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Adjuvant lipopeptide interaction with model membranes

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Abstract

The cationic lipohexapeptide $Pam_3Cys–Ser–(Lys)_4$ is a synthetic model for the triacylated N-terminal part of bacterial lipoproteins, and it is used as an adjuvant and macrophage activator. The amphiphilic lipopeptide was injected below a phosphatidylserine monolayer at the air–water interface. It interacted with the interface, as seen by a decrease in the surface potential (ΔV), and it was inserted in the monolayer, until surface charge neutralization was reached, as seen by the parallel increases of ΔV and of the surface pressure. No insertion occurred above 29 mN/m. The interaction kinetics was sensitive to ionic strength and to the nature of acidic phospholipids and of their acyl chains, but the final equilibrium was independent of these factors. Addition of the lipopeptide to large unilamellar vesicles (LUVs) induced their aggregation, and an exchange of lipids between fluorophor-labelled and non-labelled LUVs. However, no fusion was observed, just as reported for polylysine. The lipopeptide strongly inhibited calcium-induced fusion of PS LUVs, in contrast to the published effect of polylysine. This was probably due to inhibition of calcium fixation on liposomes, since it was observed that the lipopeptide efficiently displaced $^{45}Ca^{2+}$ from a PS monolayer. In addition, a phospholipid segregation was observed in SUVs for a few ten micromolar of the lipopeptide. © 1998 Elsevier Science B.V.

Keywords: Lipopeptide; Adjuvant; Fusion inhibition; Calcium competition

1. Introduction

Lipoproteins present in the cytoplasmic membrane or in the outer envelopes of Gram positive and negative bacteria share the same N-terminal *S*-glyceryl-cysteine residue bearing three fatty acyl chains: one is amide-linked and the two others are ester-linked to the 2,3-dihydroxypropyl residue attached to the sulphur atom of cysteine. The first bacterial lipoprotein described was Braun's lipoprotein [1], present in the outer membrane of *E. coli*. It has been shown that this lipoprotein stimulates B-lymphocytes [2]. Lipoproteins from other bacteria

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPA, 2,6-pyridenedicarboxylic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPX, *p*-xylene bispyridinium bromide; LUV, large unilamellar vesicle; MOPS, 4-morpholine propanesulfonic acid; NBD, 7-nitro-2-1,3-benzoxadiazol; NBD–PC, 1-acyl,2-(6-NBD-hexanoyl)-3-glycerophosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PE–NBD, egg-PE substituted by NBD on the amino group; PG, phosphatidylglycerol; PS, phosphatidylserine; SUV, small unilamellar vesicle; TNS, 2-(*p*-toluidino)naphthalene sulfonic acid

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were found to be major immunogens, for instance that from *Treponema pallidum* or from *Borrelia burgdorferi* [3,4].

It appeared that the acylated N-terminal moieties were responsible for the activity of these lipoproteins on B-cells of the immune system [5–7]. Subsequently, many lipopeptide analogues have been synthesized and tested, in order to obtain immunostimulating molecules easier to prepare than the bacterial lipoproteins [8,9]. Very few biophysical studies have been performed with such molecules: the molecular area of anionic lipopeptides at the air–water interface has been determined [10], and it has been shown that neutral and cationic lipopeptides have an affinity for liposomes and cell membranes [11].

One of the most promising synthetic lipopeptides, due to its biological activity and to its high water solubility, bears three palmitic acid residues on its N-terminal hexapeptidic moiety Cys–Ser–$(Lys)_4$. It can be abbreviated Pam_3Cys–Ser–$(Lys)_4$ (Fig. 1) and it will be designated below as the ''lipopeptide''. This cationic lipopeptide has been shown to activate macrophages and B-lymphocytes [12], to stimulate tyrosine phosphorylation in human myeloid cells [13], to activate superoxide formation in human neutrophils [14] and HL-60 leukemic cells [15]. The lipopeptide is clearly amphiphilic with three palmitic acid residues and three net positive charges. Nothing is known of its interaction with membrane lipids, especially with anionic lipids, an interaction that can be at the origin of some of the pleiotropic effects observed with this lipopeptide.

In the present work we studied the interaction of the lipopeptide with phospholipids in two model systems, i.e. monolayers at the air–water interface and large unilamellar vesicles (LUVs). Interactions with phosphatidylserine, the most abundant anionic phospholipid in animal cells, were examined. Our results are discussed with respect to literature data on oligo- and poly-lysine peptides interacting with anionic phospholipids. This comparison underscores the major influence of acyl chains of the lipopeptide on its properties.

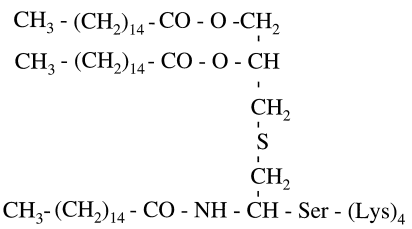

Fig. 1. Structure of the cationic lipohexapeptide Pam_3Cys–Ser–$(Lys)_4$.

2. Materials and methods

2.1. Chemicals

Bovine brain phosphatidylserine (saturated/unsaturated acyl chains = 0.95), dipalmitoyl-*sn*-glycero-3-phosphoserine, egg phosphatidylcholine and egg-derived phosphatidylgycerol (saturated/unsaturated acyl chains = 0.80), 1-palmitoyl,2-oleyl-3-phospho-*sn*-glycerophosphoglycerol (POPG), 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) called PE–NBD, phosphatidylethanolamine-*N*-(lissamine rhodamine β-sulfonyl) called PE–rhodamine and 1-acyl,2-(6-NBD-hexanoyl)-*sn*-glycero-3-phosphocholine called NBD–PC, were obtained from Avanti Polar Lipids (Alabaster, USA). 4(5)-carboxyfluorescein was obtained from Fluka (Buchs, Switzerland). Terbium (III) chloride hexahydrate and 2,6-pyridinedicarboxylic acid (DPA), 2-(*p*-toluidino)naphthalene-6-sulfonic acid (TNS), fluorescamine, trilysine (Lys_3) and 3-(*N*-morpholine)propanesulfonic acid (MOPS) were obtained from Sigma-Aldrich (St. Quentin, France). 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylene bispyridinium bromide (DPX) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes (Eugene, USA). *S*-(2,3)bis-(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-Cys–Ser–Lys_4–OH (Pam_3Cys–Ser–Lys_4) was obtained from Boehringer Mannheim-France (Meylan). ^{45}Ca (45 GBq/mmol) was obtained from Amersham-France (Les Ullis). Stock solutions (1 mM) of the lipopeptide were prepared in ultrapure water. Organic solvents were glass-distilled and stored in glass containers. Water phases for monolayers were prepared with ultrapure water (MilliQ, Millipore). Carboxyfluorescein was purified by ethanol precipitation and filtration through a Sephadex LH20 column [16].

2.2. Synthesis of the NBD derivative of the lipopeptide, $Pam_3Cys–Ser–(Lys)_3–Lys(NBD)–OH$ (called lipopeptide–NBD)

Resin and reagents were purchased from Calbiochem-Novabiochem (Bad Soden, Germany). Solvents were obtained from Fluka and *N*-palmitoyl-*S*-2,3-bis(palmitoyloxy)-(2*RS*)propyl-(*R*)-cysteine (i.e. $Pam_3Cys–OH$) was synthesized as described previously [17]. The lipopeptide was built up using the fluorenylmethoxycarbonyl (Fmoc) protocol for solid phase synthesis on an Applied Biosystems model 433A automated synthesizer. A Wang-PHB-resin loaded with Dde-protected Fmoc–lysine residue was used as solid support. Resin substitution was 0.6 mml/g and 0.1 mmol of aminoacid was used for each coupling. The following side chain protecting groups were employed: Lys (*t*-butoxy), Lys (1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl) and Ser (*t*-butyl). Aminoacids were coupled using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and hydroxybenzotriazole (TBTU/HOBt). $Pam_3Cys–OH$ was coupled in double excess to the resin-bound *N*-5mer-peptide with diisopropylcarbodiimide/HOBt in dimethylformamide/dichloromethane (1:2) for 12 h [18]. The Dde-protecting group was selectively removed by hydrazine (5% in dimethylformamide). Then 7-chloro-4-nitrobenz-2-oxa-1,3-diazole in dimethylformamide was coupled in 10 fold excess to the C-terminal lysine deprotected in the lysine side chain. The peptide and all protecting groups were cleaved from the resin with TFA containing phenol (5%), ethanedithiol (5%) and water (7%). The synthesis was monitored by electrospray ionisation mass spectrometry on a triple quadrupole instrument API III TAGA X (PE Sciex, Thornhill, Canada).

2.3. Monolayers at the air–water interface

Compression isotherms were obtained as previously described, using a Wilhelmy platinum plate to determine surface pressure with an apparatus constructed in the laboratory [19]. Briefly, 10 μl of the lipid solution ($CHCl_3/CH_3OH$, 7/3 v:v) was carefully deposited on the water phase, and a 15 min lag time was observed for solvent evaporation. A 5 mM MOPS, pH 7.4 buffered water phase was used. The compression isotherms presented are the average of at least three runs. They were reproducible within 0.04 nm^2/mol.

Insertion of the lipopeptide into phospholipid monolayers and detection of $^{45}Ca^{2+}$ at the interface were performed in a teflon trough with a fixed area (16 cm^2) and a 10 ml subphase (5 mM MOPS, pH 7.4 for lipopeptide insertion; 1 mM NaCl, pH 6.8 for Ca^{2+} detection). Films at the chosen surface pressures were obtained by successive molecule deposits. Solutions were introduced underneath the monolayer with a syringe through a side hole. The lipopeptide concentration of 7 μM in the water phase was chosen in order to reach the surface pressure plateau within 1 h with all the anionic phospholipids used to prepare monolayers. There was slow magnetic stirring of the water phase throughout the experiment.

2.4. Liposomes

Large unilamellar vesicles (LUV) were prepared by the reverse-phase method [20], except when otherwise stated for specific experiments. Briefly, lipids were dissolved in 1 volume of peroxide-free diethyl ether and 0.3 volume of buffered saline solution, calculated to obtain a 10 mM final lipid concentration, were injected rapidly. After brief sonication (in a bath), ether was removed using a rotatory evaporator at 350 mbar and 45°C, until gel formation. The gel was collapsed by a brief vortex mixing, and evaporation was continued at 150 mbar for 5 min. Then 0.7 volume of buffer saline was added and evaporation resumed for 20 min to eliminate residual diethyl ether. To achieve uniform size distribution, vesicles were extruded through a polycarbonate membrane (0.6 and 0.1 μm – Avanti Polar Lipids Mini-extruder system). Non-entrapped probes were eliminated on a Sephadex G50 column. The final phospholipid content of liposomes was calculated after phosphorus determination according to [21].

2.5. Binding to liposomes

The binding of the lipopeptide and of trilysine to POPG LUV was characterized by their respective apparent molar partition constant K = (bound)/(free)(accessible lipid) [22]. Bound and free peptide concentrations were determined by fluo-

rescamine, using sucrose-loaded LUV in order to separate peptides bound to LUV from free peptides by centrifugation [23].

2.6. Release of encapsulated material

LUVs loaded with 100 mM carboxyfluorescein in 10 mM MOPS, 5 mM Na_2SO_4, 0.1 mM EDTA, pH 7.4, were diluted to 50 μM phospholipids in the same buffer containing 105 mM Na_2SO_4. Carboxyfluorescein leakage was followed by fluorescence spectroscopy ($\lambda_{exc.}$ 470 nm, $\lambda_{emis.}$ 519 nm). 100% leak was estimated by adding Triton X100 (0.1% final concentration); leakage was expressed as the ratio $(F_x - F_0)/(F_{100} - F_0)$, with x corresponding to the fluorescence intensity of the considered assay, 0 stands for the initial intensity at time 0 and 100 for the intensity after addition of Triton X100.

2.7. Lipid mixing assay

Lipid mixing between liposomes was detected through the inhibition of energy transfer between two fluorophores inserted together in liposomes. Experiments were carried out according to [24], briefly: LUVs prepared with phosphatidylserine (10 mM final concentration) containing 1% PE–NBD plus 1% PE–rhodamine were mixed in a cuvette (10 μM PS final concentration) with non-labelled LUVs (40 μM PS final concentration) in 100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, pH 7.4. It was chosen to detect lipid mixing by measuring the NBD fluorescence increase. NBD fluorescence was monitored with $\lambda_{exc.}$ 460 nm and $\lambda_{emis.}$ 534 nm. 100% fluorescence increase was determined by addition of Triton X100 to the samples (1% final concentration), with a correction factor of 1.5 due to fluorescence quenching by the detergent. Fluorescence was measured either with a JY3 Jobin et Yvon or an Aminco SPC 500 C.

2.8. Fusion assay

Two techniques were used to monitor intermixing of the aqueous contents of the LUVs: the terbium/dipicolinic acid assay [20], and the ANTS/DPX assay [25]. In both assays, fusion of liposomes resulted in the formation of a complex, fluorescent or quenched, respectively. Each assay was performed with 50 μM phosphatidylserine, in 100 mM NaCl, 10 mM MOPS, 1 mM EDTA, pH 7.4.

2.9. TNS assay

The method used was that designed to probe the electrostatic surface potential of liposomes [26]. Briefly, TNS (0.11 μM final concentration) was added to LUVs in 100 mM NaCl, 10 mM MOPS, pH 7.4, and the lipopeptide was progressively added. Fluorescence was recorded after each addition ($\lambda_{exc.}$ 321 nm, $\lambda_{emis.}$ 445 nm).

2.10. Phospholipid segregation

Phospholipid segregation was detected according to Hoekstra [27], briefly: small unilamellar vesicles (SUV) were prepared in 0.1 NaCl, 0.01 mM MOPS, pH 7.4, by sonication (50W) of mixture of brain–PS and NBD–PC (9/1, molar ratio); segregation of NBD–PC induced either by calcium or by the lipopeptide was followed by measuring fluorescence with narrow band-pass slits and crossed polarizers to eliminate light diffusion.

3. Results

3.1. Lipopeptide solutions

The lipopeptide is clearly soluble in water up to a concentration of about 1 mM. Because of its amphiphilic structure (Fig. 1), it was examined whether the lipopeptide was able to form micelles. Increasing amounts of the lipopeptide were added into a cuvette containing diphenylhexatriene (DPH). Fluorescence intensity and polarization measurements of DPH [28] showed smooth variations between 2 and 200 μM lipopeptide concentration, but no critical concentration, corresponding to the formation of a new phase, could be observed. Thus it is quite likely that the lipopeptide does not form micelles in the tested concentration range. In addition, it is worth noting that no monolayer formation was detected at the interface between air and lipopeptide solutions, since no variation of the surface pressure was detected 1 h after

addition of the lipopeptide into the water phase (7 μM final concentration).

3.2. Lipopeptide interaction with preformed phospholipid monolayers

Lipopeptide solutions were introduced into the water phase underneath preformed phospholipid monolayers with a fixed area. Injection of the lipopeptide into the water phase resulted in an increase of the surface pressure with each of the three types of anionic monolayers used (PS, DPPS, PG), indicating an insertion of the lipopeptide in the film (Fig. 2(A)). No insertion was observed in the neutral phosphatidylcholine monolayer. The final surface pressure was the same for the three anionic lipids, but kinetics were different, indicating an influence of the nature of the polar head and of the state of acyl chain

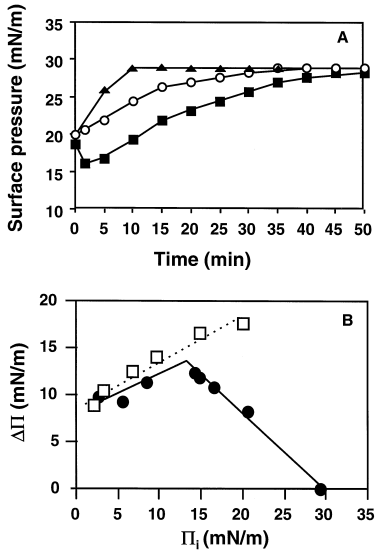

Fig. 2. Lipopeptide interaction with a preformed phospholipid monolayer. (A) Kinetics of interaction. The lipopeptide (final concentration 7×10^{-6} M) was injected in the water phase (5 mM MOPS, pH 7.4) below a monolayer prepared at a 20 mN/m initial surface pressure. Open circles: brain phosphatidylserine (PS), full squares: dipalmitoylglycerophosphorylserine (DPPS), full triangles: egg-derived phosphatidylglycerol (PG). (B) Surface pressure increase ($\Delta\pi$) due to lipopeptide/monolayer interaction. Monolayers were prepared at various surface pressures (π_i) and the lipopeptide was injected in the water subphase. full circles: $\Delta\pi$ versus π_i at the equilibrium between free and bound lipopeptide; open squares: $\Delta\pi$ calculated from compression isotherms (Fig. 3), i.e. the $\Delta\pi$ between a monolayer of PS alone and of a 1/3 lipopeptide/PS mixture.

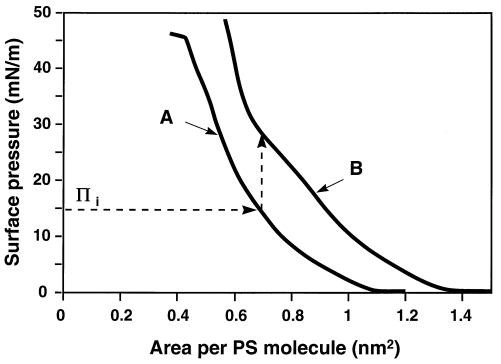

Fig. 3. Compression isotherms of PS and of a lipopeptide/PS mixture. PS alone (isotherm A) or a mixture of lipopeptide/PS (1/3 molar ratio; isotherm B) was spread on 5 mM MOPS, pH 7.4. The films were compressed and the surface pressure (mN/m) versus the molecular area (nm^2) calculated for one PS molecule was recorded. Arrows present an example of the determination of $\Delta\pi$ (vertical arrow) that could be expected from insertion of the lipopeptide within a PS monolayer at the initial lateral pressure $\pi_i = 15$ mN/m (horizontal arrow).

organization on kinetics but not on the final equilibrium state. Thus electrostatic interactions played a major role in the lipopeptide insertion in the monolayer. This type of interaction is very sensitive to ionic strength, as noted with polymyxin, an amphiphilic cationic peptide [29], or with oligo- and polylysine [30–33].

Phosphatidylserine monolayers were prepared at 15 mN/m on the 5 mM MOPS buffer with or without 300 mM NaCl. The same maximum surface pressure increase was obtained on both subphases, indicating that the final equilibrium was not dependent on ionic strength (data not shown), or in other words that electrostatic forces are not the only one involved in the lipopeptide/phospholipid interaction.

Insertion experiments were repeated with films at various initial surface pressures (Π_i) and the surface pressure increase ($\Delta\Pi$) was determined after the equilibrium was reached: no insertion of the lipopeptide in the film was detected above $\Pi_i = 29$ mN/m (Fig. 2(B)). The lipopeptide bears three net positive charges, therefore, it was checked whether the final surface pressure obtained in the above experiments corresponded to what can be estimated from compression isotherms performed either with PS alone or with a lipopeptide/PS mixture in the 1/3 molar ratio (Fig. 3). The $\Delta\Pi$ values calculated as indicated in the legend of Fig. 3 from these two isotherms are

presented in Fig. 2(B). Comparison with $\Delta\Pi$ values obtained at equilibrium between a PS monolayer and the lipopeptide injected in the subphase (Fig. 2(B)) shows a good agreement between the two set of data, up to a final pressure ($\Pi_i + \Delta\Pi$) of about 20 mN/m (i.e. up to about $\Pi_i = 10$ mN/m), while a significant deviation was observed for higher values.

In order to obtain informations on electrostatic interactions in the above experiments, the surface potential ΔV of a PS monolayer interacting with the lipopeptide was determined (Fig. 4). ΔV depends on the electric charges present at the water/lipid interface and on the permanent dipole moments of the film-forming molecules. Thus, ΔV is expected to be influenced by the orientation of the molecules within the monolayer, and by the molecules adsorbed at the interface. Monolayers prepared by spreading mixtures with known molar percentages of lipopeptide to phosphatidylserine presented a surface potential much higher above the ratio of 1 lipopeptide/3 phosphatidylserines (charge neutralization) (Fig. 4(A)). After injection of the lipopeptide in the water phase below a preformed monolayer of phosphatidylserine alone, ΔV dropped quickly, then steadily increased up to a much higher value (350 mV) than that measured at time 0 (250 mV) (Fig. 4(B)). The small initial decrease of ΔV could be due to the arrival of the lipopeptide as a counter-ion at the monolayer interface, without insertion in the monolayer, since the surface pressure did not change. Then the lipopeptide molecules were progressively inserted into the monolayer, as indicated by the increase in surface pressure, with a correlative charge neutralization of phosphatidylserine, leading to a large and progressive increase of ΔV. It is worth noting that the ΔV value obtained at the plateau (350 mV) after injection of the lipopeptide under the monolayer (Fig. 4(B)), was similar to that obtained for the lipopeptide/PS (1/3) mixture which corresponds to charge neutralization in the monolayer (Fig. 4(A)). This strongly suggests that lipopeptide molecules were inserted from the water phase into the monolayer up to charge neutralization.

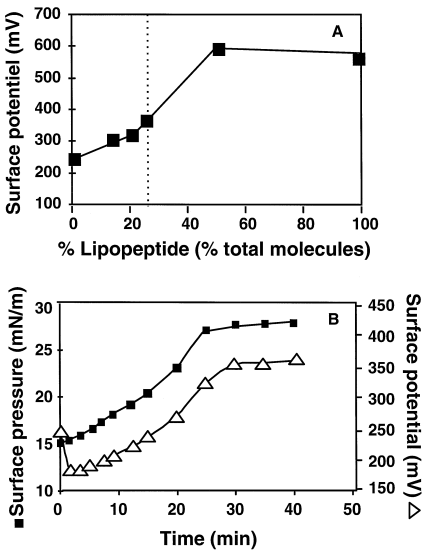

Fig. 4. Surface potential (ΔV) of lipopeptide/phosphatidylserine monolayers. (A) Surface potential of monolayers (26 mN/m) formed by spreading lipopeptide/PS mixtures (molar percentages as indicated in abcissa). Abcissa: molar percentages of lipopeptide to total molecules. The vertical dotted line indicates the molar percentages corresponding to charge neutralization of the monolayer by the lipopeptide. ΔV determinations were reproducible within 5 mV. (B) Surface potential and surface pressure variations with for a monolayer of PS, after injection of the lipopeptide into the subphase. Time 0 corresponds to the injection of the lipopeptide under the monolayer. Full squares: surface pressure; open triangles: surface potential.

3.3. Effects on liposomes

Association of the lipopeptide and of trilysine (presenting three positive net charges as the lipopeptide) with phosphatidylglycerol LUV was characterized by titrating the lipopeptide by fluorescamine. The apparent molar partition constant [22] of the lipopeptide and of trilysine were 2×10^5 and 2.5×10^2 M^{-1}, respectively.

Interaction of bacterial lipids with a phospholipid bilayer can induce an alteration of membrane passive permeability, as it was shown for mycobacterial glycopeptidolipids [34]. The three positive net charges of the lipopeptide might also facilitate fusion of negatively charged membranes, as it is well documented for polylysine molecules [35,36]. The occurrence of these two effects was investigated with the lipopeptide interacting with large unilamellar vesicles (LUVs).

LUVs entrapping carboxyfluorescein were obtained using the technique of reverse-phase evaporation followed by extrusion through polycarbonate membranes. One phospholipid (PC, PS) or binary

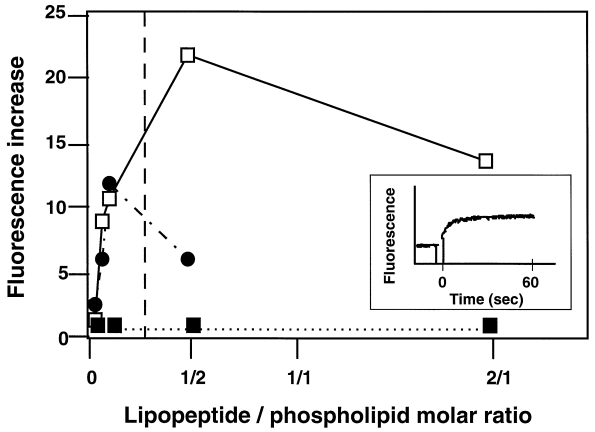

Fig. 5. Lipopeptide-induced leaks in large unilamellar vesicles (LUVs). Insert: example of an assay; time 0 corresponds to the moment of the lipopeptide addition to carboxyfluorescein-loaded LUVs. Open squares: PS LUVs; full circles: PC/PS LUVs (3/1, molar ratio); full squares: PC LUVs. The vertical dotted line indicates the molar ratio (1/6) corresponding to charge neutralization of the PS LUV outer layer. 100% fluorescence increase corresponds to the fluorescence intensity after addition of Triton X100 (1% final concentration).

mixtures (PC/PS; 3/1, molar ratio) were used. Addition of the lipopeptide to negatively charged PS-containing liposomes induced a fluorescence increase (Fig. 5), not observed with neutral PC liposomes. The leak reached a maximum for a lipopeptide/PS ratio around 1/6, a ratio corresponding to charge neutralization of half the PS molecules, i.e. the PS molecules which are present in the outer layer of the liposomes. However, this leak was limited in intensity (less than 25% of a detergent-induced leak) and transient (less than 1 min – insert in Fig. 5). Permeabilisation of liposomes as observed with the anionic carboxyfluorescein was not due to an interaction with the cationic lipopeptide, since it was also observed with terbium ions (data not shown).

In addition to the release of part of the entrapped materials, the lipopeptide induced aggregation of liposomes, as seen by light scattering measurements (data not shown). These data were reminiscent of what has been observed in liposome fusion experiments performed in the presence of polylysine [36]. The ability of the lipopeptide to induce fusion was then investigated.

Fusion was first detected by following the decrease in energy transfer between two fluorescent probes in liposomes doubly labelled (phosphatidylethanolamine–NBD plus phosphatidylethanolamine–rhodamine). This labelled liposomes were brought in contact with non-labelled liposomes [20] and the NBD fluorescence increase was measured.

Addition of the lipopeptide to liposomes induced a dose-dependent NBD fluorescence increase (Fig. 6), indicating a mixing of membranes, up to a maximum beginning at a ratio of about 1 lipopeptide to 7 PS. To correlate the extent of membrane mixing with the net external surface charge of liposomes, the known possibility to probe the electrostatic surface potential of liposomes with TNS was used [37]: a large and fast fluorescence increase occurred upon addition of lipopeptide to liposomes in the presence of TNS, followed by a progressive decrease in fluorescence (insert in Fig. 7). As it was observed that the fluorescence of TNS was greatly increased in the presence of free lipopeptide molecules (data not shown), the initial increase observed (insert in Fig. 7) is likely to correspond to an early TNS/lipopeptide interaction, followed by the progressive break down of this association due to the insertion of the lipopeptide into liposome outer layer, leading to a decrease in TNS fluorescence down to a plateau.

Successive additions of lipopeptide to liposomes progressively reduced the surface charge of liposomes; addition of the lipopeptide in excess of charge

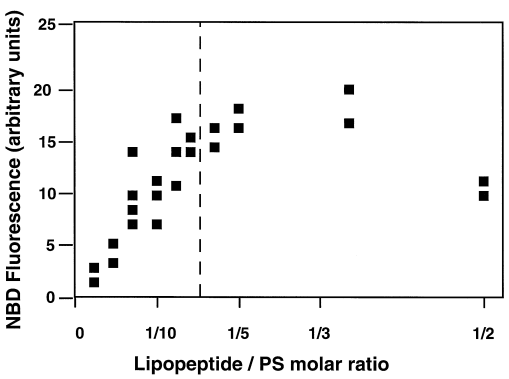

Fig. 6. Mixing of membranes of labelled and non-labelled LUVs. Mixing of membranes was determined by measuring the fluorescence of NBD–PE after addition of the lipopeptide. 100% corresponds to the fluorescence obtained after addition of Triton X100 (1% final concentration). The vertical dotted line indicates the molar ratio (1/6) corresponding to charge neutralization of the LUV outer layer.

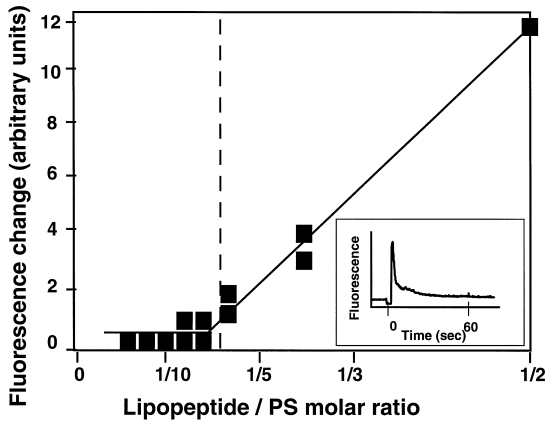

Fig. 7. Charge neutralization of phosphatidylserine LUVs by the lipopeptide, detected by TNS fluorescence. The vertical dotted line indicates the molar ratio (1/6) corresponding to charge neutralization of LUV outer layer. Insert: example of an assay; time 0 corresponds to the moment of addition of the lipopeptide.

neutralization resulted in an increase in fluorescence due to interactions of TNS molecules both with the neutral surface of liposomes and with free lipopeptide. Fluorescence at the plateau increased for lipopeptide/PS ratios above 1/7 (Fig. 7), i.e. a ratio corresponding in Fig. 6 to the lowest ratio inducing the maximum phospholipid exchange between liposomes. As the lipopeptide bears three net positive charges, it appears that the lipopeptide induced maximum mixing of liposomal membranes provided the liposome outer surface was close to electrical neutrality. However, the lipopeptide did not induce any mixing of the liposome contents, as shown by using either the terbium/DPA or the ANTS/DPX systems of fusion detection (data not shown). Thus, the lipopeptide induced an exchange of lipids between liposomes, but not liposome fusion. This was reminiscent of what has been proposed for the effect of polylysine [38].

Because polylysine has been shown to facilitate calcium-induced fusion by promoting liposome aggregation [35], a similar effect was sought for the lipopeptide. It appeared that the lipopeptide strongly inhibited calcium-induced fusion even for low lipopeptide/PS ratios, as seen with the ANTS/DPX system (Fig. 8) and with the terbium/DPA system (data not shown). Fusion was completely inhibited when the outer surface net charge of the liposomes was neutralized by the lipopeptide. A peptide (trilysine) with the same net charge as the lipopeptide had no effect on fusion, in the concentration range used for the lipopeptide.

As fusion was induced by calcium, two experiments were performed with PS monolayers to check whether a competition for binding at the interface could occur between calcium and the lipopeptide. Firstly, the surface pressure increase induced by the lipopeptide in preformed monolayers was followed in the presence of Ca^{2+}: up to 50 mM Ca^{2+}, no significant decrease of the lipopeptide interaction was detected, and a 50% reduction in the surface pressure increase was noted in the presence of 500 mM Ca^{2+} (Fig. 9(A)). Secondly, a PS monolayer was formed on a water phase containing $^{45}Ca^{2+}$. An increase in surface radioactivity due to an increase in calcium concentration at the interface was observed. Then, addition of the lipopeptide into the water phase resulted in a progressive and large decrease in radioactivity (Fig. 9(B)). This showed that the lipopeptide displaced calcium from the phosphatidylserine monolayer. As seen in Fig. 9(B), trilysine at the same concentration had a much weaker effect.

The lipopeptide bearing three positive net charges, it can interact with three PS molecules, thus it could induce a local segregation of PS molecules. An indirect experimental evidence was obtained with PS–

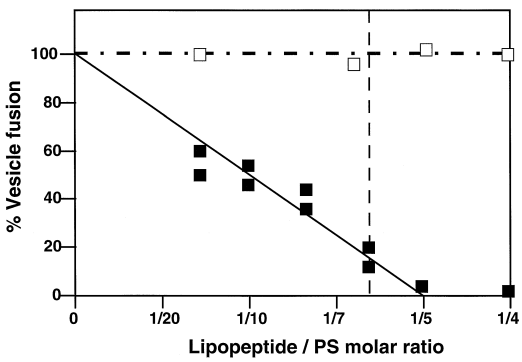

Fig. 8. Effect of the lipopeptide on calcium-induced fusion of LUVs. The fluorescence of ANTS was used to follow calcium-induced fusion (3 mM Ca^{2+} final concentration) of LUVs loaded either with ANTS or with its quencher DPX. 100% corresponds to the fluorescence measured after calcium-induced fusion, in the absence of trilysine or of the lipopeptide. Full squares: effect of the lipopeptide; open squares: effect of trilysine. The vertical dotted line indicates the molar ratio (1/6) corresponding to charge neutralization of the LUV outer layer.

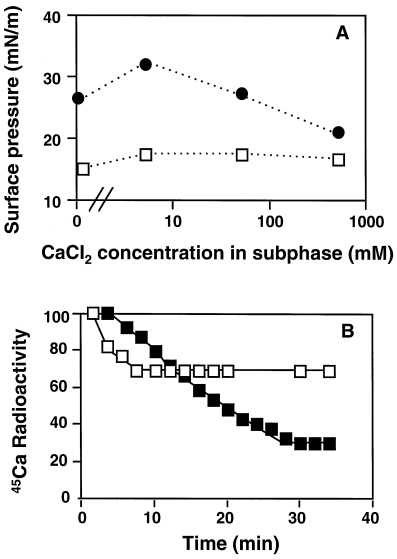

Fig. 9. Calcium and lipopeptide adsorption on a PS monolayer (A) Lipopeptide adsorption in the presence of calcium. The lipopeptide (7 mM final concentration) was injected below PS monolayers formed on water phases containing various calcium concentrations. Open squares: initial surface pressure, full circles: final surface pressure after addition of the lipopeptide. (B) Kinetics calcium displacement from a phosphatidylserine monolayer, after addition of the lipopeptide. Initial surface pressure, 20 mN/m.; 15 mM $^{45}Ca^{2+}$ in the subphase. The added lipopeptide and trilysine were at a final concentration of 0.6 mM. Time 0 corresponds to the moments of additions. Full squares: lipopeptide; open squares: trilysine.

SUV containing 10% NBD–PC. This system has been used to detect calcium-induced segregation of PS that created NBD–PC rich domains, resulting in a self-quenching of the fluorescent probe [27]. Addition of the lipopeptide in concentration above 20 μM induced up to 30% quenching of NBD fluorescence, while calcium concentrations in the millimolar range were necessary to obtain the same result (data not shown).

4. Discussion

The lipohexapeptide $Pam_3Cys–Ser–(Lys)_4$ shows strong interactions with anionic phospholipids (PG, PS), but not with the neutral PC. This behaviour was expected because of its three positive net charges. However, electrostatic interactions are not the only ones contributing to the binding of the lipopeptide to lipids, since high Na^+ concentrations (300 mM) do not inhibit these interactions, neither with monolayers nor with liposomes. Interestingly, surface pressure at the plateau (Fig. 2(A)) is identical for PG, PS and DPPS, indicating that the final equilibrium is influenced neither by the nature of the anionic polar head, nor by the presence of saturated or unsaturated acyl chains. However, the interaction kinetics is dependent of these factors. The slowest insertion rate was observed with DPPS, which formed the more condensed films due to its saturated acyl chains. This suggests that insertion of the lipopeptide in the acyl chain region of the monolayer could be the kinetically limiting step with DPPS.

It is obvious that the three palmitoyl chains of the lipopeptide can exhibit hydrophobic interactions with lipids. This is in agreement with the above determinations of apparent association constants with PS–LUV, since the lipopeptide constant is 10^3 times that of trilysine. In addition, the tetramethylene groups of lysine residues may also participate to such interactions, as it has been shown for the lysine methyl ester interacting with PG monolayers [39]. Liposomes prepared with the lipopeptide covalently linked to a NBD fluorescent group on the C-terminal lysine (lipopeptide–NBD) brought some informations on the location in the interface of the tetralysine moiety, since NBD fluorescence is very sensitive to the polarity of its environment [40,41]. When the lipopeptide–NBD was inserted in liposomes made of PC alone, its emission spectrum presented a small but significant red-shift and a higher intensity as compared to the labelled lipopeptide in PS liposomes (data not shown). This indicates that in PC liposomes, the fluorophore is in a more polar environment than in PS liposomes. It is known that lipid-linked NBD is located in the glycerol region of the phospholipid bilayer [41], thus it can be postulated that the NBD moiety of the lipopeptide–NBD resided similarly in the glycerol region in PS liposomes, with the tetralysine moiety interacting with negative charges. However, NBD was displaced towards the water phase in the absence of a net negative charge in PC liposomes, due to a tetralysine moiety dipping into water.

The three palmitoyl chains can insert within the phospholipid acyl chains, and comparison of the data obtained from compression isotherms (Fig. 3) and from lipopeptide insertion in preformed monolayers (Fig. 2(B)) strongly suggests that up to 20 mN/m,

palmitoyl residues were inserted within the hydrophobic part of monolayers. Above 20 mN/m, the surface pressure at the plateau is lower than the value expected from the compression isotherm corresponding to the lipopeptide/PS 1/3 ratio, and above 29 mN/m, the interaction is not detected. One could conclude that between 20 and 29 mN/m the two interacting molecules were present in a ratio below 1/3. However, the surface potential ΔV of a monolayer reaching a surface pressure of 27 mN/m upon interaction with the lipopeptide, showed the same value (350 mV) than a neutral preformed 1.lipopeptide/3.PS monolayer. This suggests that even at 27 mN/m, the lipopeptide interaction is able to neutralize the phospholipid layer. In this case, it must be assumed that not all lipopeptide molecules interacted in the same way with phospholipids, since the final surface pressure was lower than that expected (32 mN/m) from compression isotherms. It was not possible to analyse further the interaction with the methods used in the present work. Surface charge neutralization was also detected in the interaction of the lipopeptide with liposomes (LUV) by using the fluorescence of TNS (Fig. 7).

Four important consequences resulted from lipopeptide insertion in anionic phospholipid layers: (i) liposome aggregation; (ii) exchange of phospholipids between aggregated liposomes (Fig. 6); (iii) efficient displacement of calcium ions adsorbed on the phospholipid layer (Fig. 9(B)); (iv) inhibition of calcium-induced fusion of liposomes (Fig. 8), likely due to calcium displacement from liposome surface. In addition, the lipopeptide could induce some segregation of anionic phospholipids.

Some of the above effects have been reported in literature for polylysine, e.g. liposome aggregation and phospholipid exchange [36,38], but not with peptides shorter than pentalysine. An important difference between polylysine and lipopeptide effects is that this cationic lipopeptide inhibited the calcium-induced fusion, while polylysine facilitated it [36]. This underlines the importance of the three acyl chains in the interaction: they help to bind the lipopeptide in the membrane, anchoring the positive charges at the interface, rendering them efficient competitors to calcium ions.

It can be argueded that such an effect can take place in leucocytes, on the inner as well on the outer surface of the cell plasma membrane, since macrophages rapidly internalize this type of molecule [42]. It has already been shown that the lipopeptide induces calcium release within some blood cells [43,44]. Such an effect could disturb calcium-dependent events, and it could be at the origin of some of the observed pleiotropic activity of this lipopeptide.

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