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POLYMYXIN B-INDUCED PHASE SEPARATION AND ACYL CHAIN INTERDIGITATION IN PHOSPHATIDYLCHOLINE/PHOSPHATIDYLGLYCEROL MIXTURES

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Monolayers, fluorescence polarization, differential scanning calorimetry and X-ray diffraction experiments have been carried out to examine the effect of the polypeptide antibiotic polymyxin B on the phase behaviour of dipalmitoylphosphatidylglycerol (DPPG) either pure or mixed with dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC). It is shown that in both phosphatidylglycerol alone and phosphatidylglycerol/phosphatidylcholine mixtures, polymyxin B can induce either phase separation between lipid domains of various compositions or interdigitation of the acyl chains in the solid state, without segregation of the two lipids. Phase separation was observed by fluorescence and differential scanning calorimetry after addition of the antibiotic to vesicles composed of mixtures of DMPC and DPPG in conditions where polymyxin B did not saturate phosphatidylglycerol (DPPG to polymyxin B molar ratio, R_i, higher than 15). Phase separation was also observed in mixed monolayers of DPPC and of the 5:1 DPPG/polymyxin B complex, at high surface pressure. Acyl chain interdigitation was observed by X-ray diffraction in both 5:1 DPPG/polymyxin B mixtures and preformed 5:5:1 DMPC/DPPG/polymyxin B mixture, in which the antibiotic saturates phosphatidylglycerol (R_i 5). In both cases, raising the temperature gave rise to a complex double-peaked phase transition by differential scanning calorimetry, from the interdigitating phase to a normal L_α lamellar phase. As it is known that polymyxin B does not interact with phosphatidylcholine, the data presented show that, when phosphatidylcholine and phosphatidylglycerol are mixed together, a phase perturbation such as acyl chain interdigitation, which normally affects only phosphatidylglycerol, is also felt by phosphatidylcholine.

Introduction

Polymyxins are antibiotics isolated from various strains of *Bacillus polymyxa*, the activity of which is directed mainly against Gram-negative bacteria [1]. Polymyxins consist of one fatty acid residue attached through an amide bond to a linear tripeptide linked to a heptapeptide ring. The presence of five to six 2,4-diaminobutyric acid residues confers a net positive charge to these molecules. It is now well recognized that the primary site of action of these antibiotics is the bacterial membrane [1]. On a molecular scale, studies carried out with lipids in membrane models have revealed the marked preference of poly-

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Abbreviations: DPPG, dipalmitoylphosphatidylglycerol; DPPA, dipalmitoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; DPPC; dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; DPH, diphenylhexatriene.

myxin B for acidic phospholipids [1–7]. No interaction was found with the zwitterionic phosphatidylcholine [2–4]. Although the mechanism of these antibiotic-lipid interactions is not yet well understood, the binding of polymyxin B to acidic lipids has been shown to depend on both electrostatic and hydrophobic forces [1]. In the case of phosphatidylglycerol, monolayer studies suggest that the binding of polymyxin B to the lipid might occur by an initial electrostatic interaction between the peptide positive charges and the lipid negative charges at the interface, followed by the penetration of the antibiotic molecule into the lipid layer [4]. These interactions cause marked film expansion. At saturation, they achieve a very stable phosphatidylglycerol-polymyxin B complex in the molar ratio 5 : 1 [4]. In bilayer systems, a recent X-ray diffraction study has revealed that addition of polymyxin B to DPPG induces an interdigitation of the acyl chains of the lipid when in the gel state [8]. Polymyxin B has also been shown to interact with DPPA [5–7]. Nevertheless, no acyl chain interdigitation was observed with this lipid (Ranck, J.L., Theretz, A. and Tocanne, J.F., unpublished data). As membranes are composed of different species of neutral and charged phospholipids, the effects of polymyxin B on binary lipid mixtures is also to be considered. In this respect, polymyxin B has been shown to cause phase separation in phosphatidylcholine/phosphatidic acid mixtures [6]. Taking into account the very peculiar behaviour of phosphatidylglycerol in presence of polymyxin B, it was tempting to study the effects of the antibiotic on this lipid in the presence of phosphatidylcholine. These two lipids are known to occur in certain bacterial membranes [9], and to form very stable mixtures both in bilayer [10,11] and in monolayer [12] systems. In the present study, grounded on monolayer (π,ΔV), fluorescence polarization, differentatial scanning calorimetry and X-ray diffraction experiments, it is shown that both in phosphatidylglycerol and phosphatidylcholine/phosphatidylglycerol mixtures, polymyxin B can induce either a phase separation between lipid domains of various composition or an interdigitation of the acyl chains in the solid state, without segregation of the two lipids.

Materials and Methods

Chemicals

Dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine were purchased from Sigma (U.S.A.). Dipalmitoylphosphatidylglycerol ammonium salt was synthesized in this laboratory [13]. All these compounds were pure as considered by thin-layer chromatography. Polymyxin B sulphate was obtained from Sigma and 1,6-diphenyl-1,3,5-hexatriene from Fluka (Switzerland). Salts were of analytical grade.

Monolayer experiments

Compression isotherms were obtained by continuous compression of films using an automatic apparatus devised in our laboratory. The surface potential was measured in another set of experiments with an apparatus using two americium electrodes, the principle of which has been already described [14]. In both apparatuses, the surface pressure was measured with a platinum plate.

For both π and ΔV experiments, ultra-pure water from an industrial source (Motorola, Toulouse, France) was used. Lipids (with or without polymyxin B) were spread in the form of chloroform/methanol (5 : 1, v/v) solutions of known concentration prepared by weighing lipid samples dried under vacuum, prior to use. The experimental procedure was identical to that described elsewhere [15].

Throughout all experiments, the reference surface potentials of the aqueous phases were around 20–40 mV. Film compressions were reproducible to within 1% ($\pm 5 \cdot 10^{-3}$ nm^2), whereas the reproducibility of ΔV determinations was ± 10 mV. The data presented are the average of three to four experiments, carried out at a temperature of 20°C.

Fluorescence

Fluorescence depolarization of DPH embedded in lipid vesicles was measured as a function of temperature with a PFI apparatus [16]. The excitation wavelength was selected by means of an interference filter centered at 356 nm. Fluorescence emission was recovered through a cut-off filter transmitting light above 430 nm. Intensities were measured vertically (I_V) and horizontally (I_h) to

calculate the polarization rate:

$$p_n = \frac{I_v - I_h}{I_v + I_h}$$

The temperature was raised stepwise and was equilibrated at each step to within ± 0.1 K.

The lipids (2 mg) were dispersed in a 100 mM NaCl/1 mM phosphate buffer solution (10 ml) at pH 7.2, in the presence of the fluorescent probe at a concentration of $2.3 \cdot 10^{-6}$ M. In these conditions, the final probe to lipid molar ratio was about 1 : 100. The lipids were stirred by vortex for 2 or 3 min, then sonicated for 10 min at 80 kHz (sonicating bath). Under the electron microscope, the negatively stained suspensions [17] appeared to consist mainly of small multilamellar vesicles, about 60–100 nm in diameter. Polymyxin B was added to the lipid suspensions at concentrations such as to achieve the desired antibiotic-to-lipid molar ratio. Each sample was sonicated again for 2 min and allowed to equilibrate 3 h at 20°C before the fluorescence polarization experiments. It was checked that the absorbance of these various lipid suspensions (in the presence or in the absence of polymyxin B) did not exceed 0.15, at the used excitation wavelength of 356 nm. It should be noted that the ionic conditions used in these experiments allowed complete ionization of phosphatidylglycerol [15] without preventing it interacting with polymyxin B [4].

Differential scanning calorimetry

Calorimetric experiments were carried out on a Dupont 990 differential calorimeter thermal analyzer equipped with a differential scanning calorimeter 910 head, at scanning rates of 2–5 K/min. The lipids (0.1 mg) were weighed directly in the pans and hydrated with 10 µl pure water or 100 mM NaCl solution, at pH 5.6. Note that under these conditions, phosphatidylglycerol is fully ionized [13]. The pans were sealed and each sample was allowed to equilibrate above the phase transition temperature of the lipid, for 1 h. As usual, each sample was repeatedly scanned until reproducible thermograms were obtained. No hysteresis was observed.

X-ray diffraction

X-ray diffraction experiments were carried out on lipid samples hydrated with pure water. The experimental procedure and the interpretation of the low-and high-angle diffraction patterns have been already reported [18]. For both DSC and X-ray diffraction experiments, the various DPPG/polymyxin B and DMPC/DPPG/polymyxin B mixtures used in this study were prepared as previously described [8] by precipitation of the peptido-lipid complexes from acetone after the different mixture components had been intimately mixed by dissolution in a minimum volume of a chloroform/methanol (1 : 1, v/v) solution.

Results

Monolayer experiments

In these experiments, the effect of polymyxin B was tested on DPPG/DPPC mixtures, under

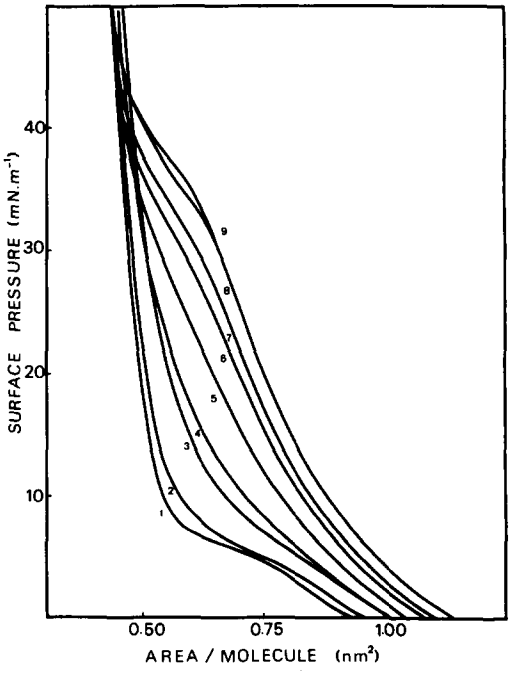

Fig. 1. Compression isotherms (recorder traces) for mixed monolayers of dipalmitoylphosphatidylcholine and 5 : 1 dipalmitoylphosphatidylglycerol-polymyxin B complex. Curves were calibrated as if the lipids were alone. Mole fraction of phosphatidylcholine: 1.0 (1); 0.9 (2); 0.8 (3); 0.7 (4); 0.5 (5); 0.3 (6); 0.2 (7); 0.1 (8); 0. (9). Subphase was 10 mM NaCl, pH 5.6, 20°C.

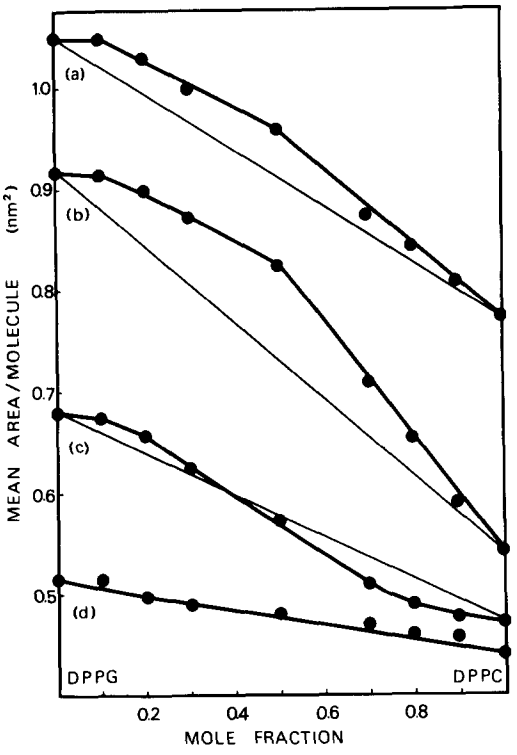

Fig. 2. Plots of mean area per molecule versus mole fraction for dipalmitoylphosphatidylcholine mixed with a dipalmitoylphosphatidylglycerol-polymyxin B complex, at various surface pressures, π. $\pi = 4$ mN·m^{-1} (a); 10 mN·m^{-1} (b); 30 mN·m^{-1} (c); 40 mN·m^{-1} (d). Subphase was 10 mM NaCl, pH 5.6, 20°C.

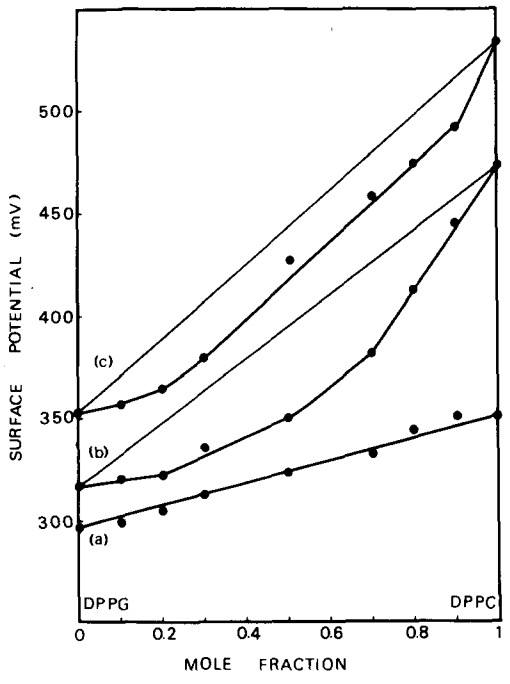

Fig. 3. Changes in surface potential versus mole fraction for dipalmitoylphosphatidylcholine mixed with 5 : 1 dipalmitoylphosphatidylglycerol-polymyxin B complex, at various surface pressures, π. $\pi = 4$ mN·m^{-1} (a); 10 mN·m^{-1} (b); 40 mN·m^{-1} (c). Subphase was 10 mM NaCl, pH 5.6, 20°C.

experimental conditions ensuring a constant DPPG to antibiotic molar ratio, R_i of 5 : 1. As previously reported, preparation consisted of mixing together the lipids and the antibiotic in the same syringe and spreading the mixture on the subphase [4]. Fig. 1 shows the various compression isotherms which were obtained on a 10 mM NaCl subphase, at pH 5.6. All these curves were plotted in terms of molecular area as if the lipids were alone [4]. Curve 1 corresponds to DPPC alone and curve 9 to the 5 : 1 DPPG/polymyxin B mixture. Curve 9 illustrates the considerable film expansion which results from the interaction of the antibiotic with the phospholipid [4]. The mean area per molecule, measured on the various π/A curves of Fig. 1, at constant surface pressure, are plotted in Fig. 2 versus the lipid mole fraction. In such phase diagrams, any straight line is to be considered as representing either ideal mixing or phase separation, while any deviation from linearity is indicative of interactions between the molecules within the film. In this diagram, linearity was obeyed only at high surface pressure ($\pi = 40$ mN · m^{-1}, curve d) with lipids in the condensed state.

Surface potentials measured on the same mixed films negatively deviated from linearity (Fig. 3) except at low surface pressure ($\pi = 4$ mN · m^{-1}, curve a) where linearity was obeyed. Nevertheless, this linear plot turned out to deviate positively after expressing the surface potential in terms of $\Delta V/N$ which corrects for changes in lipid surface density [19]. Only curve c, at $\pi = 40$ mN · m^{-1}, remained unchanged in shape after this conversion. Two breaks can be observed in this curve for DPPC mole fractions of 0.2 and 0.9. As breaks in the slope can be understood as phase boundaries [20,21], this curve and curve a in Fig. 2 suggest partial immiscibility of DPPC and the 5 : 1 DPPG-polymyxin B complex in monolayers.

Fluorescence polarization experiments

The influence of polymyxin B was tested on mixtures of DMPC and DPPG. As shown in Fig. 4 (curve a), DPPG alone exhibited a phase transition centered at 41°C, which is the usual value reported for this lipid when fully ionized [13] and in the presence of Na^+ as a counter ion. As expected [22], DMPC displayed a sharp phase transition centered at 24°C (curve not shown). DMPC/DPPG mixtures, in 3:1 and 1:1 molar ratios, exhibited phase transitions centered at 28°C (Fig. 4, curve c) and 31°C (Fig. 5, curve a), respectively.

In agreement with Sixl and Galla [6], addition of polymyxin B to DPPG liposomes lowered the lipid phase transition temperature. This is exemplified by curve b in Fig. 4, which was obtained for a lipid to antibiotic molar ratio, R_i, of 20:1. Two phase transitions were observed, one at about 35°C and one at 42°C. As already suggested [6], the lower phase transition at 35°C can be interpreted as the melting of polymyxin-lipid domains while the upper phase transition at 42°C would account for the melting of non-bound lipids. Similar results were obtained for an R_i of 15 (curve not shown). Note that this was the highest polymyxin B concentration which could be used. Attempts to increase the antibiotic concentration resulted in precipitation of the whole system.

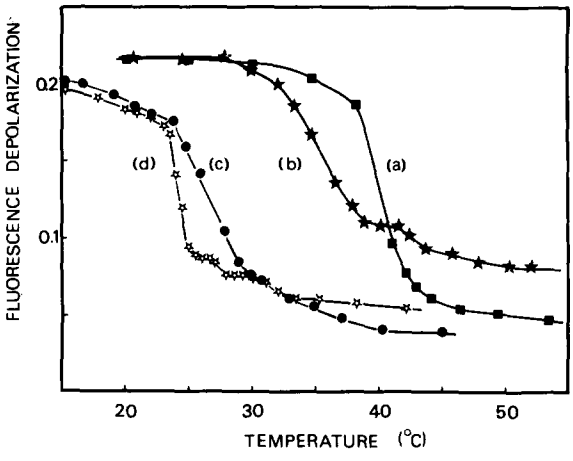

Fig. 4. Changes with temperature of the fluorescence polarization rate of diphenylhexatriene embedded in vesicles of dipalmitoylphosphatidylglycerol (a,b) and in vesicles of a 3:1 dimyristoylphosphatidylcholine/dipalmitoylphosphatidylglycerol mixture (c,d) before (a,c) and after (b,d) the addition of polymyxin B at a final antibiotic-to-phosphatidylglycerol molar ratio of 1:20. Aqueous phase was 100 mM NaCl/1 mM phosphate, pH 7.2.

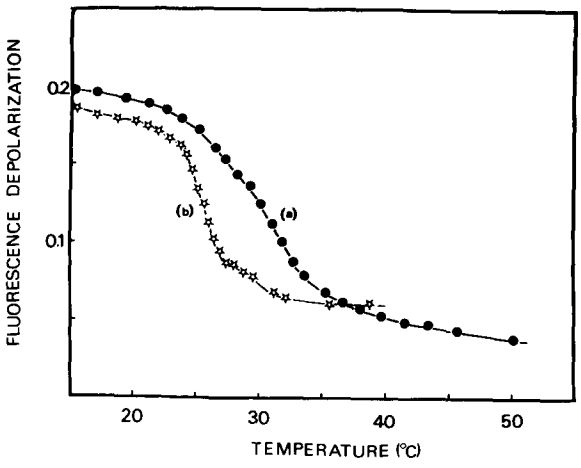

Fig. 5. Changes with temperature of the fluorescence polarization rate of diphenylhexatriene embedded in vesicles composed of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylglycerol at a molar ratio of 1:1 before (a) and after (b) the addition of polymyxin B at a final antibiotic-to-phosphatidylglycerol molar ratio of 1:20. Aqueous phase was 100 mM NaCl/1 mM phosphate, pH 7.2.

Addition of polymyxin B to 3:1 and to 1:1 DMPC/DPPG mixtures, at a final R_i of 20 with respect to phosphatidylglycerol, shifted the phase transition temperature to 24.3°C (Fig. 4, curve d) and 25.2°C (Fig. 5, curve b), respectively. Both these temperatures are very close to the value of 24°C found for dimyristoylphosphatidylcholine alone. Although less pronounced, a second transition was detectable in each curve at temperatures of about 31 and 30°C, respectively. It has been demonstrated that polymyxin B does not interact with phosphatidylcholines [2–4]. Therefore, the appearance of two phase transitions (curve d in Fig. 4 and curve b in Fig. 5) upon addition of the antibiotic to the mixed lipid membranes strongly suggests a phase separation between phosphatidylcholine enriched domains and the phosphatidylglycerol molecules interacting with polymyxin B.

Similar results were obtained for a phosphatidylglycerol-to-antibiotic R_i of 10, the lowest we could use in this case without lipid precipitation (curves not shown).

Differential scanning calorimetry

Influence of polymyxin B on DPPG alone. In agreement with a previous report [13], DPPG ammonium salt in excess water exhibited a main transition peak at temperature of 44.5°C (Fig. 6, curve a). As reported earlier [8], X-ray diffraction experiments showed that addition of polymyxin B to DPPG at a final R_i of 5 (saturating conditions) induces interdigitation of the hydrocarbon chains of the lipid, when in the gel state. A transition from the interdigitating phase to a lamellar L_α phase was detected at a temperature of about 40°C for hydrated samples (lipid/sample concentration, c, of 0.70) [8]. In the microcalorimeter, the interdigitating 5 : 1 DPPG-polymyxin B complex, in excess water, displayed two peaks: at temperatures of 42.2 and 44.5°C (Fig. 6, curve c). Such behaviour did not depend on the way the complex was prepared. Indeed, the same thermogram was recorded for the precipitate which was obtained after addition of polymyxin B to a water suspension of DPPG, in a final 1 : 5 molar ratio.

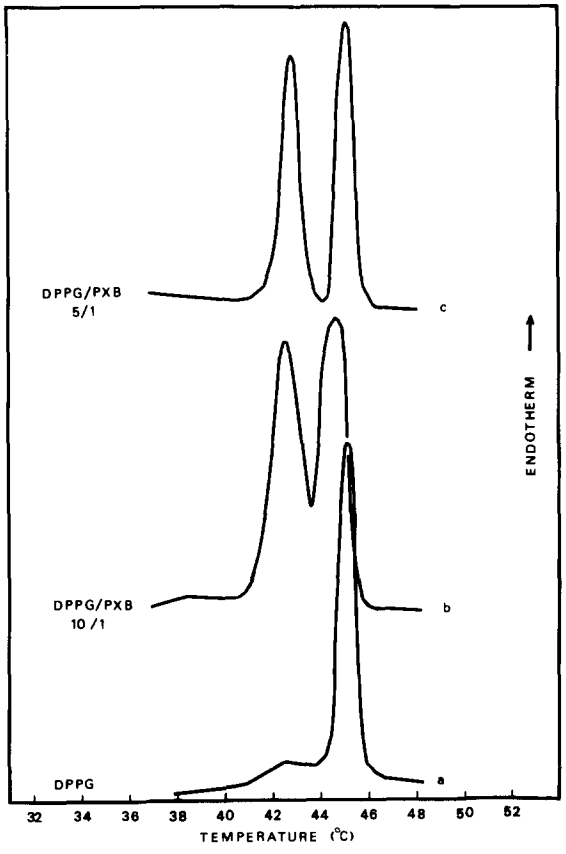

Fig. 6. Differential scanning calorimetric curves of dipalmitoylphosphatidylglycerol in the absence (a) and in the presence of polymyxin B, at lipid-to-antibiotic molar ratios of 10 : 1 (b) and of 5 : 1 (c). Lipids were hydrated as specified in Materials and Methods.

For hydrated DPPG/polymyxin B mixtures with an R_i value higher than 5 (non-saturating conditions), X-ray diffraction data showed phase separation between two lamellar phases: one with stiff and interdigitated hydrocarbon chains and the other where the conformation of the hydrocarbon chains were of type β [8]. These two lamellar phases can be reasonably assumed to correspond to the 5 : 1 DPPG-polymyxin B complex and to free DPPG, respectively. The 10 : 1 DPPG/polymyxin B mixture which showed such a phase separation [8] was tested with differential scanning calorimetry. In excess water, this mixture exhibited a thermogram (Fig. 6, curve b) similar to that of the 5 : 1 DPPG-polymyxin B complex, with two peaks, one at 41.5°C and the other at 44°C. Such a similitude between the two thermograms stems from the fact that the second peak recorded for the 5 : 1 DPPG-polymyxin B complex is nearly superimposable to the peak recorded for DPPG alone.

Influence of polymyxin B on DMPC/DPPG 1 : 1 mixtures For DMPC/DPPG 1 : 1 mixtures in excess water, a phase transition was observed at a temperature of 31.5°C (curve a, Fig. 7) nearly identical to that at 31°C measured by fluorescence polarization on vesicles of the same lipid mixture (curve a, Fig. 5). The same thermogram was recorded after hydration of this lipid mixture with a 100 mM NaCl solution (curve not shown). On the other hand, and in contrast to the fluorescence polarization data of Fig. 5, 20 : 20 : 1 DMPC/DPPG/polymyxin B mixtures (polymyxin B not saturating DPPG) in excess water showed a phase transition at 35.4°C (curve b, Fig. 7).

As the 5 : 1 DPPG-polymyxin B complex, preformed 5 : 5 : 1 DMPC/DPPG/polymyxin B mixtures (polymyxin B saturates DPPG), in excess water, showed a well-defined double peak (Fig. 7, curve c), at temperature of 35.5°C and 39°C, respectively.

In neither thermogram b nor c was a transition peak detected at 25°C, where phase transition of

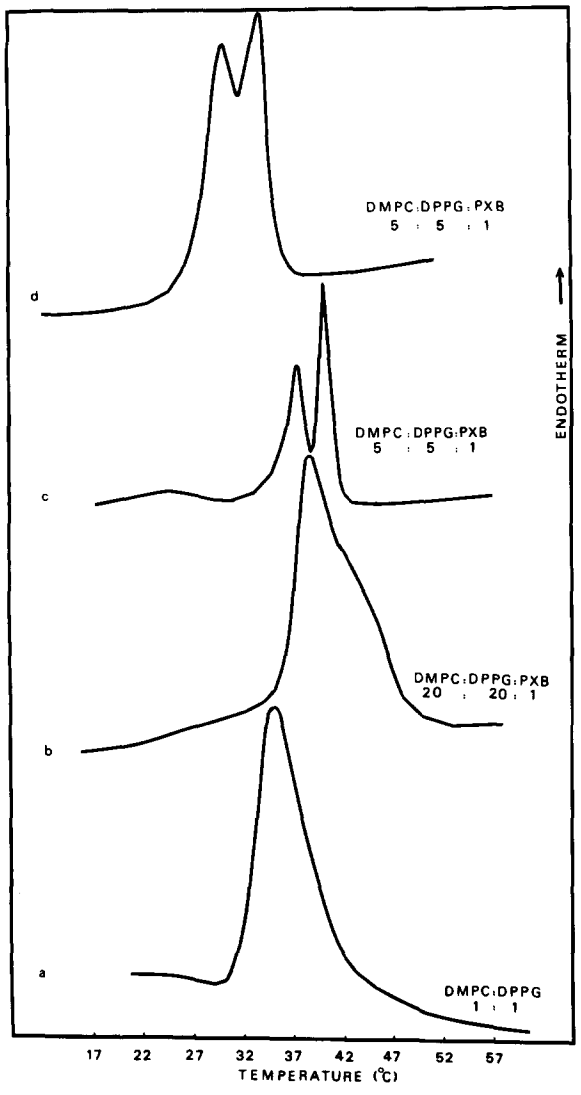

Fig. 7. Differential scanning calorimetric curves of 1:1 dimyristoylphosphatidylcholine/dipalmitoylphosphatidylglycerol mixtures in the absence (a) and in the presence of polymyxin B at lipid-to-antibiotic molar ratios of 20:20:1 (b) and 5:5:1 (c,d). Curves (b) and (c) refer to hydrated preformed mixtures. Curve (d) refers to the precipitate which was obtained after addition of polymyxin B to a water suspension of the mixed lipids.

DMPC alone is expected to occur. This strongly suggests that in these preformed 20:20:1 and 5:5:1 DMPC/DPPG/polymyxin B mixtures, no phase separation occurred.

Curve d in Fig. 7 concerns the precipitate which was obtained after addition of polymyxin B to a water suspension of a 1:1 DMPC/DPPG mixture, to a final polymyxin B-to-DPPG molar ratio of 1:5. In this case, two peaks were still observed, but at temperatures of 27°C and 30–31°C. These values were similar to those at 25°C and 30°C which were obtained by fluorescence polarization on water dispersions of the same lipid mixture and after addition of polymyxin B to a final polymyxin B-to-DPPG molar ratio of 1:20 or 1:10 (curve b, Fig. 5).

X-ray diffraction experiments

Hydrated preformed 5:5:1 DMPC/DPPG/polymyxin B mixtures ($c = 0.86$) appeared to exist in a rigid lamellar phase of type β at temperatures below 30°C, with a lamellar repeat distance, d, of 3.95 nm and in a liquid lamellar phase of type L_α at temperatures above 60°C ($d = 4.3$ nm). That chain melting occurred at a higher temperature in this case than in the microcalorimeter is due to the low water content of the lipid samples used in X-ray diffraction experiments.

From 40°C to 0°C, the organization of the chains was of type p6 and below −5°C, of type cmm. In contrast, 1:1 DMPC/DPPG mixtures at similar water concentration ($c = 0.80$) were found in a rigid lamellar L_β phase at temperatures below 35°C with a lamellar repeat distance, d, of 6.52 nm and in a liquid lamellar L_α phase at temperatures above 60°C ($d = 5.07$ nm). Similar results, in terms of chain organization, were obtained for more hydrated lipid samples ($c = 0.60$). In both cases, and for lipids in the gel state ($t < 30$°C), the lamellar repeat distance, measured for 5:5:1 DMPC/DPPG/polymyxin B mixtures, appeared to be about 2.4 nm less than the lamellar repeat distance found for 1:1 DMPC/DPPG mixtures. In addition to the temperature-induced two-dimensional chain organization changes (from p6 to cmm space group), this observation demonstrates that in preformed 5:5:1 DMPC/DPPG/polymyxin B mixtures, when in the gel state, both phosphatidylcholine and phosphatidylglycerol exist in a lamellar phase in which the hydrocarbon chains are interdigitated [8,18].

Discussion

The above results reveal two completely different behaviours of both DPPG alone and DMPC/

DPPG mixtures in the presence of polymyxin B. Addition of the antibiotic to these lipids causes either a phase separation between lipid domaines of various composition or interdigitation of the lipid acyl chains without phase separation.

In agreement with Sixl and Galla [6], phase separation between phosphatidylglycerol molecules bound to polymyxin B and non-bound lipids was observed after addition of the polypeptide to water suspensions of the lipid, in non saturating conditions ($R_i = 20$ and 15, Fig. 4). A similar conclusion was reached in the case of polymyxin B interacting with dipalmitoylphosphatidic acid [5–7]. For both lipids, the antibiotic causes a decrease of the phase transition temperature: about 7 K for DPPG and 20 K for DPPA [5]. In contrast, addition of the antibiotic to DPPG, in saturating conditions, leads to a precipitation of the lipid (associated with the polypeptide) in the form of a lamellar phase in which the hydrocarbon chains, when in the gel state, are interdigitated [8]. Upon raising the temperature, this interdigitating phase turns to a lamellar L_α phase [30], with a double-peaked endothermic phase transition, at temperatures of 42°C and 44°C, very close to the phase transition temperature of 44°C found for the lipid alone (Fig. 6). Addition of polymyxin B in saturating conditions (R_i 2.5) to dipalmitoylphosphatidic acid also precipitated the lipid. But in this case, no lamellar phase with interdigitated acyl chains was observed by X-ray diffraction (Ranck, J.L., Thèretz, A. and Tocanne, J.F., unpublished data). From a structural point of view and at the molecular level, these observations suggest different types of interaction of polymyxin B with acidic lipids. Two models have already been proposed to describe the phospholipid-antibiotic complexes [4,5,7]. In the first, polymyxin B is adsorbed at the surface of the lipid layer with only its hydrophobic hydrocarbon tail penetrating between the lipid molecules [5,7]. According to the second one, the polypeptide would penetrate more deeply the lipid layer [4]. This last model stems from the large film expansions which are observed after addition of polymyxin B to phosphatidylglycerol monolayers (Fig. 1 and Ref. 4) and which account for a destabilization of the interactions between the lipid acyl chains. This might explain the observed decrease of the phase transition temperature of the lipids in the presence of the antibiotic. At the present time, one cannot discriminate between the two models and different possibilities still have to be envisaged: either polymyxin B interacts with acidic phospholipids in different ways to achieve at least two structurally different molecular complexes, or only one kind of molecular complex is formed. The latter might be organized in either normal lamellar phases or in an interdigitating phase, depending on the lipid-to-antibiotic molar ratio, R_i, on the nature of the lipid polar head and on the temperature. As a preliminary result (Thèretz, A. and Tocanne, J.F., unpublished data), the ^{31}P-NMR spectrum obtained for large multilamellar vesicles of DPPG in 100 mM NaCl (pH 5.6), characteristic of a lipid in a lamellar phase [23], remained practically unchanged after addition of polymyxin B to the lipid suspension, even at saturation ($R_i = 5$). According to Thayer and Kohler [24]], this observation suggest that the interaction of the antibiotic with the lipid does not affect the orientation of its polar head.

Despite a remarkable tendency of phosphatidylcholine and phosphatidylglycerol to be miscible whatever the ionic conditions in the aqueous phase [10–12], the present study shows that addition of polymyxin B to water suspensions of DMPC/DPPG mixtures causes a phase separation between DMPC-enriched domains and DPPG molecules interacting with the antibiotic. Phase separation occurs in non-saturating ($R_i = 20$, fluorescence, Figs. 4 and 5) as well as in saturating ($R_i = 5$, DSC, Fig. 7) conditions of the antibiotic with respect to DPPG. Although in a less direct manner, data of Figs. 2 and 3 also suggest partial immiscibility of DPPC and the 5 : 1 DPPG-polymyxin B complex in monolayers, at high surface pressure. Polymyxin B-induced phase separations have already been reported for DPPC/DPPA mixtures [6]. Such an effect does not seem to be specific for this polypeptide. Other cationic lytic polypeptides such as mellitin and cardiotoxin have been shown to induce phase separations within various phospholipid mixtures composed of neutral and acidic lipids. As with polymyxin B [25,26], these polypeptides interact with acidic lipids by means of both electrostatic and hydrophobic forces.

In contrast to the above, it should be noted that in preformed 5 : 5 : 1 and 20 : 20 : 1 DMPC/

DPPG/polymyxin B mixtures, no phase separation was found. Instead, X-ray diffraction data demonstrate that in 5 : 5 : 1 DMPC/DPPG/polymyxin B mixtures in the solid state, both phosphatidylcholine and phosphatidylglycerol exist in a lamellar phase where the hydrocarbon chains are interdigitated.

The fact that interdigitation involves both lipids is supported by the DSC data of Fig. 7. Like the interdigitated 5 : 1 DPPG-polymyxin B complex, the 5 : 5 : 1 DMPC-DPPG-polymyxin B complex exhibits a double-peaked thermogram. In this case, phase transition starts at a temperature of about 35°C, which is higher than the temperature of 31°C found for the same mixed lipids in the absence of the antibiotic. This observation allows us to rule out the possibility of a phase separation between the two lipids after the interdigitating phase has been induced by the polypeptide. The same conclusion applies to the preformed 20 : 20 : 1 DMPC/DPPG/polymyxin B mixtures (curve b, Fig. 7) in which it is likely that part of the lipid molecules exists in the interdigitating phase. Altogether, and as a first conclusion, these data suggest that polymyxin B-induced phase separations occur only in binary lipid mixtures which are initially in a normal lamellar phase (or in monolayers).

As lateral phase separation depends on, among other things, the lateral diffusion rate of the various mixture components, this observation suggests a substantial restriction of the lateral motion of the lipids in the interdigitating phase compared to the normal lamellar phase.

As already mentioned, polymyxin B does not interact with phosphatidylcholine. Therefore, the second conclusion arising from our results is that in a binary lipid mixture, a phase perturbation which effects only one of the lipid species can be transmitted to the other one. These observations also suggest the existence of strong cohesive forces between phosphatidylcholine and phosphatidylglycerol molecules, after they have been cemented in the interdigitating phase by the cationic polypeptide.

Finally, we should mention the nature of the double-peaked main transition which seems to be a characteristic feature of lipids when in the interdigitating phase (Figs. 6 and 7). Such a point has already been questioned for lipids postulated to exhibit acyl chain interdigitation and which also display a double-peaked endothermic phase transition by DSC. This is the case for mixed-chain phosphatidylcholines in so far as their two acyl chains differ by at least four carbon atoms [27,28]. This is also the case for DPPG when interacting with the basic protein from myelin [29]. No satisfactory interpretation of this phenomenon has been developed so far. It has been tentatively suggested that the two transition peaks might arise from coexisting regions of interdigitated and non-interdigitated phosphatidylcholines within the bilayer gel phase [27,28]. Preliminary X-ray diffraction data (Ranck, J.L., Théretz, A. and Tocanne, J.F., unpublished data) concerning 5 : 1 DPPG/polymyxin B and 5 : 5 : 1 DMPC/DPPG/polymyxin B mixtures, in the temperature domains where phase transitions occur, point to the existence of an intermediate complex phase. This phase could be thought of as the superposition of the rigid interdigitating phase, of the liquid L_α phase and of the L_γ or P_γ phases. In any case, the relationship between DSC and X-ray diffraction data is far from clear and further work is necessary to elucidate this point. This problem is currently under investigation in our laboratories.

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