**Streptomyces chromofuscus** phospholipase D interaction with lipidic activators at the air–water interface

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**Abstract**

The phospholipase D from *Streptomyces chromofuscus* (PLDSc) is a soluble enzyme that interacts with membranes to catalyse phosphatidylcholine (PC) transformation. In this work, we focused on the interaction between PLDSc and two lipid activators: a neutral lipid, diacylglycerol (DAG), and an anionic one, phosphatidic acid (PA). DAG is a naturally occurring alcohol, so it is a potent nucleophile for the transphosphatidylation reaction catalysed by PLD. Concerning PA, it is a widely described activator of PLDSc-catalysed hydrolysis of PC.

The monolayer technique allowed us to define PLDSc interaction with DAG and PA. In the case of DAG, the results suggest an insertion of PLDSc within the acyl chains of the lipid with an exclusion pressure of approximately 45 mN/m. PLDSc–DAG interaction seemed to occur preferentially with the lipid in the liquid-expanded (LE) phase.

PLDSc interaction with PA was found to be more effective at high surface pressures. The overall results obtained with PA show a preferential interaction of the protein with condensed PA domains. No exclusion pressure could be found for PLDSc–PA interaction indicating only superficial interaction with the polar head of this lipid. Brewster angle microscopy (BAM) images were acquired in order to confirm these results and to visualise the patterns induced by PLDSc adsorption.

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1. Introduction

Phospholipase D (PLD) catalyses the hydrolysis of the phosphodiester bond between the phosphatidyl-moiety and the choline headgroup of phosphatidylcholine (PC) which liberates choline and phosphatidic acid (PA). This reaction involves a molecule of water for the nucleophile substitution on the phosphatidyl-enzyme intermediate to liberate PA and, if the nucleophile is an alcohol, a phosphatidyl-alcohol is produced. This latter activity is called transphosphatidylation and is specific for the PLD [1]. The hydrolytic activity catalysed by PLD occurs with P–O bond cleavage of PC as demonstrated previously [2].

The phospholipase D from *Streptomyces chromofuscus* (PLDSc) belongs to the phospholipase D superfamily. Other enzymes also belong to this family including some endonucleases, helicases, lipid synthases and many other enzymes catalysing the hydrolysis and/or the formation of phosphodiester bonds [3]. However, *S. chromofuscus* PLD from is unique among these proteins as its enzymatic activity is calcium dependent [4,5] and it has not the classical HKD catalytic site [6]. Furthermore, Stieglitz et al. [7] have demonstrated that this bacterial PLD is activated by anionic lipids, for which it also exhibits a high affinity. PA is thought to be an allosteric activator of PLDSc [8], but the nature of this activation has never been clearly identified.

Another lipidic activator of PLDSc is DAG. The effect of this neutral lipid has been demonstrated by Yamamoto et al. [9]. According to their results, DAG mixed in vesicles with PC causes an increase of the apparent $V_{\text{max}}$ for PLDSc-catalysed hydrolysis of PC. van Blittersvijk and Hilkmann [10] have shown that DAG can activate mammalian PLD...
through a transphosphatidylolation reaction leading to the formation of bis (PA). They showed that in this reaction, PC is the substrate and DAG is the alcohol required for the nucleophile substitution. Recently, the mechanism of PLDSc activation by DAG was found to be very similar to that reported for mammalian PLD [11].

Besides their activating effect on PLDSc-catalysed hydrolysis of PC, PA and DAG are both implicated as second messengers in cell signalling [12,13]. Moreover, these two lipids were found to induce lateral phase segregation in phospholipidic membranes [14,15]. Thus, even if they are only produced transiently and in small amounts in biological membranes, some domains enriched with DAG or PA may exist locally and these domains could serve as platforms for protein interaction with membranes. In the case of DAG-enriched domains, it has been suggested that they may induce a local dehydration of the membrane that could facilitate protein penetration [16].

The PLD from *S. chromofuscus* is a soluble enzyme that catalyses the hydrolysis of PC contained in macromolecular insoluble structures (i.e. the lipidic vesicles). Therefore, there must be some factors which enhance PLD interaction with such a macrostructured substance.

In this work, PLDSc interaction with PA and DAG was investigated. Three monolayer-based methods were used for this purpose, including a tensiometry assay to measure PLDSc adsorption at the air–water interface in the presence of lipids. $\pi–A$ isotherms of mixed lipid–protein were also measured to define the influence of PLDSc adsorption on the physical properties of the lipids. Brewster angle microscopy (BAM) images of these monolayers were recorded to analyse and confirm the information provided by the above methods. The combination of these approaches allowed us to elaborate a model for PLDSc interaction with its most effective lipidic activators PA and DAG.

2. Materials and methods

2.1. Chemicals

Chelex 100 was from Bio-Rad (Richmond, CA). Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol) was purchased from Boeringer Mannheim GmbH (Mannheim, Germany). Arachidic acid, $\alpha$-dimyristoyl-phosphatidylcholine (DMPC), $\alpha$-dimyristoyl-phosphatidic acid (DMPA), 1,2-dimyristoyl-rac-glycerol (DMG) and phospholipase D from *S. chromofuscus* (PLDSc) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The SDS-PAGE analysis of PLD gave the same three bands as those obtained by Geng et al. [17]. These proteins have been identified by sequence analysis as the intact PLD and its two proteolytically processed fragments [17].

2.2. Film formation and surface pressure measurements

All experiments were performed at constant temperature (21 ± 0.1 °C). The film balance was built by R&K (Riegler & Kirstein GmbH, Wiesbaden, Germany) and equipped with a Wilhelmy-type surface pressure measuring system. The subphase was aqueous buffer solutions containing 150 mM NaCl and 10 mM Tris—HCl pH 8.0. Unless otherwise specified, all experiments were performed at a final concentration of 120 $\mu$M CaCl$_2$ in the subphase. This calcium concentration is high enough to obtain maximum PLDSc enzymatic activity towards its usual substrates (according to Ref. [4], activity saturation occurs at 20 $\mu$M calcium). In all experiments, the subphase was stirred with a magnetic stirrer spinning at 100 rev/min.

2.2.1. PLDSc adsorption measured at constant surface area

Adsorption experiments were performed on a small Teflon dish (diameter, 2 cm) with a subphase volume of 8.5 ml. Phospholipids were spread at the air–water interface in chloroform at 0.175 mM to reach the desired final surface pressure. After 30-min solvent evaporation, the enzyme (15 $\mu$g protein) was injected in the subphase. PLDSc adsorption at the air–water interface was measured as an increase in surface pressure.

2.2.2. Lipid and mixed PLDSc/lipid monolayer compression

The measurement of compression–decompression–recompression isotherms has been previously described as a method giving information about the interactions between proteins and lipids [18,19]. Phospholipids were spread at the air–water interface (Langmuir trough dimensions: 165 cm$^2$ and 120 ml subphase) in chloroform at 0.545 mM to reach a final quantity of 25 nmol lipids. After 30-min solvent evaporation, the monolayer was compressed to a lateral pressure of 35 mN/m to obtain a control $\pi–A$ isotherm of the lipid alone. Then, the enzyme (15 $\mu$g protein) was injected in the subphase at zero surface pressure. Compression–decompression–recompression isotherms were measured after different times of PLDSc adsorption at 0 mN/m. The compression rate was $1.06 \times 10^{-2}$ nm$^2$/molecule/min. This low speed of compression allowed equilibrium of domains’ shape [20].

2.3. BAM measurements

The morphology of lipid and mixed PLDSc/lipid monolayers at the air–water interface was observed with a Brewster angle microscope (NFT iElli-2000, Göttingen, Germany) mounted on an R&K Langmuir trough (Riegler & Kirstein). The microscope was equipped with a frequency doubled Nd:Yag laser (532 nm, ca. 50 mW primary output), a polariser, an analyser and a CDD camera. The spatial resolution of the BAM was about 2 $\mu$m and the image size was 430 × 320 $\mu$m.
3. Results and discussion

3.1. PLDSc adsorption at the air–water interface in the presence of lipids

PLDSc activity has been measured previously in monolayers [4,11] indicating that it is not denatured upon adsorption at the air–water interface. PLDSc interaction with lipidic monolayers was measured by tensiometry on a small Teflon dish. In these experiments, we monitored the increase in surface pressure during PLDSc adsorption at the air–water interface in the presence of DMG or DMPA. These two lipids are both activators of PLDSc but cannot serve as substrates for this enzyme [21]. Furthermore, the only difference in their chemical structure is that DMPA bears a phosphate group as polar head. Different quantities of DMG or DMPA were spread at the air–water interface in order to give a definite initial surface pressure. Then, the monolayer was stabilised during 30 min to allow solvent evaporation and lipid organisation. PLDSc was then injected into the subphase and surface pressure was monitored as a function of time (insets in Fig. 1). These results clearly show that PLDSc presents a completely different behaviour when it interacts with DMG or with DMPA. For the former neutral lipid, high initial pressures lead to lower penetration of the enzyme within the monolayer (inset in Fig. 1A). So, for the lowest surface pressures, PLDSc insertion in DMG is greater than for the highest pressures. At low surface pressure, the low lipid packing allows protein adsorption at the air–water interface. At higher surface pressure, higher lipid packing would lead to a more difficult penetration of the protein within the membrane.

Fig. 1. PLDSc adsorption to lipid monolayers. The amplitude of surface pressure increase $\Delta \pi$ was calculated from the $\pi$–$t$ measurements (insets of this figure) and reported as a function of initial pressure. (A) Penetration of PLDSc into DMG (closed circles, full line) and DMG/arachidate 20:80 (mol/mol) (open circles, dashed line). (B) Adsorption of PLDSc to a DMPA monolayer. Insets: Kinetics recording of PLDSc surface adsorption at the air–water interface in the presence of DMG or DMPA. (A) $\pi$–$t$ curves of PLDSc penetration into a DMG preformed monolayer. (B) $\pi$–$t$ curves of PLDSc penetration into a DMPA preformed monolayer. First, the lipid was spread at the air–water interface to obtain the desired $\pi_0$. Thirty minutes was required for solvent evaporation and $\pi$ stabilisation. Then PLDSc (10 μg) was injected into the subphase (see arrows on the figures). (Subphase was 120 μM CaCl$_2$, 150 mM NaCl, and 10 mM Tris–HCl pH 8.0; Teflon trough, 8.5 ml, 3.14 cm$^2$).
PLDSc insertion into DMPA monolayer (inset in Fig. 1B) is also dependent on surface pressure. However, the higher the initial pressure, the more PLDSc is adsorbed at the air–water interface. It seems that PLDSc adsorption in the presence of DMPA reaches a maximum at 20 mN/m initial pressure. So the nature of the interaction of PLDSc with DMG and DMPA is quite different. Moreover, the amplitude of pressure increase is considerably larger than that obtained for DMG. It is also evident that surface pressure increase occurs at low speed for DMPA as compared to DMG. These two observations suggest that the major phenomenon is not PLDSc insertion within the acyl chains of DMPA but a direct interaction between the protein and the anionic polar head of the lipid. The penetration of the protein within the monolayer would have been less effective at high surface pressure (i.e. high lipid packing). According to these results, one can make the hypothesis that the significant surface pressure increase could be due to a change of DMPA tilt angle induced by PLDSc adsorption to its polar head.

The representation of pressure increase (Δπ) due to PLDSc adsorption at the air–water interface as a function of initial pressure for both DMG and DMPA emphasises the differences described above (Fig. 1A and B, respectively). In the case of DMG, Δπ decreases linearly with increasing initial pressure (Fig. 1A, closed circles). This is typical for a progressive exclusion of a protein from a lipidic monolayer. According to these results, we can estimate the value of surface pressure corresponding to PLDSc exclusion from the DMG monolayer: 45 mN/m. This value is quite high but it should correspond to a total exclusion of the enzyme from the interface. This exclusion pressure is considerably higher than the pressure thought to prevail within biological membranes (30 mN/m) [22–24]. One should also notice that the maximum Δπ obtained is around 5 to 6 mN/m.

As one can see in Fig. 1B, Δπ increases with growing initial pressure when PLDSc interacts with DMPA. Contrary to PLDSc insertion into DMG monolayer, the results obtained with DMPA show no linear relationship between Δπ and initial π. Such behaviour suggests that PLDSc interacts with the polar head of DMPA with no or only small insertion of the protein within the acyl chains. However, if any penetration of the protein occurs, it has only slight influence on the adsorption profile of PLDSc (Fig. 1B).

To test the influence of the anionic charge of DPMA on its interaction with PLDSc, a DMG/arachidate mixture in 20:80 molar ratio was spread at the air–water interface (DMG was used to stabilise the interfacial localisation of arachidate). The results (Fig. 1A, open circles) allowed us to estimate an exclusion pressure of about 27 mN/m. This suggests that PLDSc/DMPA interaction is not due to the negatively charged interface but seems to be specific of the phosphate group of PA. Furthermore, the exclusion pressure determined for the DMG/arachidate mixture is considerably lower than that estimated for DMG alone. This could be explained by the higher degree of membrane hydration in the presence of arachidate. Indeed, according to Ref. [16], the dehydration of membrane surface induced by DAG could facilitate protein penetration.

Since PA can complex calcium and PLDSc requires this ion for activity, another important question arose as the influence of calcium in the protein–lipid interaction. Thus, PLDSc adsorption under the DMPA monolayer was measured using subphases containing different calcium concentrations. Fig. 2 gives the resulting PLDSc-induced Δπ as a function of calcium concentration in the subphase, showing that PLDSc adsorption under a DMPA monolayer is strongly dependent upon calcium concentration. In the absence of calcium (i.e. the buffer and all the glassware were treated with Chelex 100), PLDSc adsorption under a DMPA mono-
layer induced a 2.7 mN/m pressure increase with an initial pressure of 30 mN/m (data not shown). This \( \Delta p \) is considerably lower than that observed in the presence of 0.12 mM calcium for approximately the same initial \( p \) (\( \Delta p \) of about 15 mN/m at \( p_0 = 27.5 \) mN/m). Furthermore, this pressure increase is close to that observed for DMG at 30 mN/m (observed \( \Delta p \) of 2.2 mN/m) suggesting that PA–calcium complexes are responsible for the differences observed in PLDSc interaction with PA and DAG. For PLDSc adsorption to DMG monolayers, calcium concentration ranging from 0.12 to 10 mM did not modify the kinetics (data not shown; a \( \Delta p \) of about 4 to 5 mN/m was observed with a \( p_0 = 15 \) mN/m).

Subsequently, the influence of calcium on the adsorption of PLDSc alone at the air–water interface was tested (data not shown). Increasing calcium concentration in the subphase (from 0.12 to 10 mM) leads to a higher initial rate of PLDSc adsorption (three times greater) but it does not significantly modify the final \( \Delta p \) amplitude (from 5 to 6 mN/m). Previous work suggested that \( \text{Ca}^{2+} \) could induce only small changes in PLDSc structure [6]. This might be due to the formation of neutral complexes between acidic lateral chains of PLDSc aminoacids and calcium. Another possibility could be an intermolecular protein–\( \text{Ca}^{2+} \)–protein cross-linking [25]. Both would result in an increase of the PLDSc hydrophobicity, leading to a higher interfacial

Fig. 3. Influence of PLDSc adsorption on the \( \pi-A \) isotherms of DMG (A) and DMPA (B). In the first step, the \( \pi-A \) isotherm of the lipid alone was recorded (bold line). Then, after monolayer decompression, PLDSc (12 \( \mu \)g) was injected into the subphase at \( p_0 \). Compression–decompression–recompression isotherms were measured at different times (time reference is PLDSc injection): 10 min (■), 20 min (▲), 30 min (○), 90 min (□). The subphase was 120 \( \mu \)M CaCl\(_2\), 150 mM NaCl, and 10 mM Tris–HCl pH 8.0.

Fig. 4. BAM images of a DMG monolayer before and after PLDSc injection into the subphase. A to F correspond to images of the pure DMG monolayer acquired every 5 mN/m on \( \pi-A \) isotherm recording: (A) \( \pi = 5 \) mN/m, GL = 138, OS = 50. (B) \( \pi = 10 \) mN/m, GL = 100, OS = 120. (C) \( \pi = 15 \) mN/m, GL = 122, OS = 120. (D) \( \pi = 20 \) mN/m, GL = 83, OS = 250. (E) \( \pi = 25 \) mN/m, GL = 84, OS = 250. (F) \( \pi = 30 \) mN/m, GL = 85, OS = 250. Then PLDSc was injected into the subphase and allowed to adsorb at the interface during 30 min. The monolayer was then compressed: (A’) \( \pi = 5 \) mN/m, GL = 131, OS = 50. (B’) \( \pi = 10 \) mN/m, GL = 103, OS = 120. (C’) \( \pi = 15 \) mN/m, GL = 137, OS = 120. (D’) \( \pi = 20 \) mN/m, GL = 143, OS = 120. (E’) \( \pi = 25 \) mN/m, GL = 147, OS = 120. (F’) \( \pi = 30 \) mN/m, GL = 144, OS = 120. The subphase was 120 \( \mu \)M CaCl\(_2\), 150 mM NaCl, and 10 mM Tris–HCl pH 8.0.
affinity of the protein but with no or only small modifications to protein structure.

Concerning DMPA-membrane behaviour in the presence of calcium, previous studies showed that near a neutral pH, DMPA can be doubly negatively charged and as a consequence it can form DMPA/Ca$^{2+}$ 1:1 complexes. Such complexes are organised in quasi-crystalline phases within membrane [15]. So, at the pH needed for optimum PLDSc enzymatic activity (pH 8.0), these doubly charged DMPA could coexist with singly charged ones that are involved in DMPA/Ca$^{2+}$ 2:1 complexes. These authors have also shown that increasing calcium concentration leads to a growing ratio of DMPA/Ca$^{2+}$ 1:1 complexes [15,26]. This information taken together with our results about PLDSc-calcium-dependent adsorption under DMPA monolayer raises the hypothesis that PLDSc may interact preferentially with DMPA/Ca$^{2+}$ 2:1 complexes.

3.2. Isotherms of mixed PLDSc/lipid monolayers

In order to better characterise the interaction of PLDSc with DMG and DMPA, we measured compression–decompression–recompression isotherms after different times of PLDSc adsorption at the air–water interface (Fig. 3). In a first step, the lipid alone (i.e. DMG or DMPA) was compressed at the air–water interface up to 35 mN/m. After decompression (p = 0), the protein was allowed to adsorb at the interface during the indicated times. Then the monolayer was compressed again up to 35 mN/m.

For DMG (Fig. 3A), a liquid-expanded (LE)–liquid-condensed (LC) phase transition was observed around 1 mN/m and the molecular area was estimated at 42 Å$^2$/molecule at 30 mN/m. This is consistent with the small polar head of DMG and the two acyl chains, which will obviously occupy 21 Å$^2$/chain at 30 mN/m. Ten minutes after PLDSc injection at p = 0 ($\pi_0$), the LE–LC transition occurs for 1.5 mN/m. Increasing the time of PLDSc adsorption at $\pi_0$ leads to a significant increase of this value. These results indicate that PLDSc interaction with DMG induces the retardation of LC phase appearance during the monolayer compression. This latter indication suggests that PLDSc inserts within the acyl chains of DMG in the LE phase and should be excluded from the DMG-condensed phase. At high surface pressure, the isotherms are shifted to higher molecular area, indicating a persistence of the protein within the monolayer. This is consistent with the exclusion pressure previously determined around 45 mN/m.

Concerning the isotherm of DMPA alone (Fig. 3B), it reveals an LE–LC phase transition around 5 mN/m. Its molecular area (42 Å$^2$/molecule at 30 mN/m) is consistent with literature [27,28] and is identical to that found above for DMG; the explanation is exactly the same. The first isotherm was measured 10 min after PLDSc injection at $\pi_0$. Contrary to DMG, we cannot observe a change in the value of LE–LC phase transition pressure. The situation remains unchanged until PLDSc is allowed to adsorb at the air–water interface for 60 min (Fig. 3B, open squares). Under these conditions, the pressure of the LE–LC phase transition is lowered, it occurs at 2.5 mN/m. This means that PLDSc/PA interaction facilitates the formation of the LC phase. Such a decrease with an increasing amount of PLDSc adsorbed at the interface can only be assigned to surface interaction of PLDSc with the polar head of DMPA. According to Ref. [18], protein interaction with only the polar head of lipids should lead to a decrease of LE/LC phase transition pressure. This could be explained by LC domain coalescence induced by protein adsorption. Therefore, these results confirm the previous observations derived from the pressure-dependent adsorption of PLDSc at the air–water interface. These observations raise the hypothesis of a preferential interaction of PLDSc with condensed PA domains.

3.3. Morphology of the mixed PLDSc/lipid layers during compression investigated by BAM

For both DMG and DMPA monolayers, BAM images were taken for the lipid alone and for the lipid–PLDSc mixed layer after 30-min adsorption at zero surface pressure (Figs. 4 and 5). The corresponding isotherms are shown in Fig. 3A and B, respectively: bold isotherms for pure lipid and open circles for the mixed monolayers.

BAM images of DMG alone reveal two coexisting phases at 5 mN/m (Fig. 4A). The round-shaped domains correspond to the LC phase while the black surrounding background is the LE phase. As one can see, there are also some solid phases (extremely small white dots) within the LC structures. In the case of the mixed DMG/PLDSc monolayer, image at 5 mN/m is composed of the same phases as described above but obviously the LE phase prevails. At 10 mN/m (Fig. 4B and B’), the same phases are observed with a few more solid structures on the edge of LC phases (“water lily”-shaped domains), which are also surrounded by an LE phase. In the presence of PLDSc, we can observe phases that are identical in terms of nature but the LE phase is again more important regarding the covered surface (Fig. 4B’). The LC structures are still “water lily”-shaped but they are bigger for the DMG/PLDSc mixed
monolayer. Compression up to 15 mN/m decreases the surface available for the LE phase and increases the number of solid domains observed (white points) for both DMG and DMG plus PLDSc. At this step of the isotherm, we notice that the contrast of the image is inverted in the case of the PLDSc-containing monolayer. The background suddenly turns whiter than the LC domains, indicating the squeezing out of PLDSc from the monolayer. This phenomenon has previously been observed by Rodriguez Patino et al. [29] with β-casein/monopalmitin mixed monolayers. At surface pressures above 15 mN/m, the compression of DMG results in a progressive disappearance of the LE phase and a growing ratio of solid structures surrounded by an LC phase (Fig. 4D, E and F). This behaviour at high pressure is quite different from that obtained in the presence of PLDSc (Fig. 4D', E' and F'). Bright domains of PLDSc are still visible, indicating that the protein is not totally excluded from the DMG monolayer. In other words, DMG is not able to completely exclude PLDSc from the air–water interface at pressures below 45 mN/m. Such a persistence of the protein within the acyl chains of DMG does not allow the fusion of the LC domains even at 30 mN/m. This latter result is in agreement with the estimated exclusion pressure of PLDSc interacting with DMG (45 mN/m). Furthermore, these images clearly show that PLDSc interacts with DMG in the LE phase while it does not seem to penetrate condensed DMG domains.

The BAM images of DMPA and DMPA/PLDSc monolayers (Fig. 5) are noticeably different from those observed with DMG. For DMPA alone, at 5 mN/m (Fig. 5A), the dark background corresponds to an LE phase while the small white domains are LC structures. These condensed structures must be due in part to DMPA interaction with calcium that causes the lateral segregation of the lipid as previously described [15,28]. Concerning the LE phase, it corresponds partly to DMPA free from calcium, i.e. uncomplexed DMPA molecules [15]. The image of the DMPA/PLDSc monolayer at 5 mN/m reveals the coexistence of an LE phase (dark background) with large white domains corresponding to LC structures (Fig. 5A'). The presence of such large LC domains suggests that PLDSc is adsorbed underneath the DMPA monolayer. This interaction leads to the coalescence of the LC domains and this fusion may be induced by PLDSc adsorption just underneath the monolayer under the effect of both the phosphate group of DMPA and calcium. This result clearly demonstrates that PLDSc interacts preferentially with condensed PA domains leading to their gathering at the air–water interface. Further compression of DMPA alone up to 10 mN/m induces the formation of round-shaped domains of solid phase with only small areas occupied by the LE phase (Fig. 5B). The equivalent image for the mixed DMPA/PLDSc monolayer (Fig. 5B') reveals a completely different situation. The round-shaped LC domains of DMPA appear in black while the background seems brighter. Again, this inverted contrast as compared with DMPA alone may be due to a part of PLDSc being squeezed out from the lipidic film. From 15 to 30 mN/m (Fig. 5D, E and F), the area corresponding to the solid phase in the DMPA monolayer increases while the LE phase slowly fades. We can also observe a complete fusion of the LC domains. Concerning the PLDSc-containing monolayer of DMPA, compression leads to the same features as described above but with some additional white domains remaining even at high pressure (Fig. 5D', E' and F'). These images show a progressive exclusion of PLDSc but some proteins still remain in interaction with the acyl chains of DMPA even at 30 mN/m. While some of the white domains are clearly similar to those observed for the DMG/PLDSc mixed film, there are also huge cloudy structures that are brighter than the background. These latter domains (that present a diffuse shape) seem to be located just underneath the lipidic monolayer, suggesting an interaction of large PLDSc clusters with DMPA surface.

4. Conclusions

In this work, we have provided compelling evidences that PLDSc interacts in a different way with DMG and DMPA. These two lipids are of major biological interest because they are both second messengers in cell signalling. DAG and PA are produced in low percentages (lower than 5 mol%) in membranes through the action of phospholipase C and D, respectively. However, on a local scale, these lipids can be significantly enriched. Thus, in regions where these lipids are predominant, they can induce a lateral phase separation with highly ordered domains [14,15]. Such domains were often suspected to have a biological significance for membrane interaction with proteins.

The results obtained clearly show a penetration of PLDSc within the acyl chains of DMG with an exclusion pressure around 45 mN/m. According to Refs. [22–24], the internal pressure of biological membranes is close to 30 mN/m. This suggests that PLDSc insertion into DMG-enriched domains may occur in biomembranes. However, the exclusion pressure seems extremely high when compared to other proteins except for α-lactalbumin [30]. So, further work will have to focus on that unusual behaviour.

We have also shown that calcium is required for DMPA interaction with PLDSc. Moreover, it seems that PLDSc may preferentially interact with the polar head of DMPA involved in a complex with calcium in a 2:1 stoichiometry [15]. This complex corresponds to the LC phase of DMPA, and according to the BAM images, PLDSc interaction with the monolayer provokes the coalescence of LC domains. So, this enzyme only adsorbs to DMPA-calcium-enriched domains and only weakly inserts into the hydrophobic part of the membrane. Another negatively charged monolayer of DMG/arachidate 20:80 (mol/mol) did not provide the same kind of adsorption kinetics as compared to DMPA. Therefore, PLDSc superficial interaction with DMPA seems specific to the phosphate group–calcium complexes 2:1 (mol/mol).
Such differences in the interaction of PLDSc with DMG and DMPA could be correlated with their respective activating effect. It has been suggested that DMG induces a membrane dehydration [16]. So, one can imagine that PLDSc insertion into DMG-enriched domain, where water is only poorly present, will favour the alcohol-based catalysis, i.e. the transphosphatidylolation reaction. On the other hand, the superficial interaction of PLDSc with DMPA-calcium domains should enhance the water-based activity, i.e. the hydrolysis reaction.

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