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Lysophosphatidylcholine inhibits vesicles fusion induced by the NH_2-terminal extremity of SIV/HIV fusogenic proteins

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Abstract

Intermediate lipid structures such as inverted micelles and interlamellar attachments are thought to play a crucial role in different biological processes like exocytosis, intracellular trafficking and viral infection. In the present study, we provide evidence that lipid mixing of large unilamellar lipid vesicles (LUV) mediated by the NH_2-terminal sequence of the SIV gp32 and of HIV gp41 is inhibited by external addition of lysophosphatidylcholine (lysoPC) to LUV containing phosphatidylethanolamine in their lipid bilayer. Leakage experiments confirm that lysoPC enhances the stability of the lipids organization. The temperature dependence of the two processes as well as the complementary shape of PE and lysoPC suggest that the PE–lysoPC interaction is involved in the fusion inhibition and stabilization of the bilayer.

Keywords: SIV/HIV fusion peptide; Lysophosphatidylcholine; Lipid mixing

1. Introduction

Membrane fusion is an essential event in cell biology and it has been extensively studied in the recent years, and more particularly the enveloped virus-host cell fusion event. With regard to the human immunodeficiency virus, unlike the binding of gp120 to CD4 [1] the postbinding molecular events leading to HIV-mediated membrane fusion are much less well understood even though major conformational changes of the whole gp120/gp41 complex are involved, resulting in the exposure of the gp41 N-terminal fusogenic domain [2]. This mechanism is crucial not only for the penetration of free virus into cells but also for cell fusion processes that can lead to syncytium formation [3]. Most viral fusogenic proteins contain a short amino-terminal hydrophobic segment which interacts with the lipid membrane during the fusion event [4,5]. Mutagenesis studies have confirmed that modifications disrupting the distribution of the hydrophobic amino acids in the N-terminus of Influenza hemagglutinin [6], gp41 of HIV [2,1] and gp32 of SIV [7,8] inhibit syncytium formation without affecting glycoprotein synthesis and processing or receptor binding, suggesting that hydrophobicity plays a key role in the fusion process.

Although fusion requirements for simple membrane model systems are often far from those known to be required for biological membranes, such studies have undoubtedly contributed to a molecular description of different steps of the fusion process. One of the main contributions has been to assess the role of lipid transient species like inverted micelles and hexagonal phases in the fusion mechanism. If it is accepted that fusion peptides interact with the lipids of the target membrane [4,9–11] to produce hexagonal lipid structures of negative curvature [12,13], the molecular mechanism of such a process remains unknown. Although phopholipids in biological membranes are mainly arranged as a bilayer, a fraction of the total lipids form inverted structures. This phase preference has been related to the average shape of the molecules. Bilayer preferring lipids are thought to adopt an overall cylindrical shape. In contrast, lipids which form an inverted hexagonal (H_{II}) phase are thought to be conical with the polar headgroup at the smaller end of the cone as phosphatidylethanolamine (PE). Lysophosphatidylcholine (lysoPC), with its relatively large hydrophilic moiety, prefers micellar organizations in excess water. Addition of lysoPC to the contacting monolayers of artificial planar lipid membranes was shown to inhibit the monolayer fusion [14].

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Moreover, lysoPC has been shown to inhibit diverse biological fusion processes as exocytosis and virus-mediated syncytia formation [15]. This inhibition was not related to solubilization of requisite fusion components, irreversible denaturation, or membrane lysis. The apparently universal character of the phenomenon suggested that lysolipids inhibit an intermediate step common for all fusion reactions [15].

We have previously reported that a synthetic peptide corresponding to the 12 amino acids of the N-terminus of SIV gp32 and to the 16 amino acids of the N-terminus of HIV gp41 induced the fusion of large unilamellar vesicles, provided the liposomes contained PE in their lipid bilayer [10,16]. In the present paper, to test whether the vesicles fusion induced by the SIV/HIV fusion peptide shares a common mechanistic step with the biological virus-cell fusion, we studied the role of exogenous lysoPC in the lipid fusion process induced by those peptides.

2. Materials and methods

2.1. Materials

Egg phosphatidylethanolamine (PE), egg phosphatidylcholine (PC), cholesterol (Chol), sphingomyelin (SM), lysophosphatidylcholine (lysoPC), and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). *N*-(Nitrobenzo-2-oxa-1,3-diazol)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE) were from Avanti Polar Lipids (Birmingham, AL, USA).

Calcein (Sigma) was purified by chromatography on Sephadex LH20 (Pharmacia). Calcein was loaded on the column as a sodium salt solution and eluted with water at neutral pH. The concentration of calcein was determined spectrophotometrically by using $7.0 \cdot 10^4$ as the molar extinction coefficient at 492 nm [17].

2.2. Methods

Peptide synthesis

Peptides (Fig. 1) were synthesized by solid-phase synthesis using a commercially available peptide synthesizer (model Biolynx, Pharmacia Biochrome, Cambridge, UK) and the preweighted Fmoc amino acid (Pharmacia Biochrome, Cambridge, UK). Acylation rate was monitored by the Bioplus software be measuring the release of an anionic dye (Acid violet 17.3 mg/100 ml dimethylformamide and 0.14 ml diisopropylethylamine) at 600 nm. The peptides were cleaved from the resin using trifluoroacetic acid containing 2% anisol and 2% ethanedithiol for 2 h followed by ether precipitation. Each peptide was purified to more than 95% purity by HPLC on a C18 Alltima reverse phase column (7.5 × 300 mm) (Alltech, Belgium). The peptides typically eluded at 65% acetonitrile (between 65 and 75%) using a linear gradient over 30 min from 10 to 90% acetonitrile with 0.1% trifluoroacetic acid. The sequence was verified by protein sequencing on an Applied Biosystem sequencer. The peptide was dissolved in TFA, and after evaporation of the solvent, DMSO was added to a final peptide concentration of $8 \cdot 10^{-4}$ M. The stock solutions were stored at 0°C.

Vesicles preparation

Multilamellar vesicles (MLV) were obtained by vortexing a lipid film in a Tris buffer (10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3, pH 7.4).

Large unilamellar vesicles (LUV) were prepared according to the extrusion procedure of Hope et al. [18] using an Extruder (Lipex Biomembranes, Vancouver, Canada). Briefly, frozen and thawed MLV were extruded 10 times through two stacked polycarbonate membranes with a pore size of 0.1 μm (Nucleopore, Pleasanton, CA, USA).

Assay of vesicle leakage

Dry lipid films were rehydrated to a concentration of 20 mg/ml in 10 mM Hepes pH 7.2 buffer containing 62 mM calcein purified as described above. Unencapsulated dye was removed by passing the liposome preparation over a Sephadex G50 gel filtration column, equilibrated with 10 mM Hepes, 150 mM NaCl, 1 mM EDTA buffer, pH 7.2. Liposome concentration was estimated by measuring lipid phosphorus content [19].

Release of the fluorescent dye from preloaded LUV at a final lipid concentration of $3 \cdot 10^{-4}$ M, was monitored using SLM 8000 fluorometer (SLM Instruments, Urbana, IL). Experiments were conducted in a 1 ml stirred cuvette, with right angle illumination. Excitation and emission wavelengths were set at 490 nm and 520 nm, respectively, employing a slit width of 4 nm. The addition of Triton X-100 to a final concentration of 0.1% (v/v) was used to determine maximal release. The percentage of total fluorescence was defined as

$$\%F_t = \frac{I_t - I_0}{I_{100} - I_0} \times 100$$

where I_0 is the initial fluorescence, I_{100} is the total fluorescence observed after addition of Triton X-100, and I_t is the fluorescence observed after addition of SIV/HIV fusion peptide at $t = 600$ s, corrected for dilution.

Assay of vesicle fusion

Lipid mixing was determined by measuring the fluorescence intensity change resulting from the fluorescence energy transfer between the probes NBD-PE and Rh-PE, as described by Struck et al. [20]. Fluorescence was monitored using a SLM 8000 spectrofluorimeter in the same conditions described for the leakage assay, excepted for the excitation and emission wavelengths which are 470 nm

HIV 16aa fusion peptide:

NH_2-Ala-Val-Gly-Ile-Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-COOH

SIV 12 aa fusion peptide:

NH_2-Gly-Val-Phe-Val-Leu-Gly-Phe-Leu-Gly-Phe-Leu-Ala-COOH

Fig. 1. SIV and HIV fusion peptides sequences.

and 530 nm, respectively. Both probes were added to the lipid film and LUV were prepared as described above.

Liposomes containing both probes at 0.6% (molar ratio) each, were mixed in a 1:9 mol ratio with probe free liposomes at a final lipid concentration of $3 \cdot 10^{-4}$ M. The initial fluorescence of the 1:9 (labeled/unlabeled) suspension was taken as 0% fluorescence and the 100% fluorescence was determined using an equivalent concentration of vesicles prepared with 0.06% of each fluorescent phospholipid.

3. Results

3.1. LysoPC inhibits the lipid mixing

The ability of SIV and HIV fusion peptides (Fig. 1) to induce intervesicular lipid mixing of LUV has been demonstrated by measuring the dilution of fluorescent lipids (NBD-PE, Rh-PE) occurring during mixing of fluorescently labeled and unlabeled population of vesicles [20]. SIV peptide as well as HIV fusion peptide were shown to catalyze lipid mixing of vesicles composed of PC/PE/SM/Chol (1:1:1:1.5 molar ratio) and PC/PE (1:1 or 2:1 molar ratio) with an enhancement of the fusion rate in the bilayer-hexagonal phase transition temperature range of pure egg PE [11,16]. Moreover, the fusion process was shown to increase with the concentration of PE in the lipid bilayer suggesting its direct involvement in the fusion mechanism [11]. Here, we provide evidence that addition of exogenous lysoPC to the medium resulted in a concentration dependent inhibition of the liposome fusion (Fig. 2). The PC/PE/SM/Chol composition is the most representative of the plasma membrane and has been shown to be highly fusogenic [10,16]. The inhibiting amount of lysoPC increases with the amount of PE present in the vesicles lipid bilayer, suggesting a direct relationship between these two lipids and the inhibition of the fusion step (Table 1). This inhibition was observed whether LUV were pre-incubated with lysoPC before addition of fusion peptide or whether peptide and lysoPC were added simultaneously to the vesicles suspension. At lysoPC concentration of 45 μM, inhibition is complete whatever the lipid vesicles composition. Addition of more peptide (15 μM to 40 μM) induced a high rate of vesicles aggregation but no lipid mixing, suggesting that the vesicles where unable to fuse even in the presence of an excess of fusogenic agent (data not shown). It should be mentioned that lysis of the vesicles was only observed above 350 μM lysoPC (Fig. 2)

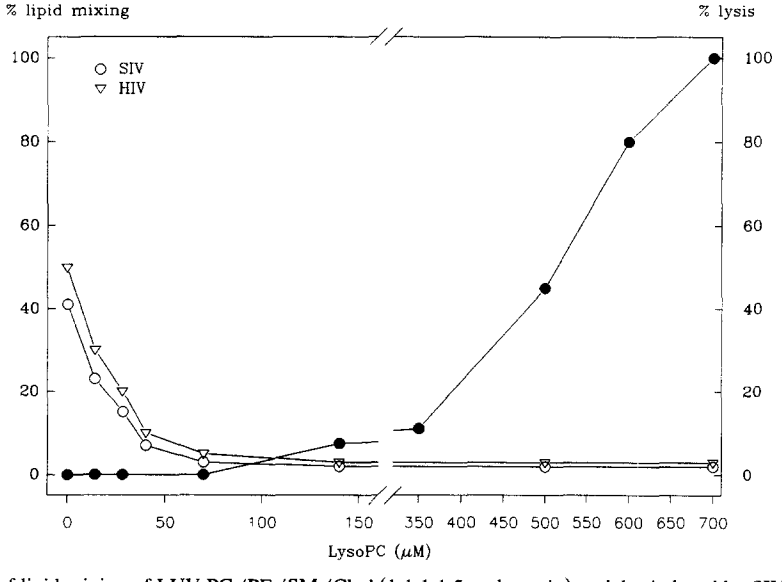

Fig. 2. LysoPC inhibition effect of lipid mixing of LUV PC/PE/SM/Chol (1:1:1:1.5 molar ratio) vesicles induced by SIV and HIV fusion peptides added from DMSO. LUV (300μM) and lysoPC (at different concentration) were preincubated at 37°C pH 7.2 in Hepes buffer, for 15 min prior the addition of peptide (13 μM). The open symbols refer to the percentage of lipid mixing while the filled circles refer to the lysis of vesicles.

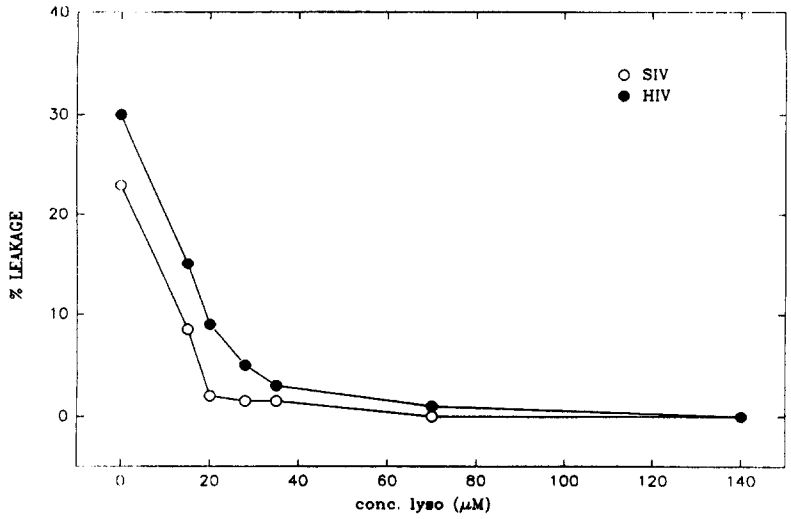

Fig. 3. LysoPC inhibition effect of calcein leakage of LUV PC/PE/SM/Chol (1:1:1:1.5 molar ratio) vesicles induced by SIV and HIV fusion peptide added from DMSO. LUV (300 μM) and lysoPC (at different concentration) were preincubated at 37°C pH 7.2 in Hepes buffer, for 15 min prior the addition of peptide (13 μM).

3.2. LysoPC stabilizes the lipid organization

We have shown that the SIV/HIV fusion peptides favor the leakage of calcein encapsulated in LUV aqueous contents of PC/PE/SM/Chol (1:1:1:1.5 molar ratio) and PC/PE (1:1 and 2:1 molar ratio) in a time-dependent and dose-dependent manner [11,16]. A rapid burst of leakage was observed approx. 30 s after addition of peptide, followed by a slower phase. Here, the leakage of liposome aqueous content was examined in the presence of exogenous lysoPC and as a function of temperature. Liposomes of PC/PE (1:1 and 2:1 molar ratio) or PC/PE/SM/Chol (1:1:1:1.5 molar ratio) were incubated with various amounts of lysoPC at 37°C. After 30 min of incubation, the peptides were added to liposomes, and the calcein fluorescence was measured. Fig. 3 demonstrates that lysoPC stabilizes the lipid organization and reduce drastically the lipid permeability. As for the lipid mixing, the inhibitory amount of lysoPC increases with the amount of PE present in the lipid bilayer (data not shown).

3.3. LysoPC modified the bilayer-non lamellar lipid structure transition

Lipid mixing and leakage induced by SIV in the absence and in the presence of lysoPC depend on tempera-

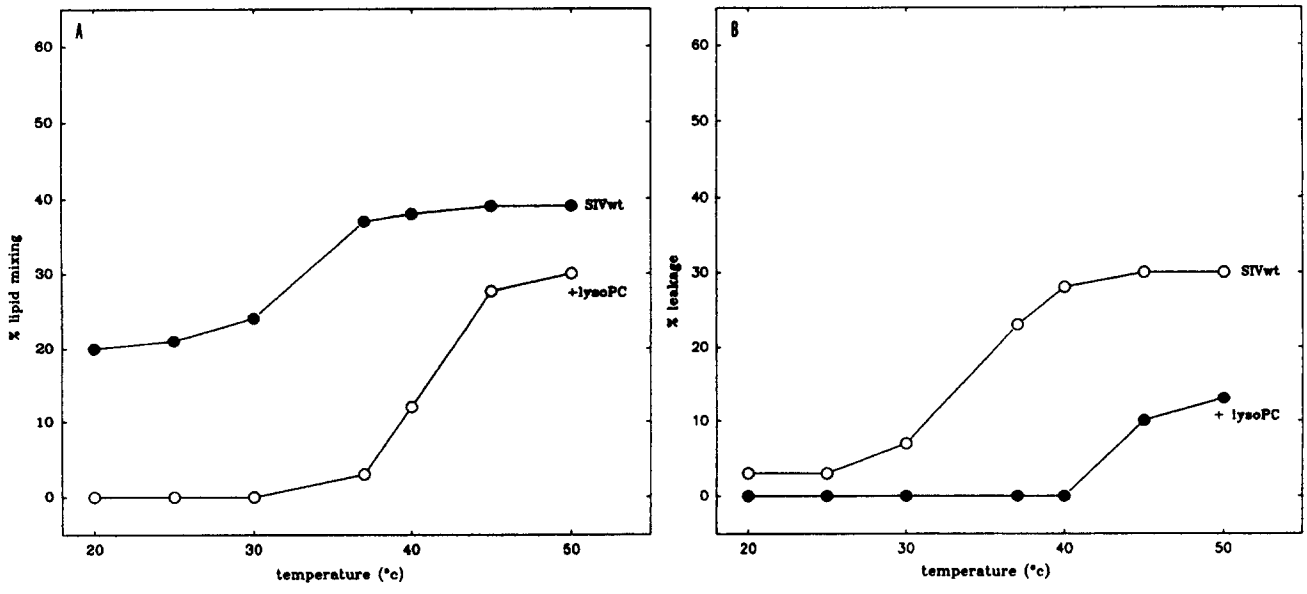

Fig. 4. Temperature dependence of lipid mixing (A) and calcein leakage (B) of LUV PC/PE/SM/Chol (1:1:1:1.5 molar ratio) vesicles induced by SIV in the absence and the presence of lysoPC. LUV (300 μM) and lysoPC (70 μM) were preincubated at 37°C pH 7.2 in Hepes buffer, for 15 min prior the addition of SIV (13 μM).

Table 1
LysoPC inhibition of lipid mixing induced by SIV and HIV fusion peptides as a function of PE concentration

LysoPC concentration (μM)	% of inhibition					
	13 μM PE		6 μM PE		3 μM PE	
	SIV	HIV	SIV	HIV	SIV	HIV
0	0	0	0	0	0	0
5	30	28	45	50	50	55
10	53	50	60	60	65	67
20	63	62	70	75	80	85
30	80	81	90	95	100	100
40	94	90	100	100	100	100
45	100	100	100	100	100	100

LUV (300 μM) and lysoPC (at different concentrations) were preincubated at 37°C pH 7.2 in Hepes buffer, for 15 min prior the addition of SIV/HIV fusion peptides (13 μM).

ture as shown in Fig. 4. Without lysoPC, the mixing of the lipid phases as well as the leakage of the encapsulated aqueous content increase steeply between 30°C and 40°C. It is in this temperature range that the transition of PE from a bilayer to a non lamellar lipid structures occurs (the transition temperature of egg-PE MLV is between 38°C and 45°C) [21]. In the presence of exogenous lysoPC, this transition temperature is shifted to the right suggesting a stabilizing effect of lysoPC. This shift was observed in the vesicle leakage and lipid mixing experiments not only for the SIV fusion peptide but also for the HIV fusion peptide (data not shown). This could be interpreted in term of the molecular shape concept. In an equimolar mixture of the inverted cone-shaped lysoPC and the cone shaped unsaturated PE, lysoPC stabilizes the lipid bilayer organization and increases of about 10°C the transition temperature from a bilayer to a non lamellar lipid structures [22].

4. Discussion

The aim of this study was to investigate the possible relationship between peptide-induced fusion of large unilamellar vesicles and the formation of non lipid bilayer structures, using the SIV and HIV fusion peptides as model peptides. We have more particularly focused on the role of the lipid bilayer in the fusion process. One important question is whether lipids which are competent to form inverted phases can enhance the protein-mediated fusion mechanism [23].

The data demonstrate that the SIV and HIV fusion peptides induce the fusion of large unilamellar vesicles containing PE in their lipid bilayer [11,16], which can form inverted phases on its own, supporting the hypothesis of the formation of the H_{II} phase during membrane fusion. PE has also been shown to enhance the rate of Influenza virus-induced membrane fusion [25]. The pH-induced fusion of peptide Sindbis virus with model membrane is also dependent on the acyl chain composition of the target membrane and is greatly enhanced by the presence of type II non bilayer lipid such as PE or cholesterol in these membranes [24]. The fact that H_{II} phase-competent lipid bilayers enhance the fusion rate of different systems strongly suggests that H_{II} phases are involved in this process.

The SIV/HIV-induced vesicle fusion is accompanied by a rapid leakage of encapsulated solute like calcein. This leakage has also been observed during LUV fusion induced by gramicidin A [25]. It has been suggested in the case of gramicidin A that the leakage is mechanistically related to H_{II} phase formation which is expected to result in a loss of vesicle integrity and thus a release of the enclosed contents [26].

In the present paper, we have shown that the fusogenic activity (lipid mixing) of the SIV/HIV fusion peptides is inhibited by exogenous lysoPC in a dose dependent and temperature dependent way. Since the sequence of the HIV fusion peptide is distinct from that of SIV, the inhibiting effect of lysoPC depends on the vesicles lipid membrane composition suggesting that the physicochemical mechanism of inhibition is directly related to membrane lipids properties.

The mechanism responsible for the lysoPC inhibition is still unclear, however our data are consistent with a lysoPC-lipid interaction. Recently, different amphiphilic molecules have been tested to determine the features of lysolipids responsible for fusion inhibition [15]. Inhibition did not correlate with any specific chemical moiety of lysolipids but rather with their specific inverted conical shape [15]. This along with the ability of lysolipids to inhibit the fusion occurring between cell membrane organelles and between organelles and plasma membrane induced by several distinct fusogenic agents like Ca^{2+}, GTP or viral fusion peptide [15] suggests that lysolipids act by altering a common motif of membrane fusion, and more particularly the ability of membranes to form highly curved 'stalk' intermediates. Regarding our results, this common motif seems also to be present in our model system suggesting a similar mechanism between SIV/HIV peptide-induced vesicle fusion and virus-induced syncytia formation.

If lipid polymorphism is important in biological processes such as fusion and if in these processes, modulation of lipid structure is triggered by lipid–protein interactions, the question arises whether proteins will be able to induce changes in lipid phase behavior upon entering the membrane. It has been proposed that the fusion process involves the penetration of the fusion peptide into the target membrane favoring inverted phase formation. The SIV fusion peptide was also shown to lower the hexagonal transition temperature of palmitoleoylphosphatidylethanolamine [27] suggesting that insertion of the fusion peptide into the target bilayer disrupts bilayer packing. Moreover, ^{31}P-NMR studies demonstrate the presence of H^{II} phase structure for MeDOPE vesicles at 37°C in

presence of SIV fusion peptide [27]. The oblique orientation of the fusion peptide into the lipid bilayer was shown to be required for the fusogenic activity. Peptides inserted parallel to a normal with respect to the lipid-water interface was not fusogenic [28] and did not affect significantly the bilayer-non lamellar phase transition temperature [29].

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