkochen, Germany). Images were registered by a CCD video camera AxioCam HRc (Carl Zeiss, Oberkochen, Germany) commanded through the Axiovision 3.1 software of the Zeiss microscope. Long distance 20× and 40× objectives were employed. The fluorescent probe was incorporated in the lipid solution before spreading (1 mol%).

For the BAM experiments we used an EP3 Imaging ellipsometer (Accurion, Goettingen, Germany) with a 20× objective. These observations were performed in order to ensure that the homogeneity on the micron scale observed in FM experiments was not due to an equal partition of the fluorescent probe used in different lipid phases. All the experiments were performed at 24 ± 1 °C.

3. Results and discussion

As already mentioned, the polar head group of DGPP is a pyrophosphate moiety (Fig. 1), and thus, the net charge on the molecule should change with pH. The pyrophosphoric acid exhibits four pK_as values: pK_{a1} = 0.91, pK_{a2} = 2.10, pK_{a3} = 6.70, pK_{a4} = 9.32 (Lide, 2005). Thus, the polar head group of DGPP may bear from 1 to 3 negative charges, depending on pH. The fourth proton dissociation will not occur because of the involvement of that oxygen atom in an ester bond with the glycerol backbone.

Taking the pyrophosphoric acid pK_as in consideration, we performed compression isotherms on subphases at pH 5 and at pH 8 with the aim of analyzing the effect of charge on the molecular packing behavior. Using the approach explained in Section 2, we calculated the surface pH on both subphases as a function of the lipid density (average molecular area). We found that for a bulk pH of 8 or 5 the surface pH drops to 5–6 or 2–3 respectively, depending on the molecular packing. At a bulk pH of 8 the equilibrium species at the surface are the two- and the three-negatively charged and α_{-3} is about 0.2 while at a bulk pH of 5 the involved species are the one- and the two-negatively charged ones and α_{-2} is about 0.8.

Fig. 2A shows the lateral pressure *vs* average molecular area isotherms for DGPP on subphases at pH 5 (dotted line) and at pH 8 (continuous line). On subphases at pH 8, DGPP forms liquid-expanded monolayers with no detectable pressure–area reorganization during the compression. The lateral pressure increases monotonically with compressibility values from 20 mN m^{-1} to 60 mN m^{-1} (Fig. 2B). The monolayer collapses at 43 mN m^{-1} and 0.57 nm^2 and the lift off area is 1.5 nm^2. Lowering the subphase pH causes a diminution in the average molecular area (Fig. 2A) and an increase of the compressibility modulus (Fig. 2B) at lateral pressures higher than 5–10 mN m^{-1} (see inset in Fig. 2B), with a slightly reduced collapse pressure (collapse point: 40 mN m^{-1} at 0.46 nm^2). The lift off area at this pH is 1 nm^2. The compressibility modulus ranges from 10 mN m^{-1} to 90 mN m^{-1}, also indicating a liquid-expanded behavior (Davies and Rideal, 1963) that becomes more condensed under compression above 5–10 mN m^{-1}, compared to the behavior at pH 8.

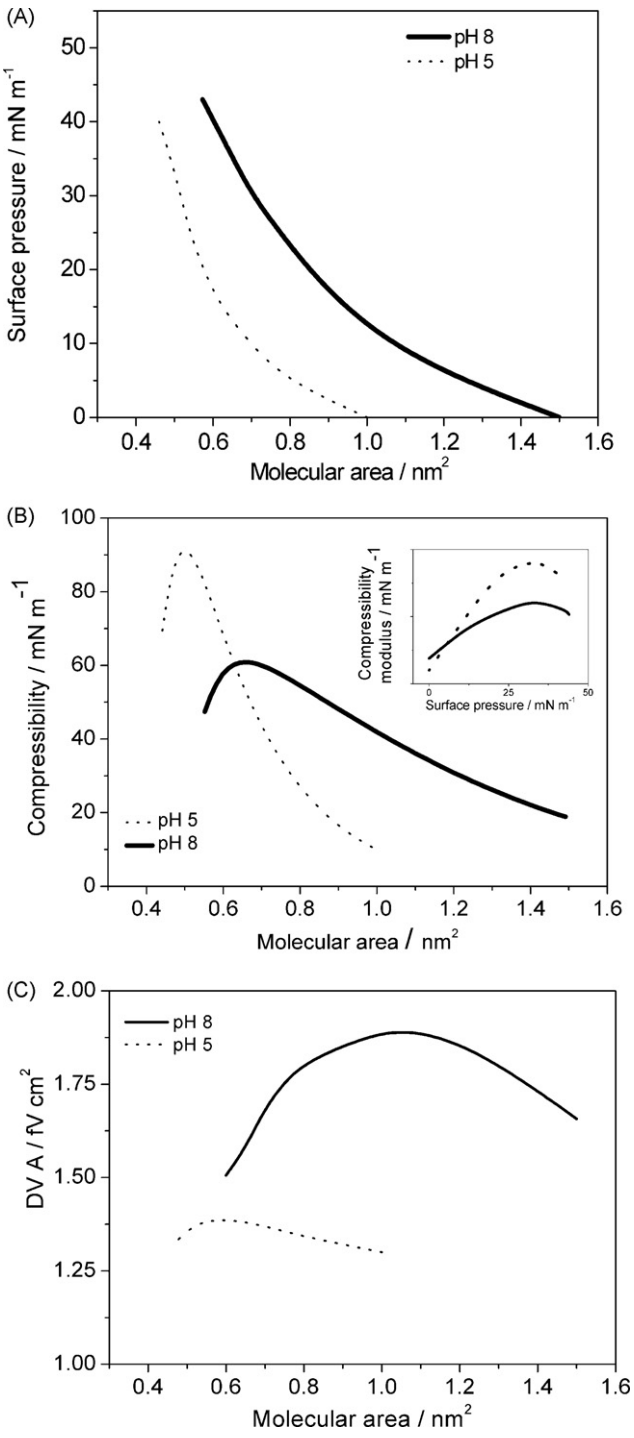

Fig. 2. Compression isotherms for monolayers of diacylglycerol pyrophosphate on subphases at the indicated pHs. Surface pressure (A), compressibility modulus (B) and surface potential density (C) as a function of the average molecular area. The inset in (B) shows the compressibility modulus as a function of the lateral pressure for the indicated in the main panel.

The compression isotherms of monolayers for a particular lipid species depend on the length and unsaturation of the hydrocarbon chain and on the bulkiness and charge of the polar head group. Long and saturated hydrocarbon chains would interact through Van der Waals attractions, promoting more condensed monolayers. By contrast, ionization of the lipid head groups should result in repulsive interactions, leading to loosely packed monolayers (Brown and Brockman, 2007). Thus, the observed compression isotherms of

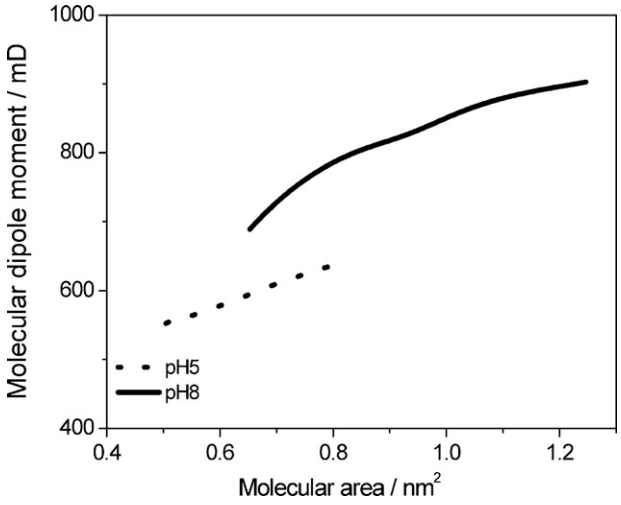

Fig. 3. Resultant perpendicular dipole moment as a function of the mean molecular area at different pHs.

DGPP at pHs 5 and 8 are in agreement with an increased electrostatic repulsion at pH 8 where a higher net charge (about −2.2 at pH 8 and about −1.8 at pH 5) is expected, according to the pK_as values for the pyrophosphate acid. The dielectric constant decreases abruptly from about 80 in the bulk aqueous medium to between 3 and 10 in the polar head group–hydrocarbon interface, depending on the type, charge and proportion of lipid polar head groups (Montich et al., 1985). This means that the negative charge on the lipids signifies an increased electrostatic potential (McLaughlin, 1989) with an effect on the distribution and ionization of nearby charged components such as lipid dipoles that can act as sensitive sensors of the surface electrostatics (Seelig et al., 1987); this potential includes the contribution of mobile ions in the immediate aqueous milieu (the double layer potential). To analyze the effect of charge on the interface electrostatics, we measured the surface potential/molecular density compression isotherms on subphases at each pH. Fig. 2C shows the surface potential/molecular density as a function of the average molecular area at pH 8 (continuous line) and at pH 5 (dotted line). At pH 8, the surface potential/molecular density at the lift off area is 1.65 fV cm^2/molecule (Fig. 2C, continuous line). This parameter increases upon compression reaching a maximum value of 1.85 fV cm^2/molecule at 1 nm^2; afterwards, it decreases up to 1.5 fV cm^2/molecule at collapse. By contrast, at pH 5 the surface potential/molecular density is about 0.5 fV cm^2/molecule lower and it remains relatively constant upon compression. The combined contributions of the fundamental lipid dipoles (from the polar head group and hydrocarbon moieties), their variation by relative orientations perpendicular to the surface, the changes of the ionic double layer potential, and/or alterations of the polar head group hydration shell are all included in the parameter calculated as surface potential/molecular density (Gaines, 1966; Brockman, 1994). Only one of those components namely the contribution of the ionic double layer to the surface potential (which is proportional to the surface charge density and, therefore, to the molecular packing directly accessible at each surface pressure from the compression isotherm), can be rather adequately accounted for by the Gouy–Chapman theory. With the latter, such contribution can be subtracted and the overall resultant perpendicular dipole moment to the surface can be obtained (Gaines, 1966; Brockman, 1994); this is still a very complex parameter that includes all the other contributions mentioned above but it is more closely related to changes pertaining to the interfacial dipoles directly involved with the lipids.

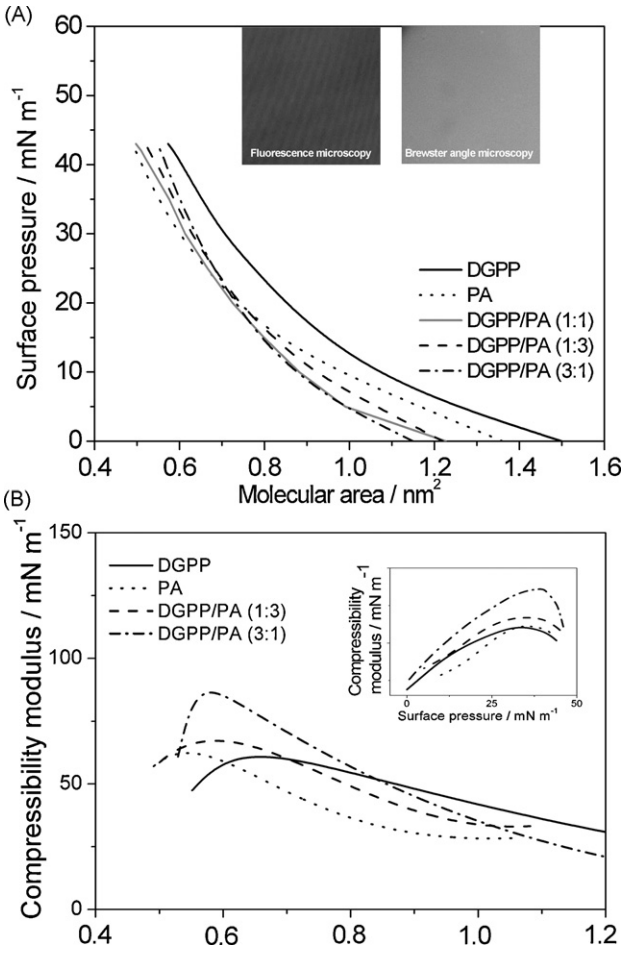

Fig. 4. Compression isotherms for monolayers of diacylglycerol pyrophosphate, phosphatidic acid and their mixtures on subphases at pH 8. Surface pressure (A) and compressibility modulus (B) as a function of the average molecular area. The inset in (A) shows the FM and BAM images for DGPP/PA (1:1) at 20 mN m^{-1}. The inset in (B) shows the compressibility modulus as a function of the lateral pressure for films with the same proportions than in main panel.

Fig. 3 shows the resultant perpendicular dipole moment at both pHs as a function of the monolayer packing. This figure shows that, once the ionic double layer potential is subtracted, considerable differences in the resultant perpendicular dipole moment contribution are revealed for films at both pHs under compression, due to the change in the expanded state of the film or to hydration water rearrangement. On the other hand, the dipole moment for the molecules at pH 8 is higher than the value found on pH 5, as expected considering the increase in the average molecule charge with the increase on the bulk pH.

Besides, the presence of inflections of the dipole moment on subphases at pH 8 (at about 1.0 nm^2) reveals rearrangements of the dipoles upon compression that is absent at pH 5.

In biological membranes, DGPP formation after stimulus takes place after a transient increase of the PA levels (Munnik et al., 1995). As a consequence, a temporary and local accumulation of DGPP and its precursor in the membrane is expected. This may affect the phospholipid packing during signaling processes. Since the monolayer packing properties are affected by the interactions with neighboring lipids (Maggio, 2004), we have studied the packing properties of mixed films of DGPP and PA on subphases at pH 8. This pH is within the physiological pH range (which can vary between 4 and 10 in proximity of negatively charged membranes, depending on the surface charge density) and in addition both lipids are mostly ionized at pH 8 and repulsive electrostatic interactions should be maxi-

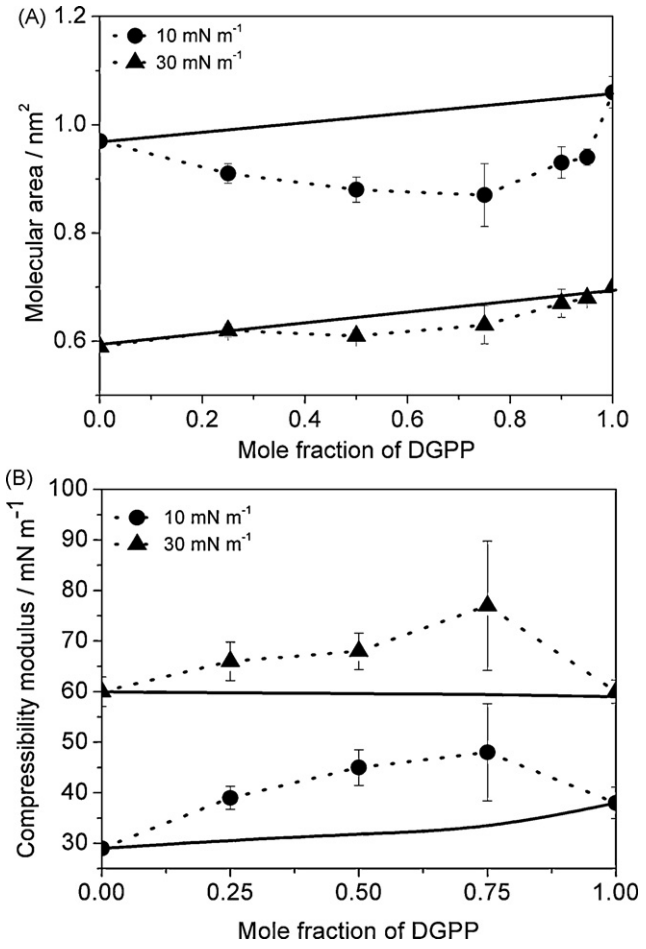

Fig. 5. Average molecular area (A) and compressibility modulus (B) as a function of the mole fraction of DGPP in the mixture. The lateral pressure is 10 mN m^{-1} (circles) and 30 mN m^{-1} (triangles). The straight lines correspond to the ideal behavior.

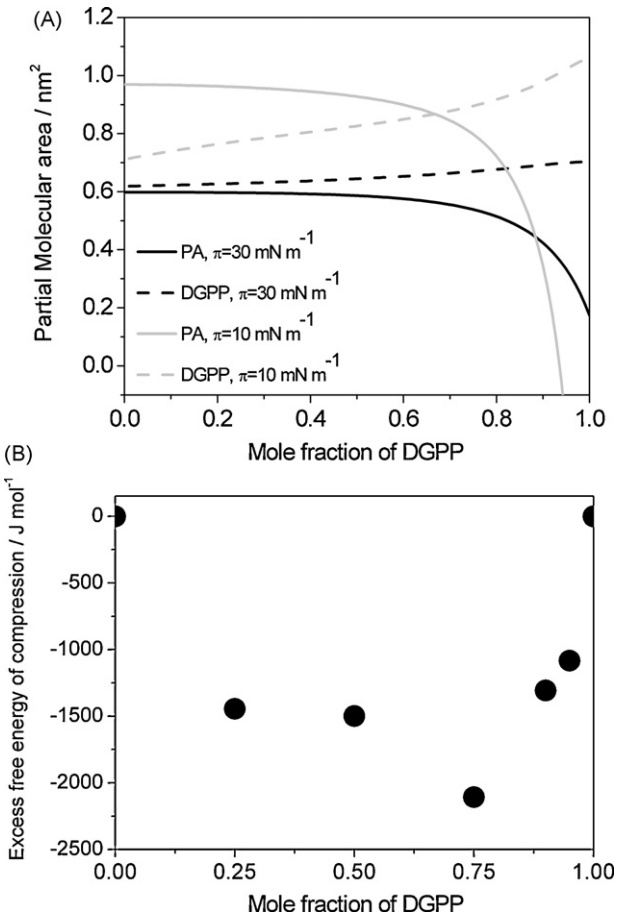

Fig. 6. Partial molecular area for DGPP (dashed line) and PA (continuous line) at 10 mN m^{-1} (black line) and 30 mN m^{-1} (grey line) (A), and excess free energy of compression (B) as a function of the mole fraction of DGPP in the mixture.

mal (Kooijman and Burger, 2009). Fig. 4A shows the compression isotherms for monolayers of PA, DGPP and their mixtures. The mixtures are homogeneous on the micron scale (0.25 μm^2), as revealed by either FM or BAM (see inset in Fig. 4A) indicating that there is no formation of microscopic phase-segregated domains. The compression isotherm of monolayers of pure PA (Fig. 4A, dotted line) is typical of a liquid-expanded monolayer. The lift-off area is 1.36 nm^2 and the collapse occurs at 42 mN m^{-1} and 0.50 nm^2, in agreement with results reported by others (Brockman et al., 2003).

Mixed films of DGPP with its precursor PA also form liquid-expanded films (Fig. 4A). However, the compression isotherms are shifted to lower average molecular areas, compared to either of the pure lipids at lateral pressure below 20 mN m^{-1}. At higher lateral pressures, the mean molecular areas of the mixed films approach to those of DGPP and are higher than those of PA. This leads to an increased compressibility modulus of the mixtures compared to films of the pure lipids. This is clearly shown in Fig. 4B, where the compressibility moduli for some DGPP/PA proportions are shown as a function of the average molecular area (and of the lateral pressure, see inset). The packing behavior of the mixed films is non-ideal at low lateral pressures, as revealed from plots of the average molecular area (Fig. 5A) and compressibility (Fig. 5B) *vs* the mole fraction of DGPP. In these figures, the straight lines correspond to the behavior of an ideal mixture.

However, it is important to note that at high surface pressure (30 mN m^{-1}) and at low and high mole fraction of DGPP the behavior of the mixture correspond to that of an ideal mixture. At intermediate proportion at this pressure the behavior is not clear since the values for the real and ideal average molecular areas are the same within errors. The deviation of the ideal behavior of both, the compressibility modulus and the average molecular area indicate that the interaction between PA and DGPP are stronger than for ideally mixed components, forming a more condensed and rigid monolayer than expected from purely entropic mixing. In order to analyze the influence of each lipid in the observed molecular packing, we calculated, from the data in Fig. 5A, the partial molecular area that is contributed by each lipid to the mean molecular area of the mixture. The results obtained at 10 and 30 mN m^{-1} are shown in Fig. 6A. This figure shows that, at relatively low surface pressure (π = 10 mN m^{-1}), the presence of PA in the mixed film gradually promotes a contribution of DGPP reaching about 40% area expansion with respect to pure DGPP; at the higher pressure of 30 mN m^{-1} increasing proportions of PA affects little the mean molecular area contribution of DGPP to that of the mixture, which is an expected result since the monolayer is already close to the limiting area. On the other hand, the mean molecular area contributed by PA to the mixture, either at 10 or 30 mN m^{-1}, remains quite insensitive to the amount of DGPP until proportions of the latter reach above about 75 mol% where it dramatically contributes to condensation. This may be understood considering that at such proportions, the presence of a PA molecule in a closely packed (and more highly charged) enriched DGPP matrix can induce a non-proportional reduction of surface charge density and consequently of electrostatic molecular repulsions which would explain an increasing thermodynamically favorable con-

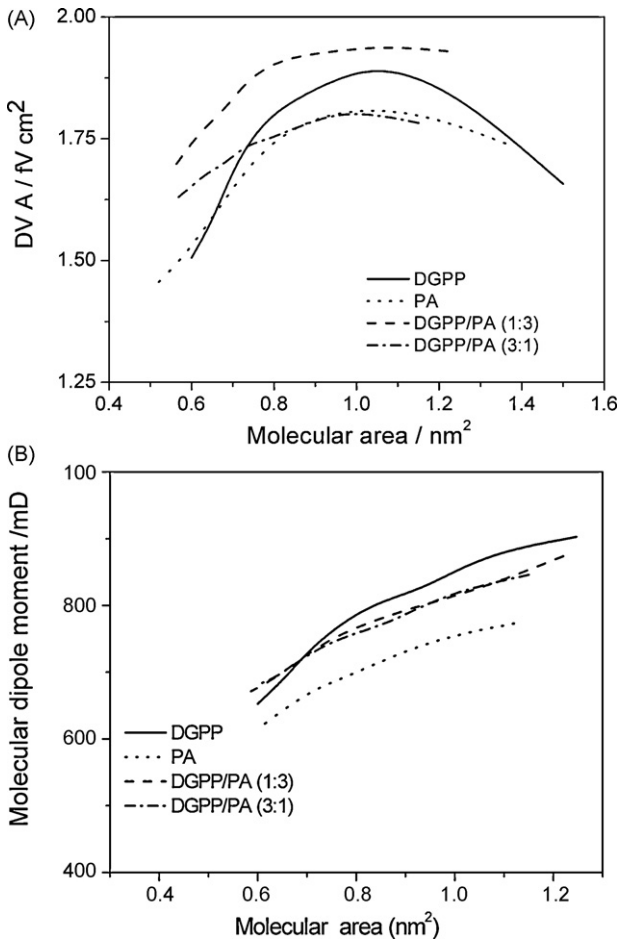

Fig. 7. (A) Surface potential density as a function of the average molecular area for monolayers of diacylglycerol pyrophosphate, phosphatidic acid and their mixtures on subphases at pH 8. (B) Resultant molecular dipole moment as a function of the average molecular area for monolayers of diacylglycerol pyrophosphate, phosphatidic acid and their mixtures on subphases at pH 8.

densed intermolecular packing, as observed (see Figs. 5 and 6). However, for mole fraction of DGPP below 0.05, the contribution of the DGPP molecules to the total mean area of the mixture is 0.6 nm^2 at 30 $mN\,m^{-1}$ and 0.7 nm^2 at 10 $mN\,m^{-1}$, which are lower than the average molecular area of the mixed monolayers at 10 and 30 $mN\,m^{-1}$ (from 0.95 to 0.80 nm^2 at 10 $mN\,m^{-1}$ and from 0.60 to 0.70 nm^2 at 30 $mN\,m^{-1}$, see Fig. 5A). This suggests that other factors such as hydrogen bonding (or charge screening by ions) may contribute some additional reduction of repulsive interactions (Kooijman et al., 2009).

The sign and magnitude of the excess free energy of compression (the difference between the compression free energy of the mixed film and that of the corresponding ideal mixture in which mixing results from purely entropic effects) reflects the favorable lipid–lipid interactions. As can be appreciated in Fig. 6B, for all the investigated proportions the excess free energy of compression values are negative. Thus, the mixed monolayers are more stable, from a thermodynamic point of view, than the films expected for ideally mixed components in which no intermolecular interactions occur. The minimum of excess free energy of compression observed at $X_{DGPP} = 0.75$ for DGPP/PA indicates that this monolayer composition bears the most favorable arrangements. This may explain the high tendency for DGPP synthesis when PA levels are increased during signaling processes, since a high X_{DGPP} would stabilize locally the whole membrane system through variations of the lateral packing.

Fig. 7A shows the surface potential/molecular density as a function of the average molecular area for monolayers prepared with the pure lipids and some mixtures. At pH 8, the surface potential/molecular density of DGPP and PA are similar, within errors (e.g. at an area of 1 nm^2 the values are 1.9 $fV\,cm^2$/molecule for DGPP and 1.8 $fV\,cm^2$/molecule for PA, with the error being of about 0.1 $fV\,cm^2$/molecule). The value of the surface potential/molecular density for monolayers prepared with the mixture of DGPP and PA are also similar, within errors. The resultant perpendicular molecular dipole moment was calculated after subtracting the double layer potential. Fig. 7B shows the molecular dipole moment as a function of the average molecular area for different DGPP/PA proportions. This figure shows that the mixed films present dipole moment values intermediate between that of the pure lipid monolayers. The dipole rearrangement observed at 1.0 nm^2 for pure DGPP monolayers is absent in the mixed monolayers.

4. Conclusions

In this work we investigated the molecular behavior and structural properties of the minor cellular lipid component, DGPP, at the air–water interface on subphases at pHs 5 and 8. It is well known that the intracellular pH varies among compartments of eukaryotic cells; besides, the surface pH in the proximity of membranes depends on the interfacial charge density. Since the polar head group of DGPP has a pyrophosphate moiety and the pK_{a3} of this moiety is about 7, the net charge on the molecule could change during biological processes inside the cell and also differ from a compartment to another having different packing or phase state. We found that DGPP on subphases at pH 8 forms expanded isotherms; by contrast, at pH 5 the isotherms were more condensed and with a lower surface potential per unit of molecular density at the surface. Marked variations of the surface electrostatics and of the resultant perpendicular dipole moment contributions of the oriented molecules to the dipole potential of the interface take place which can be induced by local changes of pH and the surface pressure.

On the other hand, we explored the lateral organization in mixed monolayers of DGPP and the parent compound PA to provide some molecular basis that may aid interpreting its potential role as a signaling molecule in cell membranes. Langmuir films were chosen as experimental model system in order to obtain quantitative information on the intermolecular packing, the surface potential and the in-plane elasticity (compressibility modulus) under controlled surface organization, which are often more difficult to access in bilayers (Clarke, 2001).

Even though the overall molar concentration of DGPP in biological membranes is low (van Schooten et al., 2006), its local concentration may be much higher. Besides, it is not known what type of signaling involving PA could trigger the local increase of DGPP. It is likely that during signaling events, i.e., on the activation of PLD (phospholipase D), the local concentration of PA increases and subsequently the local concentration of DGPP also increases. We found that the mixtures of DGPP with its precursor PA show a non ideal behavior. The excess free energy of compression is negative for all the proportions analyzed and the most favorable interaction occurs at a DGPP/PA 3:1 ratio. The interaction among these lipids leads to a diminution of the mean molecular area and to an increase of the compressibility modulus at the closest packings.

Our results indicate that the surface behavior of the individual lipids can be modified by changes of the relative lipid proportions, indicating their inherent capability for transducing membrane events through dynamic variations of molecular packing, in-plane elasticity, electrostatic interactions and compositional changes. All these effects can constitute structural-electrostatic signaling events involving DGPP that may be sensed both along the

membrane surface and into the surrounding aqueous environment whereupon regulate the recognition and activity of bioactive ligands.

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