Journal of Thermal Analysis and Calorimetry, Vol. 63 (2001) 47–57

INTERACTIONS BETWEEN ANTIVIRAL PROSTAGLANDINS AND BIOMIMETIC MEMBRANES
A Langmuir films study

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(Received June 26, 2000; in revised form September 28, 2000)

Abstract

Cyclopentenonic derivatives of prostaglandins are able to interfere at different levels with virus infection but the complex mechanism of their antiviral effect is still unresolved. As the antiviral activity was shown to be influenced by the cyclopentenonic structure, the interaction with biomimetic membrane of two types of prostaglandin having different antiviral activity were studied by thermal technique and completed with epifluorescence microscopy. The surface-pressure *vs*. molecular area isotherms showed an increase in the lateral packing density of the liquid-crystalline phases in the presence of prostaglandin A_1-type, whereas the prostaglandin E_1-type seems to penetrate without producing any significant modifications in the molecular organization of the biomimetic membrane.

Keywords: DPPC, epifluorescence, Langmuir films, prostaglandin

Introduction

Prostaglandins are cyclic oxygenated fatty acids involved in the control of many physiological processes. Cyclopentenonic derivatives such as A_1-type (PGA) prostaglandins have been shown to inhibit specifically the *in vitro* virus replication and to suppress proliferation of tumour cells. This antiviral activity was attributed to an α,β-unsaturated carbonyl group in the cyclopentane ring structure (Fig. 1), and was effective against a wide variety of naked or enveloped, DNA or RNA viruses [1–7]. The antiviral activity of PGA was observed to affect different levels in the early phase of virus infection [5, 8–10]. Not only PGA could act on the viral gene expression [11, 12] but also inhibited the RNA polymerase activity [13, 14] or altered the mRNA expression [15, 16]. In late events of the infection process, PGA inhibited the glycosylation of viral glycoproteins in the viral replication [17].

Cyclopentane derivatives were shown inefficient in inhibiting viral infection in the concentration range of that of PGA. Prostaglandins E_1 are cyclic oxygenated fatty acids with no cyclopentenonic group, which are involved in many physiological

1418–2874/2001/ $ 5.00

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events such as the inflammatory process, hemolysis or flexibility in erythrocyte membranes [18–21].

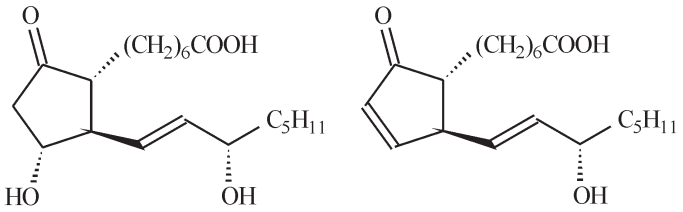

Fig. 1 Schematic structures of PGE_1 and PGA_1 molecules

Although the precise mechanisms by which PGA induces its antiviral activity are still unresolved, many lines of evidence suggest that the amphiphilic properties of cyclopentenonic prostaglandin are of major importance in the inhibition of the viral replication.

First, the requirement of a cyclopentenonic ring in prostaglandin molecules to induce an antiviral activity suggested that the inhibitory process was dependent on the structural feature of PGA. A cyclopentane derivative such as PGE, known to dehydrate to PGA, was required at 10-fold higher concentration than PGA for antiviral activity. Furthermore, the antiviral potency of cyclopentenonic prostaglandin was shown to be dependent on the structure, the presence of the 15-OH group being necessary for high potency, although varying the position of the keto group or the double bound in the pentene cycle did not influence the antiviral potency [14]. These latter results lead to the hypothesis of an amphiphilic contribution of PGA in the antiviral process.

In this paper, we analyzed the interactions occurring between PGA and PGE with a lipid monolayer as membrane model. In order to investigate the in-plane interactions between prostaglandin and phospholipids, Langmuir film technique were used, the compression isotherms providing information on the molecular packing density in the monolayers. These thermal results are completed by an epifluorescence microscopy study.

Experimental

Materials

*L*α-dipalmitoylphosphatidylcholine (DPPC), prostaglandins PGE_1 ([11α, 13*E*, 15*S*]-11, 15-dihydroxy-9-oxoprost-13-enoic acid), and synthetic PGA_1 ([13*E*, 15*S*]-15-hyroxy-9-oxoprosta-10, 13-dien-1-oic acid) were purchased from Sigma-Aldrich Chimie. Fluorescent lipid probe NBD-C_6-HPC (2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) and the esterification reagent ADAM (9-anthryldiazomethane) were supplied by Molecular Probes.

The purity of DPPC was greater than 99% based on thin-layer chromatographic analysis. Stock solutions of lipids and prostaglandins 10^{-3} M, were prepared in chlo-

roform and were diluted at the desired molar concentrations. Water, acetate and tris buffers were used as subphase. Pure water (pH 5.7) was obtained from deionised water purified with an UHQ2 Elgastat system (resistivity>18 MΩ cm). Acetate buffer consisted of 0.05 M sodium acetate in addition to 0.2 M acid for acetate buffer (pH 4), and tris buffer (pH 7.4) were realized with 0.2 M tris (hydroxymethyl)-aminomethan which final pH was achieved with chlorhydric acid (35%). Acetic acid, sodium salt, anhydrous C.P. was obtained from Acros, acetic acid glacial G.R. from Merck, tris from Fluka and chlorhydric acid from Fisher Scientific. Methanol and chloroform, used as spreading solvents, were purchased from Carlo Erba Regenti and Fisher Scientific respectively. All solvents were high purity reagents for HPLC applications.

Langmuir balance

Isotherm diagrams of surface pressure π *vs.* molecular area A were obtained with a Langmuir type balance (Riegler and Kirstein). The Langmuir trough was equipped with two symmetrical barriers for monolayer compression and a Wilhelmy balance for surface pressure determination. The surface pressure was measured with a differential uncertainty of about 0.1 mN m^{-1} and the molecular area, $2 \cdot 10^{-3}$ nm^2. Compressions of DPPC films were proceeded after a 20 min waiting time for equilibration, at a speed of $3 \cdot 10^{-2}$ nm^2 mol^{-1} min^{-1}. The trough was filled with ~100 ml of pure water. All the experiments were done at a temperature of 25±0.5°C.

Increasing amounts of prostaglandins were injected in the subphase of a same lipidic film, the total concentration in the subphase being 2.5, 5, 7.5, 10, 15 and $20 \cdot 10^{-7}$ M respectively. These values represent the concentrations range of antiviral effect for PGA. In order to prevent the collapse of the monolayer, each experiment was realized in a surface pressure range 0–25 mN m^{-1}.

A compression/expansion cycle was first realized on each DPPC film. A second compression was then realized up to an area 100 nm^2 from which the prostaglandin was deposited outside the monolayer, between the side of the trough and the barriers. The film was then totally expanded and film compression was initialised after a 45 min delay for prostaglandin diffusion. The lipid mean molecular area of the two component monolayers was evaluated at a given surface pressure by dividing the total surface film area by the total number of DPPC molecules applied to the surface, in the absence or in the presence of prostaglandin.

Epifluorescence

Fluorescence microscopy observations were made by means of an Olympus-BX30 microscope, set on a R&K Langmuir trough. An AIS (MXRi2) video camera with an image intensifier, which allows for a very high sensitivity (10^{-6} lux), enabled us to visualize the film. Images were then digitalized and analyzed using the software Optimas v.5.1 (Optimas Corporation). The Langmuir isotherms were observed using lipid solutions containing mol% 0.5 of NBD-C6-HPC. The probe concentration did not significantly alter the π–A diagrams nor the phase transitions of the monolayer,

since π–A isotherms of pure monolayers and monolayers containing the fluorescent probe were similar.

HPLC and Langmuir-Blodgett (LB) techniques

Films of DPPC containing PGA were transferred onto a glass substrate (20×20×1 mm) at a stabilized surface pressure of 25 mN m^{-1}, by lifting the substrate vertically out of the subphase (Z-type deposition) at a speed of 80 μm s^{-1}. Before transfer, the glass plates were cleaned by immersion in an Hellmanex solution ultrasonicated and then rinsed with deionised pure water. All of the transfers were performed at a temperature of 25±0.5°C and the transfer ratios were always larger than 95%. A single monolayer was stacked on both sides of each substrate and the resulting Langmuir–Blodgett (LB) film was dissolved in methanol. Each solution was realized with ten transfers from DPPC monolayers containing $15 \cdot 10^{-7}$ M of prostaglandin. These solutions were then concentrated and the quantitative determination of prostaglandin was realized by high-performance liquid chromatography (HPLC). A volume of 10 μl of each sample was injected onto a C_{18} column (Kromacil KR125 4.6×125 mm) at room temperature. The flow rate was 1 ml min^{-1}. The mobile phase consisted on a filtered and degassed mixture of 63% acetonitrile, 18% methanol, and 19% deionised water. The quantitative determination of prostaglandin was realized by fluorescence, using the esterification reagent ADAM [22, 23]. The wavelengths were 364 and 418 nm for excitation and emission respectively. The calibration device was made with standard solutions composed with various DPPC/prostaglandin molar ratios. Chloroform was removed and the residue was then dissolved in 1 ml of ethyl acetate and 10 μl of ADAM $1.15 \cdot 10^{-2}$ M. Optimal esterification was obtained at 40°C after a 30 min delay.

Results and discussion

We analyzed the interfacial behaviour of DPPC monolayers containing increasing amounts of PGA or PGE in a water subphase. Prostaglandin molecules being soluble in water, we evaluated in the condensed phase, the partitioning of prostaglandin molecules between the water subphase and the lipid monolayer. Langmuir–Blodgett method associated with high-performance liquid chromatography (HPLC) allowed to quantify the amount of prostaglandin molecules in the phospholipidic film. Langmuir–Blodgett transfers were realized in the condensed phase of the DPPC films, at a surface pressure of 25 mN m^{-1}. Such measurements were not realized at lower surface pressures, phospholipid transfers being impossible. The molar ratio (mole prostaglandin/mole DPPC) of incorporated prostaglandin (PGA or PGE) were estimated between 3 and 5% for an initial prostaglandin concentration $2 \cdot 10^{-6}$ M in the subphase. These results show that the structural differences between PGA and PGE had no influence on the prostaglandin distribution between the lipidic interface and the water subphase. The amount of prostaglandin penetrated within the monolayer being lower than 5% for the highest concentration of prostaglandins, it can be assumed that the error made on the molecular area is less than a few percents.

The surface pressure (π) *vs*. mean area per lipid molecule (A) isotherms of pure DPPC monolayers and DPPC/prostaglandin films are presented in Fig. 2. The spreading isotherms of DPPC on a pure water subphase were in good agreement with those previously observed [24]. Experiments were realized with the following concentrations of prostaglandin injected in a subphase of pure water: 2.5, 5, 7.5, 10, 15 and $20 \cdot 10^{-7}$ M. This corresponds to antiviral activities of prostaglandin revealed by *in vitro* studies [1–15]. For the sake of simplicity, isotherm diagrams are only presented for results obtained at prostaglandin concentrations of $5 \cdot 10^{-7}$, 10^{-6} and $2 \cdot 10^{-6}$ M. We noticed that several compression/expansion cycles on a same mixed DPPC/prostaglandin monolayer produced reproducible diagrams.

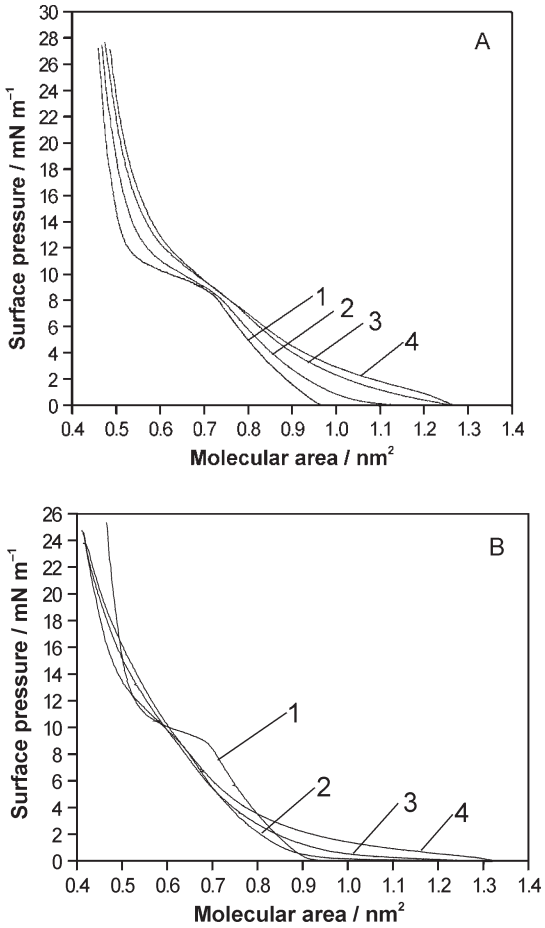

Fig. 2 Surface pressure *vs*. apparent lipid molecular area. Isotherms were recorded at 25°C. **A** – shows DPPC with increasing amounts of PGE: 1 – 0; 2 – $5 \cdot 10^{-7}$ M; 3 – 10^{-6} M and 4 – $2 \cdot 10^{-6}$ M; **B** – shows DPPC with increasing amounts of PGA: 1 – 0; 2 – $5 \cdot 10^{-7}$ M; 3 – 10^{-6} M and 4 – $2 \cdot 10^{-6}$ M

LC phase

The liquid condensed (LC) phase of pure DPPC monolayers was observed between π of 12.5 and 25 mN m^{-1}. The presence of PGE induced a shift of isotherm diagrams towards higher molecular areas (Fig. 2A). This effect was concentration dependent. The slope values of isotherms being nearly identical, it can be assumed that the lipidic structure of the monolayer was not modified by the presence of PGE inside the film. Figure 2B shows the effect of increasing amounts of PGA in DPPC film. The slope values of the isotherms decreased in the presence of PGA, which is indicative of a more compressible state than in pure DPPC films. Experiments being reversible, the variation in the compressibility of the film cannot be explained by a micellization process, mixed DPPC/PGA layer isotherms being reversible.

In order to characterize the prostaglandin effect within the lipidic film, the molecular area variation $\Delta A = A_{(DPPC+PG)} - A_{(DPPC)}$ was studied *vs*. surface pressure. Figure 3 shows that, for a concentration of PGE $2 \cdot 10^{-6}$ M injected in the subphase, ΔA decreased from 0.08 to 0.03 nm^2 at 12.5 and 25 mN m^{-1} respectively. At an equivalent amount of PGA injected in the subphase (Fig. 4), the variation range of ΔA was increased by 50% compared to that of PGE. From π of 12.5 to 25 mN m^{-1}, the ΔA values were significantly lower than that of PGE, as they decreased from 0.032 to a negative value –0.052 nm^2. For all the PGA concentrations studied, ΔA was observed to increase with the prostaglandin content at 12.5 whereas it remained approximately constant at 25 mN m^{-1}.

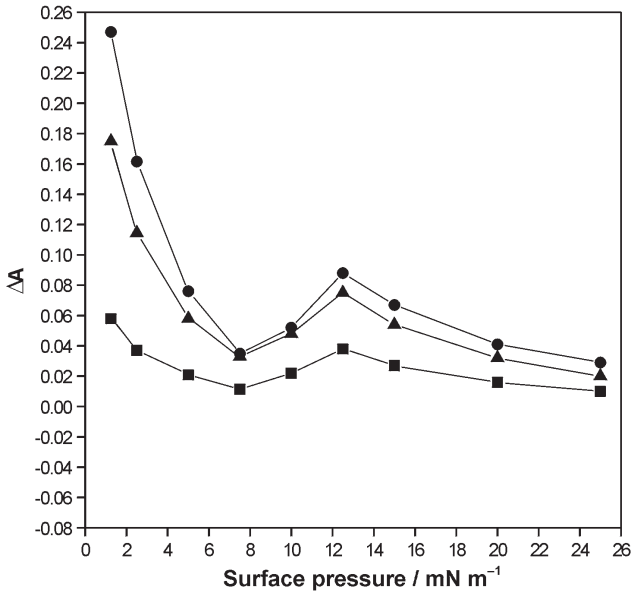

Fig. 3 Molecular area variation (ΔA) *vs*. surface pressure with increasing amounts of PGE in the subphase. The concentrations of PGE are ■ – $5 \cdot 10^{-7}$ M; ▲ – 10^{-6} M and ● – $2 \cdot 10^{-6}$ M

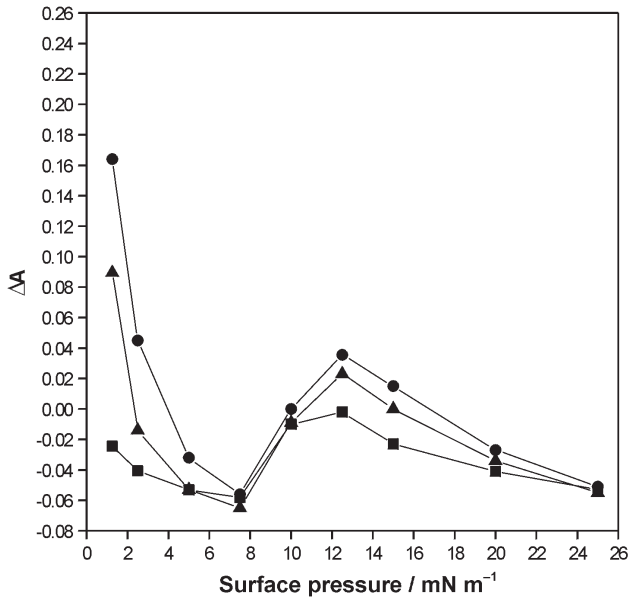

Fig. 4 Molecular area variation (ΔA) *vs*. surface pressure with increasing amounts of PGA in the subphase. The concentrations of PGA are ■ – $5 \cdot 10^{-7}$ M; ▲ – 10^{-6} M and ● – $2 \cdot 10^{-6}$ M

As the amount of PGA penetrated within the monolayer was shown to be approximately equivalent to that of PGE, the structural differences of these two types of prostaglandin are directly involved in the variations observed in the liquid-crystalline organization of the corresponding mixed films. Compared to PGA, the structure of PGE is composed with an additive hydroxyl group inducing higher polarity and solubility. These PGE properties enabled an incorporation thermodynamically stable within the lipid interface without any apparent modifications in the organization of the condensed lipidic monolayer. The ΔA negative values, from DPPC/PGA mixed films are indicative of a PGA condensing effect on the condensed monolayer. This condensation may be explained by the strong van der Waals attraction from the ordered acyl chains with the cyclopentenonic structure resulting in a compacting effect of PGA on the DPPC monolayer. For PGA concentration above $2.5 \cdot 10^{-7}$ M, each isotherm converged to a same molecular area value at 25 mN m^{-1}. This means that the effect of PGA on the densely packed film is not dependent of its concentration and it can be proposed that the presence of PGA induced some alterations in the lipidic lattice, which propagated in all the monolayer.

LE/LC phase transition

The presence of prostaglandin modified the profile of the DPPC isotherms so as the quasi-plateau region was no longer observable. Epifluorescence microscopy enabled

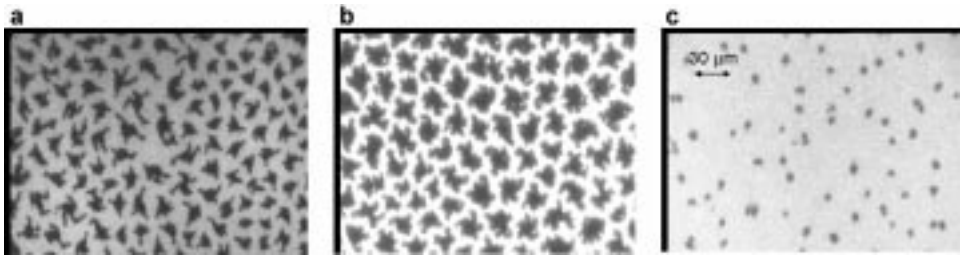

Fig. 5 Typical images of DPPC in the presence of prostaglandin. The monolayers contained 0.5 mol% NBD-C_6-HPC, and 99.5 mol% DPPC. All the micrographs were observed at a fixed molecular area of 0.54 nm^2. The light areas indicate the phases containing the fluorescent probe. a – represents a micrograph of a DPPC monolayer; b – represents a micrograph of a DPPC monolayer with $1.5 \cdot 10^{-6}$ M PGE injected in the subphase; c – represents a micrograph of a DPPC monolayer with $1.5 \cdot 10^{-6}$ M PGA injected in the subphase. The scale bar is: 30 μm

us to visualize lateral domain formation in pure lipidic films and mixed prostaglandin/DPPC films, in the molecular area range corresponding to the LE/LC phase transition of DPPC. The NBD fluorescent probe partitions preferentially into liquid expanded domains, giving a bright fluorescence from fluid phases, while it is excluded from more packed liquid condensed domains giving a darker appearance.

Figure 5 shows micrographs of DPPC with a prostaglandin concentration of $2 \cdot 10^{-6}$ M observed at a molecular area of 0.54 nm^2, corresponding to the middle of the classic LE/LC phase transition of DPPC. Micrograph 5-A showed classic patterns of dark condensed domains of DPPC coexisting with lightly expanded areas, representative of a first order transition. Figure 5b showed a micrograph of a mixed DPPC/PGE film. The dark domains were interdigitated and composed with a biggest core, compared to those of DPPC. Compared to the phase transition of DPPC, the beginning of the LE/LC transition was detected at higher molecular area, but all the transition took place in an equivalent molecular area interval. In the presence of PGA (Fig. 5c), condensed phase domains appeared as dark distorted circles. Interdigitated dark structures were observed at higher molecular areas during the transition. Furthermore, although the beginning of the LE/LC phase transition was detected at equivalent molecular areas than in DPPC films, the end of the transition was observed at lower molecular areas. This effect was PGA-concentration dependent.

These results showed that the LE/LC phase transition still exists in mixed DPPC/prostaglandin films. The presence of PGE in the liquid expanded phase compressed the DPPC, leading the LE/LC transition to begin at higher molecular area. The equivalent molecular area interval of the transition between DPPC and mixed DPPC/PGE films is in agreement with a lipidic organization, which is not modified by the presence of PGE. In DPPC/PGA film, the increase in the molecular area interval of the transition is coherent with the apparition of a new more condensed LC phase compared to that of DPPC.

LE phase

Comparing diagrams of pure DPPC films to those obtained on a subphase containing PGA molecules, the beginning of the LE phase was detected at higher molecular areas when the concentration of PGA was above $5 \cdot 10^{-7}$ M (Fig. 2B). The same observation was made with the π–*A* isotherms of PGE-containing films (Fig. 2A) with a more pronounced effect in the same experimental conditions. This increase was concentration dependent and can be explained by a prostaglandin incorporation within the DPPC film. The amount of prostaglandins incorporated is probably higher than in the LC phase. Indeed, LE phase lipid monolayers probably accommodate those soluble prostaglandins more effectively than highly ordered films in condensed liquid-crystalline phase.

Figure 3 shows the molecular area variation ΔA *vs*. surface pressure in the presence of PGE for a surface pressure range corresponding to the LE phase. For all the PGE concentrations studied, from $5 \cdot 10^{-7}$ M to $2 \cdot 10^{-6}$ M, ΔA remained positive and increased *vs*. concentration. In the presence of PGA (Fig. 4), the same variation profile was observed, but the minimum value are negatives. These ΔA negative values observed in the PGA/DPPC mixed film at the end of the LE phase, are indicative of an increase of the compressibility compared to pure DPPC films, as previously observed in the LC phase.

To investigate the contribution of an electrostatic effect from the PGA carboxylic group (p*Ka* 4.8), experiments were realized on a buffered subphase at pH 7.4 where prostaglandins exist predominantly as carboxylate anions. Injections of 10^{-7} M or 10^{-6} of PGA or PGE did not modify significantly the pattern of the isotherm curves. At pH 4, a larger percentage of prostaglandin is protonated. Using an acetate buffer subphase, isotherm diagrams in the presence of prostaglandin showed similar profile than those observed on pure water subphase. As previously observed for PGE [25], these experiments verified that the protonated form of both prostaglandins PGE and PGA were more able to incorporate lipidic films and that consequently electrostatic interactions of the carboxylate anion are not favourable to a penetration process.

Conclusions

Thermal analysis of prostaglandins on lipid films were performed in order to characterize if the amphiphilic properties of the cyclopentenonic derivatives could be involved in their ability to inhibit viral infection. The Langmuir-Blodgett technique associated with HPLC measurements enabled us to quantify the molar ratio of incorporated prostaglandins between 3 and 5% for each type of prostaglandin (PGA or PGE) for the highest prostaglandin concentration studied.

Surface pressure–molecular area isotherms and fluorescence microscopy were realized with increasing amounts of PGA or PGE injected in a water subphase of DPPC films. Our observations suggested that the incorporation of PGE within the DPPC monolayer had no noticeable effects on the organization of the liquid-crystalline phases. Their penetration induced the compression of the DPPC molecules, lead-

ing the isotherms to be shifted towards higher molecular area values. On the other hand, the incorporation of PGA seemed to increase the compressibility of each liquid-crystalline phase and induced a more condensed state of the lipidic organization for the highest surface pressure. It is known that cholesterol is also able to induce a condensing effect on the LC phase, but with a decrease in the LC phase compressibility. Furthermore this effect is observed at higher mole ratio (30% mole cholesterol/mole DPPC) compared to the few percents of PGA used in our experiments. A possible mechanism of action for the PGA could be an alteration effect in the liquid-crystalline lattice, which would induce a cooperative modification of the lipidic organization.

These results support our hypothesis of an amphiphilic contribution of the cyclopentenonic prostaglandin PGA in the antiviral process and open a new interesting way to investigate antiviral mechanisms.

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We thank Dr. P. Dansette (UMR CNRS 8601) for HPLC measurements, Dr. S. Bernard and Dr. M. Picquart (GRPB) for very helpful discussions.

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