Biochimica et Biophysica Acta 1828 (2013) 2056–2063

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Surface mixing of products and substrate of PLA_2 in enzyme-free mixed monolayers reproduces enzyme-driven structural topography

Luisina De Tullio, María L. Fanani, Bruno Maggio *

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC-CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 2 January 2013
Received in revised form 2 May 2013
Accepted 20 May 2013
Available online 29 May 2013

Keywords:
Porcine pancreas phospholipase A_2
Dipalmitoylphosphatidylcholine
Lipid monolayer
Surface mixing
Lipid domain morphology

ABSTRACT

It was proposed that topographic changes in lipid monolayers hydrolyzed by lipolytic enzymes such as Phospholipase A_2 (PLA_2) are a consequence of enzyme activity at the surface. Lateral packing defects that arise from lipid phase coexistence were suggested as places at which PLA_2 activity is preferably localized. Our work employs a method for mixing two lipid monolayers in order to simulate lipid mixing of products and substrate at the surface in the absence of enzyme. In such enzyme-free mixed films, a topographic pattern similar to that actively generated by PLA_2 is observed. The main conclusion from our experiments is that mixing–demixing properties of substrate and products generated by PLA_2 can determine the evolution of the surface topography.

1. Introduction

Widespread in nature, phospholipases are a group of soluble, secretory or membrane-associated enzymes, whose enzymatic activity is modulated directly at the interface by the substrate organization. Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the sn−2 fatty acyl ester bond of phospholipids, producing fatty acids and lysophospholipids.

Several studies have related "membrane lateral defects" with enhancement of enzymatic activity of different PLA_2s [1–8], but the details by which this occurs at the molecular level have yet to be elucidated. Grainger et al. [9] proposed that *Naja naja* PLA_2 hydrolysis started at the edge of the LC domains present in DPPC monolayers, over the range of surface pressures where there is coexistence of LE–LC phases. This interpretation was based on the appearance of nicks at the edge of the LC domains present within the LE phase in monolayers of DPPC hydrolyzed by *N. naja* PLA_2. This was supported by the occurrence of bell-shaped surface pressure-enzyme activity curves having an optimum enzymatic activity coinciding with the phase coexistence region. In 1998 Grandbois et al. [5] reported similar observations by AFM using solid-supported DPPC bilayers in which the topographic changes indicated that the enzymatic activity started at lateral packing defects. However, in those studies the enzyme was not localized during hydrolysis and the interpretation reached was made on the basis of the topographic changes observed in the lipid domains, as visualized by differential fluorescence probe partitioning. Dahmen-Levison et al. [10] have shown that fluorescein-labeled *Naja naja naja* PLA_2 preferably partitions at the LE–LC phase coexistence boundaries of the domains in D-DPPC (non-hydrolysable analog) monolayers. This work also supported the idea that membrane lateral defects can activate PLA_2 with the involvement of direct physical contact of the enzyme with boundary defects. On the other hand, another study showed that *Crotalus atrox* fluorescent-labeled PLA_2 was homogeneously distributed in the LE phase of POPC/DPPC GUVs [11]. More recently, Gudmand et al. [12] published a detailed study of the topographic localization of porcine pancreas PLA_2 ($ppPLA_2$) in a L-DPPC and in a D-DPPC monolayer at the single molecule level showing that the enzyme had a different behavior when localized in different regions of the monolayer. These authors found that in monolayers of DPPC showing the LC–LE phase coexistence that was hydrolyzed by $ppPLA_2$, nicks of fluid phase began to spread from one side of the LC domains resulting in a product-enriched area that located next to the domains. Regarding the fluorescent-labeled $ppPLA_2$ it was concluded that the enzyme preferably partitions into the more fluid phase and a very low signal was detected in the LC phase, with an enrichment of $ppPLA_2$ at the fluid/gel phase coexistence boundaries where the product-enriched area was localized [12]. Interestingly, single-particle studies of the enzyme acting against D-DPPC and L-DPPC revealed differences between the enzyme residence times in fluid/gel boundaries for both enantiomers. This suggests that the topographic localization of $ppPLA_2$ can be modified by the presence of reaction products, besides the involvement of lateral defect.

Using different microscopy techniques, there are examples in the literature on how lipolytic enzymes can modify the surface topography

* Corresponding author. Tel.: +54 351 4334168×3437.
E-mail addresses: luisinadetullio@mail.fcq.unc.edu.ar (L. De Tullio), lfanani@fcq.unc.edu.ar (M.L. Fanani), bmaggio@fcq.unc.edu.ar (B. Maggio).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.05.018

of lipid monolayers and bilayers [5,9,13–15]. These observations may be interpreted in at least two different manners. One of them is that the topographic evolution of a lipid interface being hydrolyzed is entirely enzyme-driven, in other words, that enzyme molecules themselves have a major active role in the development of the surface pattern and morphology of domains. Another possibility is that the enzyme acts mainly as a modifier of the surface composition and, depending on the rate of activity, the system can be taken out of the equilibrium state for miscibility thus leading to phase separation and/or domain topographic changes [14,16,17]. Under this conception two temporally coupled processes are participating: the compositional change due to enzymatic activity which changes the substrate/product ratio at the surface at a defined rate, and the structural equilibration of the new lipid mixture that depends on substrate–product mixing–demixing. This was reported by our group [13,18,19] in several studies on the generation of ceramide from sphingomyelin by sphingomyelinase activity; the domain shape and topography were different in films generated by different levels of enzyme activity when compared to enzyme-free mixed monolayers of sphingomyelin/ceramide in the same proportion [16].

Demixing kinetics (phase separation) from an out of equilibrium condition [20,21] is a scarcely explored area of lipid research. Such situation might be generated by some sudden change, namely temperature jumps, fast lateral compression and/or compositional changes caused by fast or slow enzyme activity. A recent study [16] has described in detail that the time-course of the surface topography generated by sphingomyelinase corresponds to an out-of-equilibrium nucleation process of LC ceramide domains that became demixed from the sphingomyelin-enriched phase; this is driven by the overcoming of the miscibility limit of these lipids that occurs during the change of surface composition at the interface. This effect obviously depends on the relative time of product generation by the enzymatic activity compared to that for compositional equilibration. In agreement with this, it was demonstrated [22] that physico-chemical changes induced by SM to Cer conversion by SMase in LUVs remained after the enzymatic reaction has halted.

The aim of the present study was to investigate the modification of the membrane surface topography induced by an enzyme-free compositional change, involving the substrate and products of $ppPLA_2$ action. To approach this study we investigated the differences and similarities of: a) enzymatically-generated, b) enzyme-free (pre-equilibrated) mixed lipid monolayers and c) non-equilibrium surface mixing of substrate and products of the $ppPLA_2$ reaction in enzyme-free films. The manner in which the mixture of these lipids evolves to equilibrium under a change of composition indicates that $ppPLA_2$ activity appears to act as a modifier of surface lipid composition. Independent on the abundant evidence for the involvement of boundary defects in the enzyme activation at the surface, our results clearly show that the evolution of the monolayer topography can also be determined by substrate–product lipid mixing–demixing processes in the absence of enzyme.

2. Materials and methods

2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylcholine (dlPC), lysophosphatidylcholine (LPC) and palmitic acid (Pm) were purchased from Avanti Polar Lipids (Alabaster, AL) or Sigma-Aldrich (St. Louis, MO). The lipids were 99% pure by HPTLC and were used without further purification. The lipophilic fluorescent probe 1,1′-didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate ($DiIC_{12}$) was purchased from Molecular Probes (Eugene, OR). Phospholipase A_2 from porcine pancreas ($ppPLA_2$) was purchased from Sigma-Aldrich (St. Louis, MO). Solvents and chemicals were of the highest commercial purity available. The water was purified by the Milli-Q system to yield a product with a resistivity of ~18.5 MV/cm. Absence of surface-active impurities was routinely checked as described elsewhere [23].

2.2. Epifluorescence microscopy of monolayers

Monolayers of different composition doped with 0.5 mol% $DiIC_{12}$ (lipophilic probe that preferentially partitions into the LE phase), were spread from preformed lipid solutions in chloroform–methanol (2:1) over a subphase of 10 mM Tris/HCl, 100 mM NaCl, 20 mM $CaCl_2$, pH 8 until reaching a surface pressure of less than ~0.5 mN/m. After solvent evaporation (10 min), the monolayer was slowly compressed to the desired surface pressure. The all-Teflon zero-order trough (Kibron m-Trough S; Kibron, Helsinki, Finland) was mounted on the microscope stage of an epifluorescence microscope (Zeiss Axiovert 200 or Zeiss Axioplan; Carl Zeiss, Oberkochen, Germany) with a mercury lamp (HBO 50) or a green laser, a 20× LD objective, and a rhodamine filter set. Images with exposure times of 60–100 ms were recorded with a CCD video camera controlled by Metamorph 3.0 software, or with a CCD video camera (Andor, IXON). All experiments were carried out in an air-conditioned room (22 ± 2 °C).

2.3. Determination of phospholipase activity

In lipid monolayers the short chain dlPC is usually employed as the substrate of $ppPLA_2$ enzymatic activity because the latter can be measured in real time under zero-order kinetics following the reduction of monolayer area at constant surface pressure [7]. This is due to the immediate desorption of the short-chain products into the aqueous subphase. On the contrary, when DPPC is used as the substrate both products of the reaction (Pm, LPC) remain at the interface in equimolar proportions (at least during a sufficiently long time, compared to the duration of the experiment). Because of that, and due to the interactions between the substrate and the products in a complex ternary system, the enzymatic activity cannot be followed simply by a change of the surface area. In the latter case, enzymatic activity was determined by collecting the lipid interface with a hydrophobic paper (Whatman 1PS according [24–26]) and quantifying the substrate and the products by High Performance Thin Layer Chromatography (HPTLC). The film was collected at: 0, 5, 10, 15 and 20 min after $ppPLA_2$ injection (final subphase concentration 100 ng/ml). The completion of film harvest was monitored and ascertained by the fall of surface pressure to 0 mN/m. Briefly, the film collection was carried out by gently depositing the hydrophobic filter paper onto the film for a few seconds after which it was rapidly removed and immersed in a chloroform–methanol (2:1) solution for 15 min under stirring. The filter paper was removed, washed with the same solvent, and the latter was evaporated under N_2 flow until reaching a volume of ~20 μl, which was spotted on a HPTLC plate. Chromatography was run with chloroform/methanol/acetic acid/water 30:15:3:2 (v/v/v/v). Two monolayers were collected for each point. The quantification was made by digital densitometry with ImageJ software [27] after revealing the plate by the oxidation of organic compounds (8% v/v phosphoric acid–3% p/v cupric acetate, solution) [28].

2.4. Surface mixing

The rapid surface mixing of lipid monolayers with different composition was performed in a home-made specially modified all-Teflon zero-order trough that was driven by the mechanic and electronic set-up provided by a Kibron μ-Trough S unit (Kibron, Helsinki, Finland). The special trough has three different compartments (see Fig. 1): two lateral ones (denominated 1 and 2) that are surface-connected by a narrow and shallow slit to a circular central compartment (3 ml, 3.14 cm^2; denominated reaction compartment), with barriers controlled by an automated surface barostat. In general

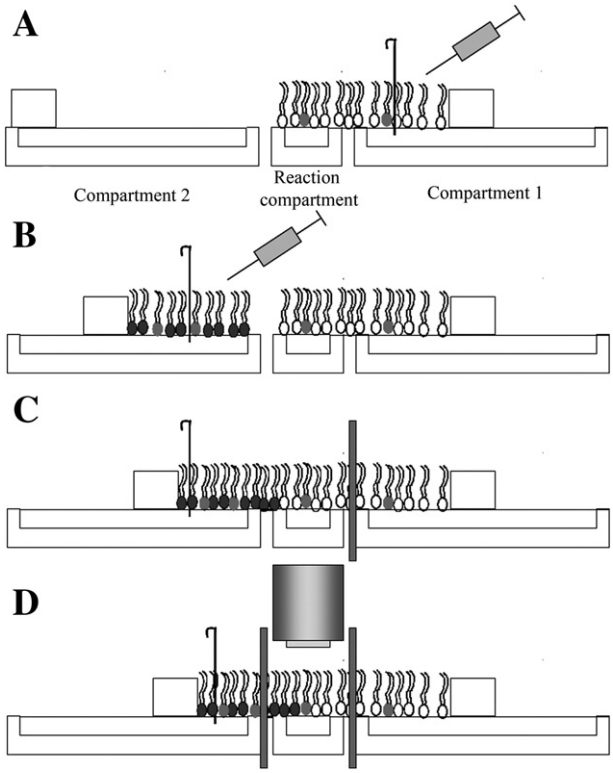

Fig. 1. Diagram of the surface mixing procedure. A) The first monolayer of composition 1 (LPC:Pm (1:1)) was spread on the reaction compartment and compartment 1, until a surface pressure ~0.5 mN/m was reached. After solvent evaporation (10 min) the monolayer was slowly compressed to ~7–10 mN/m. B) The second monolayer (DPPC:Products 75:25 (mol%)) was spread to 0.5 mN/m on compartment 2 that was kept disconnected from the reaction compartment by a septum. After solvent evaporation it was slowly compressed to ~10 mN/m. C) The reaction compartment was disconnected from compartment 1 and connected to compartment 2 until a surface pressure of ~10 mN/m was reached in the reaction compartment by compensation with the surface barostat. At this moment the connection with compartment 2 was interrupted, leaving the reaction compartment isolated. D) Observation of the surface mixing was carried out continuously.

terms the surface mixing procedure was as follows. Initially, the reaction compartment was connected with compartment 1. The first monolayer with a defined lipid composition (LPC:Pm (1:1) in this particular case) was spread onto this compartment until a surface pressure of ~0.5 mN/m was reached. After solvent evaporation (10 min) the monolayer was slowly compressed to the desired surface pressure of 7 or 10 mN/m (Fig. 1A). Then a second monolayer of different composition (DPPC enriched monolayer, particularly in this work) was spread over compartment 2 that was disconnected with a septum from the reaction compartment at this time, and this film was brought by slow compression to the desired surface pressure of 10 mN/m (Fig. 1B). Once the two monolayers were spread on both compartments the latter septum is quickly removed and the reaction compartment becomes surface-connected to compartment 2, while at the same time, compartment 1 is now disconnected from it by another septum (Fig. 1C). After reaching a surface pressure of 10 mN/m, compartment 2 is again disconnected, thus leaving the reaction compartment isolated (Fig. 1D). The changes of surface topography were continuously observed by epifluorescence microscopy while the surface mixing was taking place. Note that when the reaction compartment is isolated after surface mixing, there is a difference between the surface pressure of monolayer 1 (7 mN/m) and monolayer 2 (10 mN/m). This was to allow the second monolayer to enter the surface of the reaction compartment until the latter is isolated in order to observe the evolution of mixing in a confined place. In the experiments in which the topography of the surface-mixed monolayer is viewed after hours of mixing, the reaction compartment was not isolated and, instead, the surface was allowed to equilibrate. In the latter case both monolayers were compressed to a surface pressure ~10 mN/m before opening the septum for mixing (the final proportion of substrate:products is accurately determined). In both cases (monolayer 1 compressed to a surface pressure of 7 mN/m or 10 mN/m before surface mixing), when a DPPC enriched monolayer was surface-mixed with a product monolayer, the alteration of DPPC LC domains was similar.

3. Results

For simplicity, in the following sections ppPLA_2 (porcine pancreas PLA_2) is denominated PLA_2 and L-DPPC (hydrolysable analog) is abbreviated as DPPC.

3.1. Topographic evolution of DPPC monolayers hydrolyzed by PLA_2

Fig. 2 shows the topographic evolution of DPPC monolayer hydrolyzed by PLA_2 at a surface pressure where LE–LC phase domain coexistence occurs (~10–12 mN/m). After minutes following the injection of the enzyme into the subphase some nicks appeared at the edge of the LC domains (see arrows in Fig. 2E) which became progressively indented, in full agreement with the experiment described by Grainger et al. [9]. In addition, in our experiments, several small round LC domains appeared over some period, dispersed in the LE phase, after PLA_2 injection in the subphase (particularly noticeable in Fig. 2C, E and H). We performed this assay with PLA_2 (Fig. 2), already described by Grainger et al. [9], in order to have a direct comparison with our following experiments in which surface mixing of the substrate and the products was carried out in the absence of enzyme.

In order to confirm that enzymatic activity was actually occurring (producing a change of lipid composition in the monolayer hydrolyzed by PLA_2 that exhibited the topographic changes (Fig. 2), a quantification of the substrate and the products of the reaction was made as a function of time, by collecting the lipids from the interface and analyzing the composition by HPTLC (Fig. 3). The comparison of Fig. 2 and Fig. 3, led to the conclusion that both, the variation of monolayer composition (due to enzymatic degradation of DPPC) and the consequent changes of surface topography, occur over a period of 0–30 min. Product formation began earlier than alteration of the LC domain edge morphology, meaning that the generation of products and the appearance of the alteration of domain morphology were not temporally coupled to the initial part of the reaction. Approximately between 14 and 17 min after enzyme injection in the subphase, some small LC domains appeared in the LE phase (Fig. 2C, E, H), when product formation reached about 20% (Fig. 3). Such domains may reflect the existence of product-enriched clusters in the LE phase, formed in the presence of $CaCl_2$ in the subphase, due to PLA_2 enzymatic activity after PLA_2 absorption to the LE phases as described for other lipolytic enzymes [14]. In the following sections some experiments are described to support this idea.

3.2. Premixed monolayers of DPPC and LPC:Pm (1:1)

To compare enzymatically generated monolayers with enzyme-free mixed monolayers of the same composition, we inspected the monolayer topography of films under equilibrated conditions formed by pre-mixing solutions with different proportions of substrate and products before spreading the monolayer. As described in Materials and methods section, after solvent evaporation the monolayers were slowly compressed to a surface pressure of ~10–12 mN/m. Fig. 4 shows a sequence of epifluorescence micrographies of DPPC with increasing proportions of an equimolar mixture of the products (LPC:Pm (1:1)).

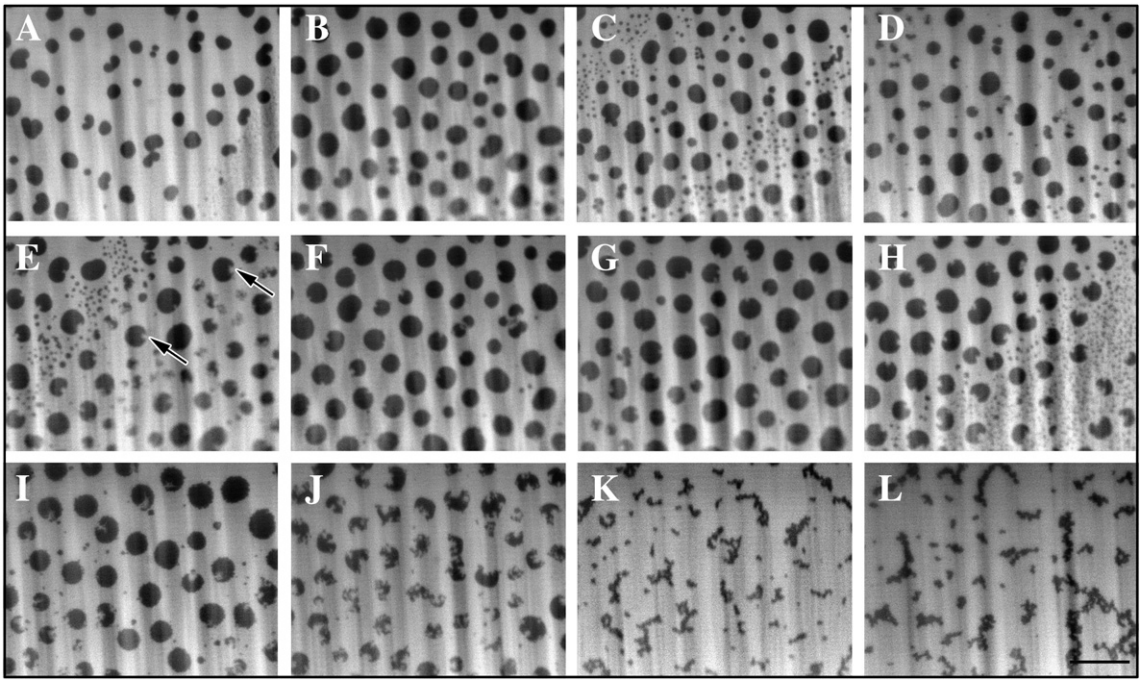

Fig. 2. Representative epifluorescence images of the time course of PLA_2 action against pure DPPC labeled by 0.5 mol% of $DiIC_{12}$ monolayer at a surface pressure ~12 mN/m. A) Before PLA_2 injection. B–L) After enzyme injection (100 ng/ml final concentration) at B: 13 C: 14 D: 15 E: 17 F: 18 G: 18.5 H: 19 I: 20 J: 23 K: 26 and L: 27 min. Arrows indicate representative topographic changes due to enzymatic activity. The images are representative of at least two independent experiments. Scale bar, 100 μm.

None of the mixed films showed changes reproducing the enzymatically generated surface topography.

On the other hand, as described above for Fig. 2 (clearly noticed in panels C, E and H), some small LC domains appeared at relatively earlier times after PLA_2 injection in the subphase. These domains are similar in aspect to the premixed monolayer of pure products (LPC:Pm (1:1)) in the presence of $CaCl_2$ in the subphase (Fig. 4D). In order to explore whether these domains could represent product-enriched clusters deriving from the composition change generated by the PLA_2 enzymatic activity, a mixed lipid solution of LPC:Pm (1:1) was spread over the subphase and compressed to lateral surface pressures of 7, 10 and 12 mN/m, with $CaCl_2$ (Fig. 5A to C) or without $CaCl_2$ (Fig. 5D to F) (EGTA 5 mM) in the subphase. The pattern found (Fig. 5A to C) reveals LC domains similar to the small ones shown in Fig. 2C, E, and H during enzymatic activity. It should be recalled that the larger domains in the first panels of Fig. 2 correspond to the LC domains of DPPC coexisting with the LE phase of this lipid in the phase transition region that occurs isothermally at the surface pressure studied. As a control, in monolayers of LPC:Pm (1:1) spread over subphases without $CaCl_2$ (lateral surface pressures of 7 and 10 mN/M (Fig. 5D, E)), the interface was homogeneous, without LC domains. At 12 mN/m a diffuse, smooth lipid phase transition was evidenced by the presence of large round LC domains (Fig. 5F) that are different in aspect than the LC domains of DPPC, or their variation due to the PLA_2 activity against the monolayer. These experiments suggest that relatively early enzymatic degradation of DPPC by PLA_2 (whose activity requires $CaCl_2$ in the subphase) appears to be accompanied by LC product clustering that may take some time to equilibrate. This type of effects could also help understand why those small domains might be present at some time but disappear at others and appear again subsequently if waves of product slowly equilibrate by mixing with the substrate and distorting the shape of LC domains of DPPC. Ascertaining these possibilities will require extended investigations of kinetically-limited surface processes.

3.3. Surface-mixed monolayers of the substrate and the products of PLA_2 reaction, in the absence of enzyme

We inspected if it was possible to generate a topography similar to that generated by PLA_2 but in enzyme-free films under out of equilibrium mixing conditions. For that purpose, a procedure for "surface mixing" lipid monolayers of different composition directly at the interface was devised (see Section 2.4, Fig. 1). A substrate-enriched monolayer (DPPC:Products, 75:25 mol%) was surface-mixed with a monolayer of the mixed products (LPC:Pm (1:1)). A mixed film of DPPC:Products (75:25 mol%) was chosen instead of pure DPPC in this particular assay, because of the convenient round morphology that helps in the detection of domain edge alteration. Fig. 6 shows the topography resulting from mixing both monolayers directly at the interface (final surface pressure ~ 10 mN/m). The morphological change of LC domains generated during surface mixing resembled those generated in films hydrolyzed by PLA_2 (distortions of domain's edges, see Fig. 2 panels I, J for example). To ensure that morphological domain alteration arose from the mixing of substrate-enriched and product monolayers and is not due to the procedure of mixing itself, a control experiment was made with a film obtained by surface mixing two monolayers of the same composition (DPPC:Products 75:25 mol%) at a final surface pressure of 10 mN/m. No alteration of LC domain morphology was observed in this experiment (Fig. 7).

Fig. 8 shows snapshots of different regions of the monolayer minutes after mixing in order to illustrate the changes of LC domain morphology induced by the change of surface composition. The alteration of LC domain boundaries shown in Fig. 8A was similar to that found in enzymatically generated films at ~20 min after PLA_2 injection (Fig. 2H to J). With our method, the surface mixing of two monolayers of different composition starts at one side of the film surface thus generating a gradient of surface composition, and topography, evolving from the lateral region of contact. Fig. 8B, C clearly shows the gradient regarding the degree of domain shape modification, depending on the proximity of them to the region of contact.

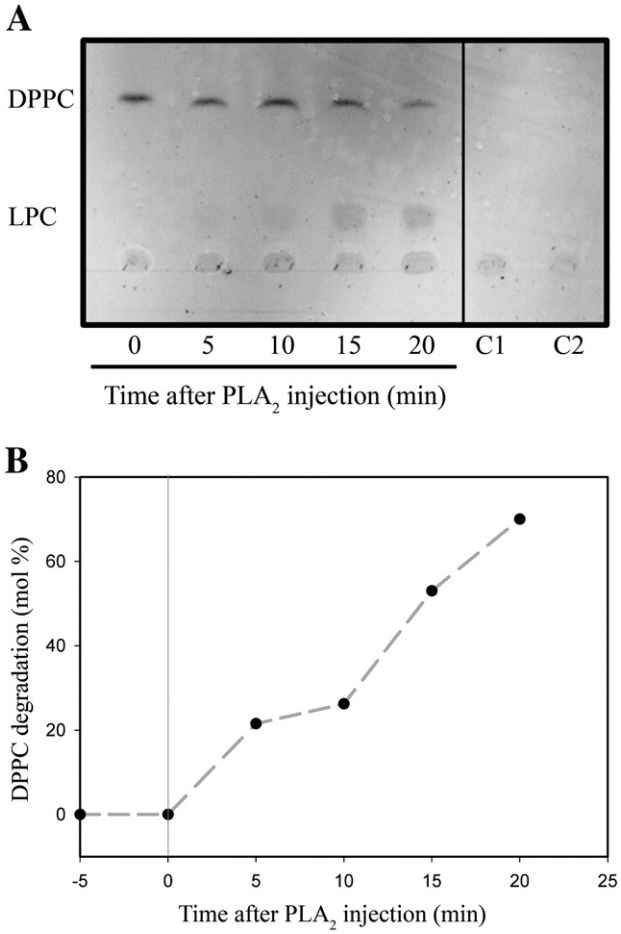

Fig. 3. Quantification of DPPC hydrolysis by PLA_2. A) Representative HPTLC of the time course of PLA_2 action against pure DPPC monolayer at a surface pressure ~12 mN/m. Each point represents a pool collected from two independent monolayers. The lanes correspond to the collection at different times (as indicated) after enzyme injection in the subphase (100 ng/ml). Lane C1 (panel A left) represents the collection of a clean interface (lipid free) after 20 min of the injection of PLA_2 in the subphase and lane C2 corresponds to the hydrophobic paper extract after solvent treatment without collecting any interface. B) Densitometric evaluation of A with ImageJ software [27]. The maximal variation of mean values was less than 10% in all cases.

3.4. Surface-mixed monolayers: domain shape evolution

In previous sections it was demonstrated that the pattern generated during PLA_2 action could be mimicked, in the absence of enzyme, mixing substrate and products of the reaction directly at the interphase, at high lateral surface pressure (see Fig. 6 and Fig. 8). This section evaluates the topographic evolution of a monolayer generated by surface mixing a pure-substrate (Fig. 9A) and a pure-product (Fig. 9B) monolayer in different final proportions. Fig. 9C to E shows the result of surface mixing DPPC and LPC:Pm (1:1), in 70:30 or 55:45 (mol%) final proportions. Some indentations in LC domains could be observed (as previously described in Fig. 6), in both final proportions, minutes after interfacial mixing. After 2–3 h of interfacial mixing, the topographies found were mostly rounded LC domains (Fig. 9E, 70:30 (mol%) substrate:products) and star-like LC domains (Fig. 9F, 55:45 (mol%) substrate:products) similar to the ones found in premixed equilibrated monolayers (Fig. 4B, C). Note that in these experiments the reaction compartment was not isolated (see Section 2.4). Instead, after the spreading and compression (10 mN/m) of both individual monolayers, the surfaces of both compartments were connected and the lipid composition was allowed to equilibrate during 2–3 h at a constant surface pressure ~10 mN/m.

4. Discussion

In 1990 an important report in the field of regulation of surface topography by lipolytic enzymes was published [9]. These authors showed by epifluorescence microscopy the topographic changes of DPPC monolayer hydrolyzed by *N. naja* PLA_2 at a surface pressure where LE–LC phase coexistence occurs. It was concluded that lateral interfaces (arising from the coexistence of lipid phases in different physical states) were initial points of enzymatic activity, presumably due to a physical contact of *N. naja* PLA_2 with the domain boundaries at which the hydrolysis would appeared to start, and proceed from the edge to the center of the LC domains. On this basis, the topographic evolution of the monolayer hydrolyzed by *N. naja* PLA_2 was related not only to a role of the enzyme actively generating topography but also to the surface localization of the enzyme.

Interestingly, in the present work, in Fig. 2E (the beginning of domain morphology alterations) there was only an average of one nick of LE phase in the gel phase per the LC domain (as detected by the probe partitioning). Gudmand et al. [12] also reported that structural changes induced by PLA_2 action on DPPC monolayers started from one side of the LC domains. If domain edges were initial places of enzymatic activity, each domain would have to be hydrolyzed by only one enzyme (or a very few) at a single particular point on the domain edge. Nevertheless, Gudmand et al. [12] have also shown that the topographic localization of PLA_2 in DPPC monolayers corresponded to a uniform distribution of enzyme around the perimeter of the LC domains mainly because of nucleation of a product-enriched phase at the LC domain boundary that could act as an enzyme trap. It is very important to point out that the nicks observed at the edge of the LC domains represent only an increased partitioning of the lipophilic fluorescent probe $DiIC_{12}$ into the product-enriched LE phase. Considering Gudmand's result, it is worth noticing that the accumulation of PLA_2 in those product-enriched areas does not imply that the reaction takes place only at that location where additional product inhibition could also be enhanced. Therefore, with the methodology of fluorescent probe partitioning that was so far employed, it is not possible to rule out the occurrence of enzymatic activity in the LE phase and that the mixing–demixing of the substrate and the products formed

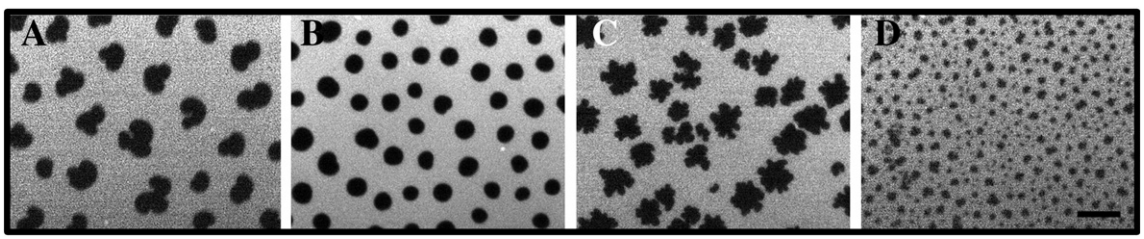

Fig. 4. Epifluorescence micrograph of $DiIC_{12}$ 0.5 mol% doped DPPC/(LPC:Pm (1:1)) monolayers at a surface pressure ~12 mN/m. A) Pure DPPC. B–C) Premixed monolayers of DPPC:(LPC:Pm, (1:1)) in different proportions: B) 75:25 mol%, C) 50:50 mol%. D) LPC:Pm 50:50 mol%. The images are representative of at least two independent experiments. Scale bar, 50 μm.

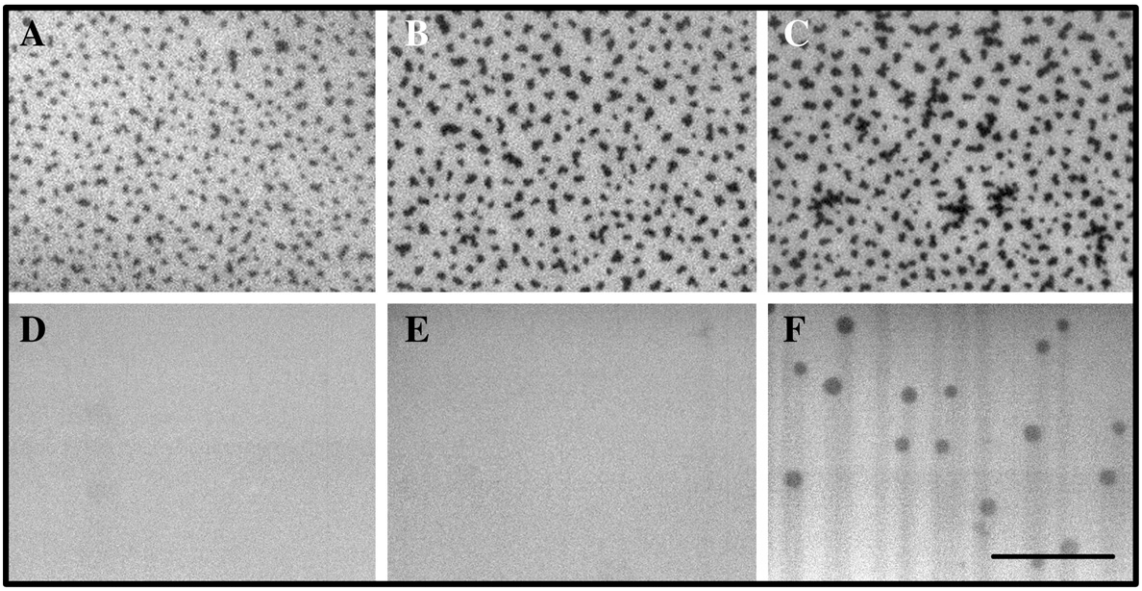

Fig. 5. Epifluorescence micrograph of LPC:Pm (1:1) monolayers doped with $DiIC_{12}$ 0.5 mol%. A–C) LPC:Pm (1:1) at 7 mN/m (A), 10 mN/m (B) and 12 mN/m (C) over a subphase of 10 mM Tris/HCl, 100 mM NaCl, 20 mM $CaCl_2$, pH 8. D–E) LPC:Pm (1:1) at 7 mN/m (D), 10 mN/m (E) and 12 mN/m (F) over a subphase of 10 mM Tris/HCl, 100 mM NaCl, 5 mM EGTA, pH 8. The images are representative of at least two independent experiments. Scale bar, 100 μm.

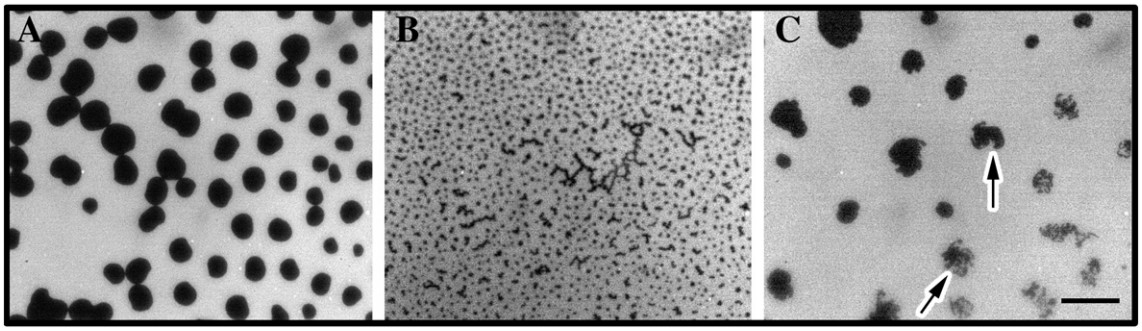

Fig. 6. Epifluorescence micrographs of enzyme-free, surface-mixed monolayers. A) Substrate-enriched monolayer: DPPC:(LPC:Pm, (1:1)) 75:25 mol% (surface pressure ~ 10 mN/m). B) Product monolayer: LPC:Pm (1:1) (surface pressure ~ 7 mN/m). C) Surface topography as a result of surface mixing monolayers A and B (surface pressure ~ 10 mN/m). Arrows show morphological changes of LC domains. The images are representative of at least two independent experiments. Scale bar, 100 μm.

in the LE phase could result in alterations of the domain edge morphology.

Fig. 4 shows pre-equilibrate substrate–product mixtures in different proportions. None of them showed a surface topography similar to that enzymatically generated. One possible explanation is that, when the monolayer is enzymatically generated, relatively rapid out-of-equilibrium and non-homogeneous transient changes of surface compositions and organization occur that are not possible to observe in the pre-mixed enzyme-free lipid monolayers because these films represent a near equilibrium condition. These results raised

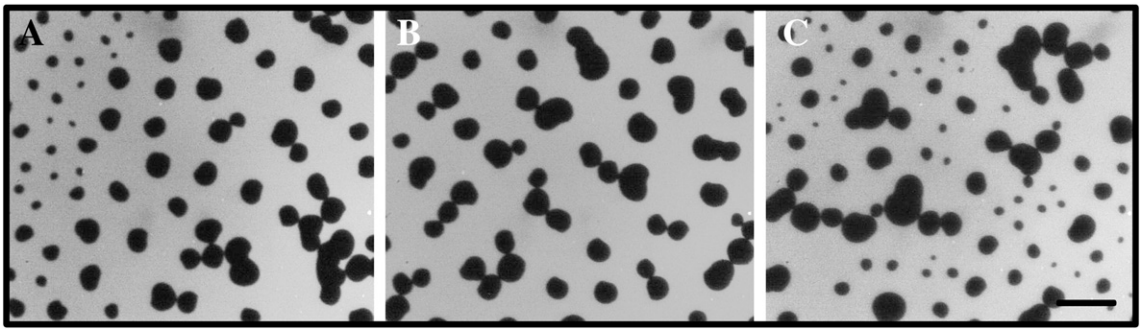

Fig. 7. Control experiment. Epifluorescence micrographs of enzyme-free, surface-mixed monolayers. A) DPPC:Product (75:25 mol%) at a surface pressure ~10 mN/m. B) DPPC:Product (75:25 mol%) at a surface pressure ~7 or 10 mN/m (isolated or open reaction compartment, see Section 2.4). C) Representative image of the result of surface-mixed A and B monolayers. Scale bar, 100 μm.

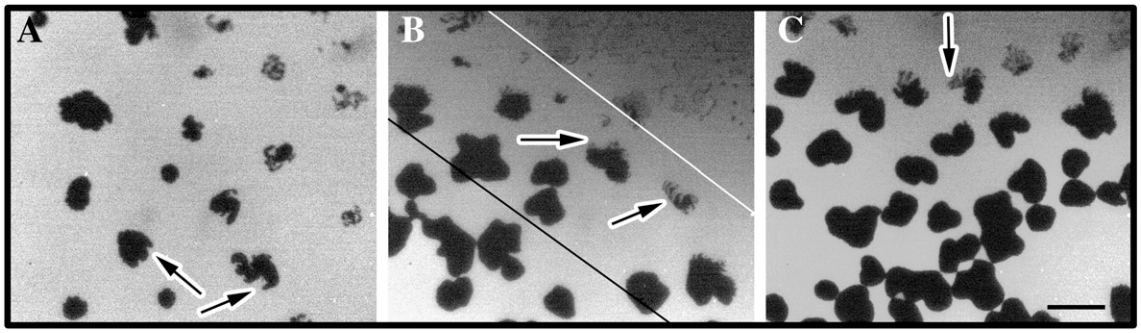

Fig. 8. Epifluorescence micrographs showing different regions of surface mixing a substrate-enriched monolayer (DPPC:Products, 75:25 mol%) and a product monolayer (LPC:Pm (1:1)). A) LC domain boundary alteration similar to Fig. 2H to J. B–C) Compositional gradient generated by the surface mixing procedure. In B) three different zones can be observed. The center represents the region where the mixing is occurring. Beyond the black line to the bottom left: DPPC:Products 75:25 mol%; beyond the white line to the upper right: LPC:Pm (1:1). Arrows show different morphologic LC domain alterations. Scale bar, 100 μm.

the testable possibility that the topographic changes occurring at the interface might be a consequence of a non-equilibrium generation of products in the substrate film. In this sense, the generation of products (LPC:Pm (1:1)) in a PLA_2 hydrolyzed monolayer is localized in the enzyme-enriched LE phase regions, meaning that in the surroundings of a particular LC domain there is a gradient of product concentration. The localization of a nick in LC domains could be a consequence of a local liberation of products by PLA_2. The vectorial discharge of products imposed by our surface mixing method and the manner in which the edge of the domains become affected when

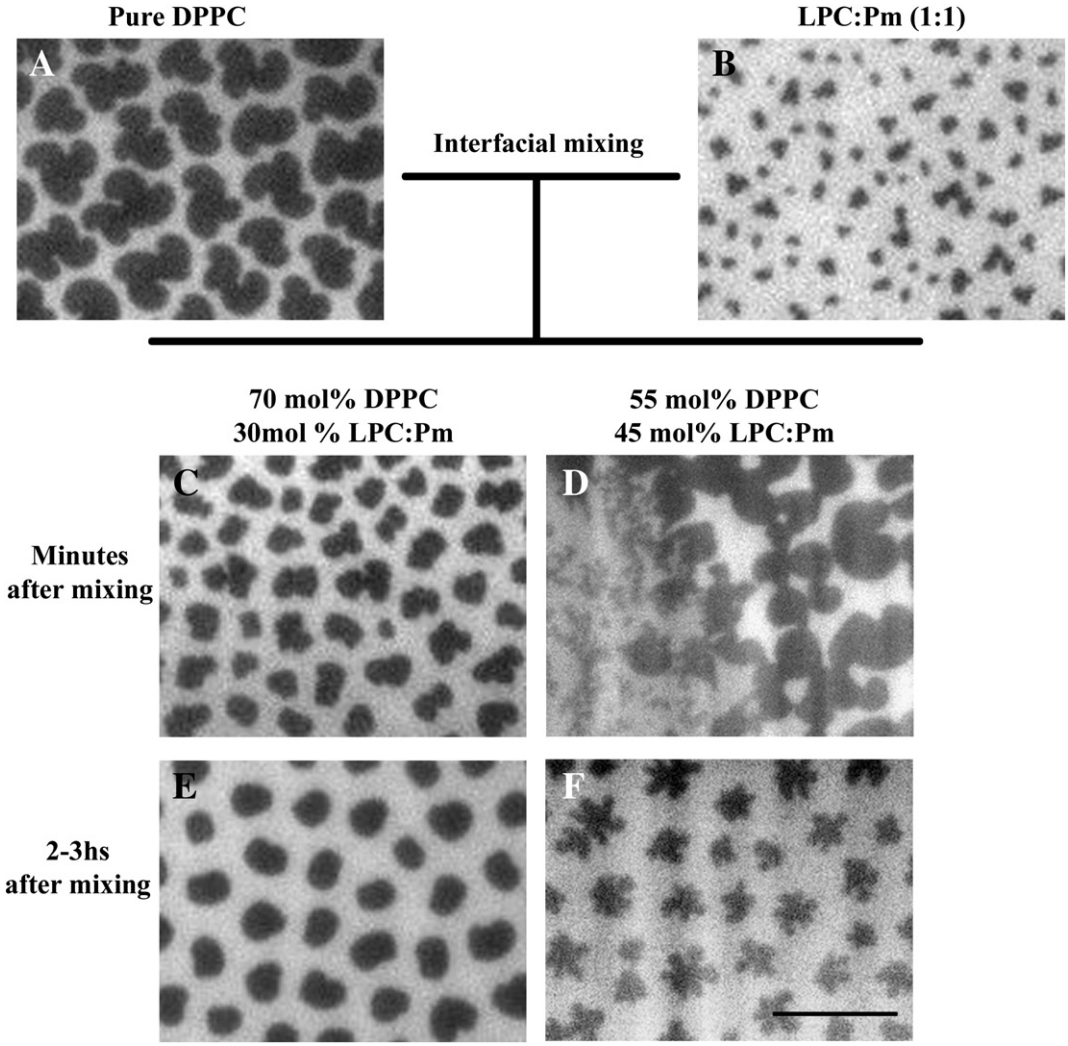

Fig. 9. Topographic evolution of surface-mixed monolayers. A DPPC monolayer (surface pressure ~ 10 mN/m) and LPC:Pm (1:1) monolayer (surface pressure ~ 10 mN/m) were surface-mixed obtaining final proportions of DPPC:Products 70:30 mol% (C, E) and DPPC:Products 55:45 mol% (D, F) at 10 mN/m (final surface pressure). C–D) Epifluorescence micrographs minutes after surface mixing. E–F) Epifluorescence micrographs 2–3 h after surface mixing. Scale bar, 50 μm.

they are in contact with a monolayer of products suggest that the liberation of enzyme-generated products should also be vectorial; this would be in keeping with an out-of-equilibrium local formation of concentrated products by PLA_2 in the LE phase by which mixing of waves of products near the LC domains could affect their morphology.

Regarding the stability of the surface-mixed monolayers, after hours from mixing, the topography mainly found is similar to that of the pre-equilibrated films with corresponding proportions of substrate and products. This supports the idea that the alterations at the edge of the LC domains are due to out-of-equilibrium concentration of products in the LE phase that subsequently become more slowly mixed with the substrate thus generating time-dependent topographic alterations in the lipid film.

5. Conclusions

Our work showed that similar changes of the LC-domain morphology can be observed when the lipid composition of the monolayer is changed at the surface, under out-of-equilibrium conditions, either by PLA_2 activity or by surface mixing of monolayers of substrate and products in enzyme-free systems. It is straightforward to conclude that the enzymatic activity must be changing the lipid composition by forming products at some relative speed until a critical composition point is reached above which the surface topography reorganizes according to substrate–product miscibility. The idea that membrane lateral defects (arising from phase coexistences of lipid domains in different physical states or from packing defects), regulate lipolytic enzymes is well accepted in the literature. However a direct physical contact of the enzyme with the domain boundaries does not appear to be required to induce the topographic changes occurring during the enzyme action.

Acknowledgements

This work was supported by SECyT-UNC, CONICET and FONCyT (Argentina). B.M. and M.L.F. are Career Investigators of CONICET. L.D.T. is postdoctoral fellow of CONICET.

References

[1] M.K. Jain, J. Rogers, D.V. Jahagirdar, J.F. Marecek, F. Ramirez, Kinetics of interfacial catalysis by phospholipase A2 in intravesicle scooting mode, and heterofusion of anionic and zwitterionic vesicles, Biochim. Biophys. Acta 860 (1986) 435–447.
[2] B. Maggio, Control by ganglioside GD1a of phospholipase A2 activity through modulation of the lamellar–hexagonal (HII) phase transition, Mol. Membr. Biol. 13 (1996) 109–112.
[3] J.D. Bell, M. Burnside, J.A. Owen, M.L. Royall, M.L. Baker, Relationships between bilayer structure and phospholipase A2 activity: interactions among temperature, diacylglycerol, lysolecithin, palmitic acid, and dipalmitoylphosphatidylcholine, Biochemistry 35 (1996) 4945–4955.
[4] H.W. Huang, E.M. Goldberg, R. Zidovetzki, Ceramide induces structural defects into phosphatidylcholine bilayers and activates phospholipase A2, Biochem. Biophys. Res. Commun. 220 (1996) 834–838.
[5] M. Grandbois, H. Clausen-Schaumann, H. Gaub, Atomic force microscope imaging of phospholipid bilayer degradation by phospholipase A2, Biophys. J. 74 (1998) 2398–2404.
[6] C. Leidy, L. Linderoth, T.L. Andresen, O.G. Mouritsen, K. Jorgensen, G.H. Peters, Domain-induced activation of human phospholipase A2 type IIA: local versus global lipid composition, Biophys. J. 90 (2006) 3165–3175.
[7] B. Maggio, I.D. Bianco, G.G. Montich, G.D. Fidelio, R.K. Yu, Regulation by gangliosides and sulfatides of phospholipase A2 activity against dipalmitoyl- and dilauroylphosphatidylcholine in small unilamellar bilayer vesicles and mixed monolayers, Biochim. Biophys. Acta 1190 (1994) 137–148.
[8] K. Wagner, B. Desbat, G. Brezesinski, Liquid–liquid immiscibility in model membranes activates secretory phospholipase A2, Biochim. Biophys. Acta 1778 (2008) 166–174.
[9] D.W. Grainger, A. Reichert, H. Ringsdorf, C. Salesse, Hydrolytic action of phospholipase A2 in monolayers in the phase transition region: direct observation of enzyme domain formation using fluorescence microscopy, Biochim. Biophys. Acta 1023 (1990) 365–379.
[10] U. Dahmen-Levison, G. Brezesinski, H. Mohwald, Specific adsorption of PLA2 at monolayers, Thin Solid Films 327 (1998) 616–620.
[11] S.A. Sanchez, L.A. Bagatolli, E. Gratton, T.L. Hazlett, A two-photon view of an enzyme at work: *Crotalus atrox* venom PLA2 interaction with single-lipid and mixed-lipid giant unilamellar vesicles, Biophys. J. 82 (2002) 2232–2243.
[12] M. Gudmand, S. Rocha, N.S. Hatzakis, K. Peneva, K. Mullen, D. Stamou, I. Uji, J. Hofkens, T. Bjornholm, T. Heimburg, Influence of lipid heterogeneity and phase behavior on phospholipase A2 action at the single molecule level, Biophys. J. 98 (2010) 1873–1882.
[13] M.L. Fanani, S. Hartel, R.G. Oliveira, B. Maggio, Bidirectional control of sphingomyelinase activity and surface topography in lipid monolayers, Biophys. J. 83 (2002) 3416–3424.
[14] L. De Tullio, B. Maggio, M.L. Fanani, Sphingomyelinase acts by an area-activated mechanism on the liquid-expanded phase of sphingomyelin monolayers, J. Lipid Res. 49 (2008) 2347–2355.
[15] G. Basanez, M.B. Ruiz-Arguello, A. Alonso, F.M. Goni, G. Karlsson, K. Edwards, Morphological changes induced by phospholipase C and by sphingomyelinase on large unilamellar vesicles: a cryo-transmission electron microscopy study of liposome fusion, Biophys. J. 72 (1997) 2630–2637.
[16] M.L. Fanani, L. De Tullio, S. Hartel, J. Jara, B. Maggio, Sphingomyelinase-induced domain shape relaxation driven by out-of-equilibrium changes of composition, Biophys. J. 96 (2009) 67–76.
[17] M.L. Fanani, S. Hartel, B. Maggio, L. De Tullio, J. Jara, F. Olmos, R.G. Oliveira, The action of sphingomyelinase in lipid monolayers as revealed by microscopic image analysis, Biochim. Biophys. Acta 1798 (2010) 1309–1323.
[18] L. De Tullio, B. Maggio, S. Hartel, J. Jara, M.L. Fanani, The initial surface composition and topography modulate sphingomyelinase-driven sphingomyelin to ceramide conversion in lipid monolayers, Cell Biochem. Biophys. 47 (2007) 169–177.
[19] S. Hartel, M.L. Fanani, B. Maggio, Shape transitions and lattice structuring of ceramide-enriched domains generated by sphingomyelinase in lipid monolayers, Biophys. J. 88 (2005) 287–304.
[20] K. Jorgensen, A. Klinger, R.L. Biltonen, Nonequilibrium lipid domain growth in the gel-fluid two-phase region of a DC16PC–DC22PC lipid mixture investigated by Monte Carlo computer simulation, FT-IR, and fluorescence spectroscopy, J. Phys. Chem. B 104 (2000) 11763–11773.
[21] R.F. de Almeida, L.M. Loura, A. Fedorov, M. Prieto, Nonequilibrium phenomena in the phase separation of a two-component lipid bilayer, Biophys. J. 82 (2002) 823–834.
[22] J.M. Holopainen, M. Subramanian, P.K. Kinnunen, Sphingomyelinase induces lipid microdomain formation in a fluid phosphatidylcholine/sphingomyelin membrane, Biochemistry 37 (1998) 17562–17570.
[23] I.D. Bianco, B. Maggio, Interactions of neutral and anionic glycosphingolipids with dilauroylphosphatidylcholine and dilauroylphosphatidic acid in mixed monolayers, Colloids Surf. 40 (1989) 249–260.
[24] B. Maggio, The surface behavior of glycosphingolipids in biomembranes: a new frontier of molecular ecology, Prog. Biophys. Mol. Biol. 62 (1994) 55–117.
[25] W.E. Momsen, H.L. Brockman, Recovery of monomolecular films in studies of lipolysis, Methods Enzymol. 286 (1997) 292–305.
[26] M.A. Perillo, R.K. Yu, B. Maggio, Modulation of the activity of *Clostridium perfringens* neuraminidase by the molecular organization of gangliosides in monolayers, Biochim. Biophys. Acta 1193 (1994) 155–164.
[27] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[28] R.G. Oliveira, R.O. Calderon, B. Maggio, Surface behavior of myelin monolayers, Biochim. Biophys. Acta 1370 (1998) 127–137.