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Interaction of Influenza Virus Fusion Peptide with Lipid Membranes: Effect of Lysolipid

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Abstract. The effect of lysophosphatidylcholine (LPC) on lipid vesicle fusion and leakage induced by influenza virus fusion peptides and the peptide interaction with lipid membranes were studied by using fluorescence spectroscopy and monolayer surface tension measurements. It was confirmed that the wild-type fusion peptide-induced vesicle fusion rate increased several-fold between pH 7 and 5, unlike a mutated peptide, in which valine residues were substituted for glutamic acid residues at positions 11 and 15. This mutated peptide exhibited a much greater ability to induce lipid vesicle fusion and leakage but in a less pH-dependent manner compared to the wild-type fusion peptide. The peptide-induced vesicle fusion and leakage were well correlated with the degree of interaction of these peptides with lipid membranes, as deduced from the rotational correlation time obtained for the peptide tryptophan fluorescence. Both vesicle fusion and leakage induced by the peptides were suppressed by LPC incorporated into lipid vesicle membranes in a concentration-dependent manner. The rotational correlation time associated with the peptide's tryptophan residue, which interacts with lipid membranes containing up to 25 mole % LPC, was virtually the same compared to lipid membranes without LPC, indicating that LPC-incorporated membrane did not affect the peptide interaction with the membrane. The adsorption of peptide onto a lipid monolayer also showed that the presence of LPC did not affect peptide adsorption.

Key words: Viral fusion peptide — Membrane fusion and leakage — Peptide adsorption onto membranes — Fluorescence and monolayer studies

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Abbreviations: ANTS, 1-aminonaphthalene–1,3,6-trisulfonic acid, sodium salt; DOPC, dioleolyphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine DPX, N,N′-p-xylene-bis-pyridinium bromide; E11V/E15V fusion peptide, GLFGAIAGFIVNGWVGMIDG-amide; LPC, 1-stearoyl-2-hydroxy-phosphatidylcholine; LUV, large unilamellar vesicles; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; NCB, 0.15 M NaCl/10 mM Na citrate, pH 4.7 or 5.0; NHB, 150 mM NaCl/10 mM HEPES, pH 7.3 or 7.4; Rh-PE, N-(lissamine rhodamine B sulphonyl)- phosphatidylethanolamine; SUV, small unilamellar vesicles; NTB, 0.15 M NaCl/10 mM Tris, pH 7.4; WT-fusion peptide, GLFGAIAGFIENGWEGMIDG-amide.

Introduction

It is known that the fusion of influenza virus with target membranes is mediated by the hemagglutinin glycoprotein (HA) on influenza viral envelopes [10, 20, 29, 30]. After the HA binds to a receptor molecule of the target membrane, the change in pH from neutral to acidic pH (e.g., pH 5) appears to induce a conformational change in the HA molecule [5, 29] so that the "fusion peptide", which is a stretch of about 20 amino acids at the N-terminus of the HA, penetrates into the target membrane and initiates fusion of the viral envelope with target cell membranes [11, 15, 19, 31]. It was shown that the fusion peptide of influenza virus has virtually no ability to induce lipid vesicle fusion at neutral pH; however, the extent of the vesicle fusion increases greatly at lower pH (e.g., pH 5 or below), compared with that at neutral pH [12, 34]. Recent studies indicated that some other peptide regions (e.g., heptad repeats in viral fusion

protein) greatly enhance lipid vesicle fusion induced by the fusion peptide possibly by helping to facilitate close approach of viral envelope membranes to target membranes [5, 6, 12]. It was reported recently [18] that a modified fusion peptide (E11V/E15V) of the influenza HA, in which glutamic acid residues at positions 11 and 15 were replaced with valine residues, exhibited much greater fusion of lipid vesicles regardless of medium pH, compared to the wild type. It is also known that many of the fusion peptides found in viral fusion proteins have the ability to lyse cells. The ability of vesicle fusion and cell lysis by these peptides seems to correlate with the strong interaction of these peptides with membranes. The degree of insertion of influenza virus fusion peptide (wild type as well as mutated peptides) into lipid membranes has been studied by various methods [8, 9, 13, 21]. One pertinent fluorescence study [8] showed that the degree of insertion of the wild-type influenza virus fusion peptide into the membrane is pH-dependent; at pH 5.0 the peptide appears to penetrate deeper than at neutral pH, which corresponds to the pH dependence of the ability of this peptide to induce fusion of lipid membranes. It was also shown that lipid mixing between lipid vesicles induced by some other viral fusion peptides (e.g., HIV and SIV) was inhibited by incorporation of lysolipid (LPC) into lipid vesicle membranes [22]. The ability of LPC to form a spontaneous positive-curved membrane surface has been suggested to explain the inhibition of membrane fusion [7, 27].

In this study, we report the effect of LPC on vesicle fusion and leakage induced by the influenza virus fusion peptides (wild type and its mutated form (E11V/E15V)). We use fluorescence and surface tension measurements to demonstrate that the strong interaction of these fusion peptides with lipid membranes correlates with the extent of vesicle fusion and leakage induced by the same peptides. We discuss how the incorporation of LPC into lipid membranes affects the interaction of viral fusion peptides with respect to membrane fusion and membrane leakage.

Materials and Methods

Materials

Dioleolyphosphatidylcholine (DOPC), dioleolyphosphatidylethanolamine (DOPE), and stearoyl lysophosphatidylcholine (LPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was from Matreya, Inc. (Pleasant Gap, PA). Fluorophore-attached phospholipids: *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B Sulfonyl)- phosphatidylethanolamine (Rh-PE), and other fluorescence probes 1-aminonaphthalene–1,3, 6-trisulfonic acid, sodium salt (ANTS) and the quenching agent, *N*,*N*′-*p*-xylene-bis-pyridinium bromide) (DPX) were all obtained from Molecular Probes (Eugene, OR). The peptides, GLFGAIAGFIENGWEGMIDG-amide (WT) and GLFGAIAGFIVNGWVGMIDG-amide (E11V/E15V) were synthesized by Biosource (Hopkinton, MA) and purified to > 95% by HPLC. Hexane used for lipid monolayer spreading solution was obtained from Baker Chemical (Baker Instra-Analyzed grade). All other chemicals were of reagent grade. Water was doubly distilled through a glass distillation apparatus. Triply distilled water, including the process of alkaline-permanganate treatment, was used for the lipid monolayer experiments.

Preparation of Lipid Vesicles

Large Unilamellar Vesicles (LUV)

The mixture of DOPC/DOPE/Cholesterol (1:1:1 in mole ratio) was dissolved in chloroform and then the solvent was evaporated to form a lipid film and traces of solvent were then removed completely under vacuum. The dried lipid mixture was then hydrated with 0.15 M NaCl/10 mM Tris, pH 7.4 (NTB) at the total lipid concentration of 3 mM. The hydrated lipids were vortexed for 10 min to form a multilamellar vesicle suspension. The multilamellar vesicle suspension was then passed 20 times through two polycarbonate membranes with 0.1 μm pore diameters by use of an extrusion apparatus (LiposoFast, Avestin Co., Ottawa, Canada) to produce large unilamellar vesicles (LUV). The LUV size was determined with a submicron particle analyzer (Coulter N4). The average diameter was 150 ± 20 nm. For the vesicle fusion study, 1% each of NBD-PE and Rh-PE were dissolved in the lipid mixture solution mentioned above, before hydration with NTB. Thus, the final LUV was composed of 3 mM of DOPC/DOPE/cholesterol bearing 1% each of NBD-PE and Rh-PE. For vesicle leakage studies, the vesicles encapsulating ANTS and DPX were prepared as above using the DOPC/DOPE/cholesterol mixture that was hydrated with the ANTS/DPX buffer composed of 25 mM ANTS/90 mM DPX/10 mM Tris, pH 7.4, followed by extrusion to form LUV. These LUVs were also passed through a Sephadex G-75 column using NTB as the eluting buffer to remove unencapsulated fluorophore and DPX. LUVs containing lysophospholipid were prepared from the above mentioned lipid mixtures containing LPC at different mole % before hydration.

Small Unilamellar Vesicles (SUV)

The mixture of DOPC/DOPE (2:1) was hydrated with NHB (0.15 M NaCl/10 mM HEPES, pH 7.4 or 7.3) or NCB (0.15 M NaCl/10 mM Na citrate, pH 4.7) and vortexed for 10 min to form multilamellar vesicles (MLV). The MLV suspension was then sonicated in a bath-type sonicator (Laboratory Supply, Hicksville, NY) for about 30 min until the suspension became completely clear. The average vesicle diameter was about 40 nm. The detailed protocol for forming the SUVs was described elsewhere [23]. LPC-incorporated SUV were also prepared by mixing LPC in a 12.5% and 25% molar ratio with DOPC/DOPE (2:1) similar to the above. The average sizes of these liposomes with and without LPC were identical within our measurement precision.

Lipid-Mixing Fusion Assay for Vesicle Fusion

Lipid mixing between two lipid vesicles was followed to determine the extent of fusion of vesicles. A 1:4 ratio of fluorescently (NBD/Rh) labeled vesicles to unlabeled vesicles was mixed in a cuvette at the total lipid concentration of 75 μM in NTB (pH 6.0 or 7.4) or NCB (150 mM NaCl/10 mM Na citrate, pH 4.7 or 5.0) prewarmed at 37°C. The energy transfer efficiency of the fluorescently (NBD/Rh) labeled vesicles was initially high. However, when an aliquot of

fusion peptide solution (1 mM in DMSO) was added to such a vesicle suspension, the peptide induced lipid mixing between the vesicles, which is considered to be due to vesicle fusion. When the fluorescently labeled lipids are transferred into unlabeled lipid vesicles due to lipid mixing, the labeled lipids are diluted and the energy transfer efficiency is reduced. Thus, the donor's fluorescence will increase. The fluorophore was excited at 460 nm and the emission signal of NBD (donor) was measured at 525 nm to determine the degree of energy transfer of NBD emission signal to Rh (Resonance Energy Transfer Fluorescence) with time. From the fluorescence energy transfer efficiency, one can determine the extent of vesicle fusion [17]. Thus, the extent of vesicle fusion, F, was defined as follows:

$$F = [I_t - I_0]/[I_{tri} - I_0] \times 100\% \quad (1)$$

where I_t refers to the fluorescence intensity (at 525 nm) at a given time, t, I_0 the fluorescence at the initial time, and I_{tri} the maximum fluorescence when 2% Triton X-100 was added to the vesicle suspension. With this method, the extent of vesicle fusion can be monitored as a function of time. To reduce the effect of scattered excitation light, a sharp-cut filter (CS# 3–70), which effectively cut the light below 500 nm, was placed before the emission monochrometer. As a control, the effect of peptide solvent, DMSO, on the fluorescence dequenching measurements was examined; the addition of a volume of 0.25% (v/v) DMSO into the vesicle suspension at pH 5.0, 37°C, which corresponded to the case of 2.5 μM peptide addition, caused approximately 2% fluorescence dequenching for 10 min.

Leakage Measurements of Lipid Vesicles

Leakage of ANTS/DPX encapsulated in LUV (DOPC/DOPE/Cholesterol (1:1:1)) was monitored as the dequenching of ANTS fluorescence as ANTS and DPX leaked out from the liposomes and became diluted [14]. The ANTS/DPX solution encapsulated in the vesicles has a residual fluorescence of ANTS but was mostly quenched. When an aliquot of fusion peptide was added, usually lipid vesicle leakage occurred. The leakage was measured by exciting ANTS at 360 nm and monitoring the emission of ANTS fluorescence ≥530 nm using a sharp-cut filter (CS# 3–69), which cut the light below 520 nm. Then, the extent of vesicle leakage, L, was defined as:

$$L = [I_t - I_0]/[I_{tri} - I_0] \times 100\ \% \quad (2)$$

where I_t refers to the fluorescence intensity ≥530 nm measured at a given time, I_0 to that measured at the initial time, and I_{tri} to the maximum fluorescence intensity after the addition of Triton X-100 (2%) into the vesicle suspension. Unless otherwise mentioned, vesicle fusion and vesicle leakage experiments were performed at 37°C.

Fluorescence Studies of Intrinsic Tryptophan in Fusion Peptides

Steady-state and time-resolved fluorescence studies on tryptophan of viral fusion peptides were performed in an aqueous solution in the absence and presence of lipid vesicles, using instrumentation described elsewhere [2, 4]. To avoid scattered light from the use of LUV, SUV had to be used for these experiments. The SUV used were composed of DOPC/DOPE (2:1) with 0, 12.5 or 25 mole % LPC. Viral fusion peptides used were the wild type of influenza virus (WT) and the E11V/E15V mutant. An aliquot of each peptide dissolved in 1.0 mg/ml in DMSO was suspended at 10 μM in 150 mM NaCl/10 mM HEPES, pH 7.3 or 7.4 (NHB), or 150 mM NaCl/10 mM citrate, pH 4.7 (NCB) with or without lipid vesicles (1 mM) and the intrinsic fluorescence properties of tryptophan of the peptides (e.g., excitation and emission spectra for the steady-state fluorescence and the time-resolved intensity and anisotropy decays) were measured. These experiments were performed at room temperature (24°C).

Surface Tension Measurements of Lipid Monolayers

Th lipid monolayer-forming solution was composed of DOPC/DOPE (2:1) dissolved in hexane at 1 mM. Lipid monolayers were formed on the surface of an NHB (150 mM NaCl/10 mM HEPES, pH 7.4) solution of a fixed area (63.6 cm^2) in a glass dish by applying the monolayer-forming solution with the use of a Hamilton micro-syringe at various areas per molecule of surfactant. Surface tension was measured by the Wilhelmy plate method [25]; the Wilhelmy plate was made of a microscope glass plate (18 mm × 18 mm × 0.16 mm). The plate was inserted vertically through the surface film into the subphase solution at about a 1.0 mm depth and the downward force exerting onto the plate, in addition to the force of gravity, was measured with time, using an electromicrobalance. For each experiment, water (subphase aqueous solution) was measured to insure cleanliness of the aqueous surface. After spreading the monolayer-forming solution, the surface tension for each surface film reached a stationary value within a few minutes unless desorption from or adsorption onto the surface film occurred. The film surface tension, γ, the water surface tension, γ_w, and the film pressure, π, have the following relationship:

$$\pi = \gamma_w - \gamma \quad (3)$$

To study the adsorption of lysophospholipid on to the lipid monolayers, after a constant film tension was attained for a lipid monolayer, an aliquot of the LPC solution (5.0 mM in NHB) was injected into the subphase solution, the subphase solution was stirred well, and the change (decrease) in the film tension was recorded on a strip chart recorder vs. time. To measure LPC desorption from membranes, PC/PE monolayer-forming solutions with or without different concentrations of LPC were used to form monolayers. An aliquot of monolayer-forming solution containing the same amount of lipids was spread onto the NHB surface. After a transient change in surface tension, the surface tension stabilized within a few minutes. All the experiments were performed at room temperature (24°C) unless otherwise specified.

Results

Fusion peptide-induced lipid mixing between lipid vesicles, which may be considered to be due to vesicle fusion, was measured by monitoring the efficiency of fluorescence energy transfer of donor to acceptor fluorophores incorporated initially in lipid vesicles of one type, "fluorophore-labeled vesicles". This process requires both liposome aggregation as well as the mixing of the membrane lipids between two vesicles. It has been shown that the rate of growth of vesicle size is well correlated with the rate of lipid mixing [32]. The lipid mixing between the fluorophore-labeled vesicles and non-labeled vesicles induced by the wild-type fusion peptide (WT) of influenza virus was pH-dependent. At neutral pH, virtually no lipid mixing was detected, while at a lower pH (4.7 and 5.0), a small but definite extent of lipid mixing was observed. This pH-dependent lipid mixing has been

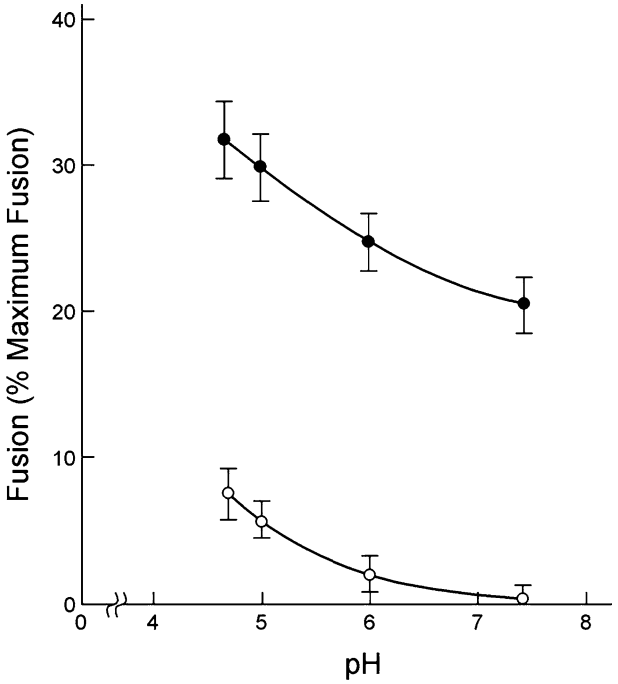

Fig. 1. The extent of vesicle fusion at different pHs determined from lipid mixing monitored with the NBD-Rh lipid-mixing fusion assay. The fluorescently labeled LUV and unlabeled LUV (1: 4 mole ratio) were suspended in 2 ml NTB or NCB at 37°C, both of which consisted of DOPC/DOPE/Cholesterol (1:1:1). The total lipid concentration of the LUVs was 75 μM. Then, the influenza virus fusion peptides of either WT (*empty circles*) or the mutated (E11V/E15V) peptide (*filled circles*) were added into the above vesicle suspension at 2.5 μM. Thus, the ratio of virus fusion peptide to lipid was 30. The fluorescence was measured at 10 min after the addition of the peptide.

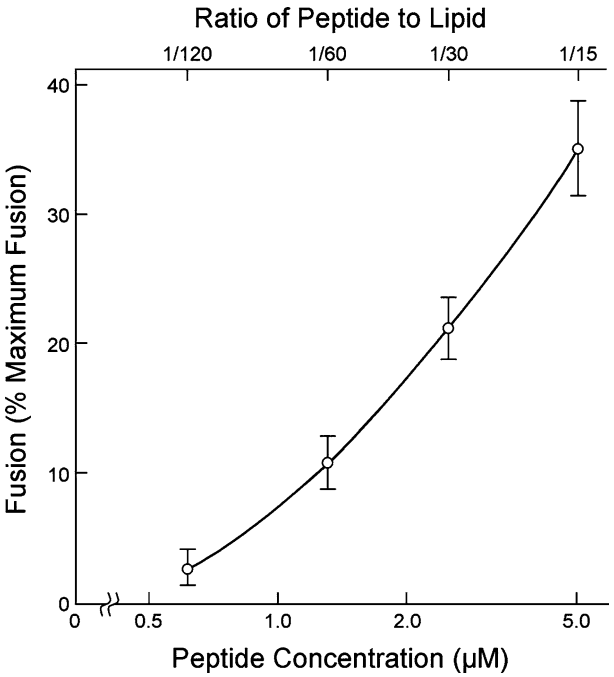

Fig. 2. The extent of vesicle fusion as a function of the mutated fusion peptide concentrations. The vesicles and the experimental conditions were the same as those indicated in Fig. 1. The lipid concentration of the vesicles was 75 μM and the peptide concentrations were varied and pH of the vesicle suspension solution (NTB) was 7.4.

reported by other workers [12, 34]. On the other hand, the fusion peptide modified to have two of the glutamic acid residues substituted with valine (E11V/E15V) induced vesicle fusion at acidic and neutral pH. The extent of lipid mixing for this peptide also exhibited some pH dependence (lower pH induced more lipid mixing compared to higher pH). The extent of vesicle fusion by the E11V/E15V peptide was much greater (approximately 5-fold) when compared to the WT peptide at low pH (4.7–5.0) at 37°C. These results are shown in Fig. 1.

The lipid mixing between vesicles induced by the above mutated fusion peptide was dependent on the peptide concentration for a given concentration of lipid vesicles, as shown in Fig. 2. The WT peptide also showed a similar trend (*results not shown*). The extent of lipid mixing depended on the concentration of peptide in the vesicle suspension and the peptide to lipid concentration ratio in the experimental solution.

The lipid mixings induced by the peptides were inhibited by LPC incorporated in the lipid vesicle membranes. The degree of inhibition was dependent on the LPC concentration incorporated. The higher the LPC concentration, the greater extent of inhibition. For example, incorporation of 20 mole% of LPC into lipid vesicles (DOPC/DOPE/cholesterol (1:1:1)) virtually inhibited lipid mixing completely for the case of 2.5 μM mutated peptide (E11V/E15V) and lipid vesicles of 75 μM lipids in the solution (Fig. 3).

These peptides also caused the leakage of lipid vesicles. Fig. 4 shows the time-dependent leakage of ANTS/DPX from the PC/PE/cholesterol (1:1:1) vesicles induced by E11V/E15V at various concentrations. The peptide-induced vesicle leakage correlated well with the lipid-mixing results. The time course of lipid mixing and vesicle leakage paralleled each other and the relative extents of lipid mixing and vesicle leakage were similar, although there were some differences. During the initial interaction period, the extent of vesicle mixing rate was faster (two to three times faster) in comparison to vesicle leakage. It is considered that the peptides interacting with lipid membranes caused both lipid mixing and membrane leakage. In this case also, the LPC-incorporated lipid vesicles suppressed the vesicle leakage induced by the peptides (Fig. 5). Although LPC alone has detergent-like properties and can lyse membranes at high concentrations, at the concentrations used in the present work we observed no leakage induced by the addition of LPC. LPC inhibits peptide-induced leakage. The peptide promotes vesicle leakage as a consequence of

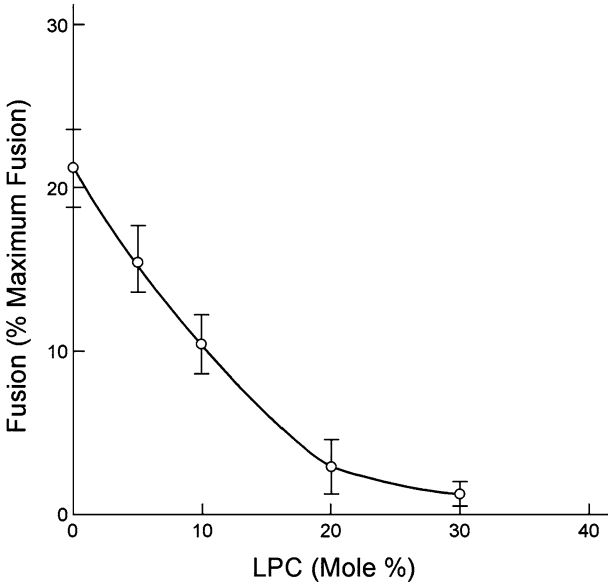

Fig. 3. The extent of vesicle fusion as a function of LPC concentration in vesicle membranes. The experimental procedures were similar to those in Fig. 1. The total lipid of vesicles suspended was 75 μM and that of the mutated fusion peptide was 2.5 μM. The experiments were performed in NTB, pH 7.4, at 37°C.

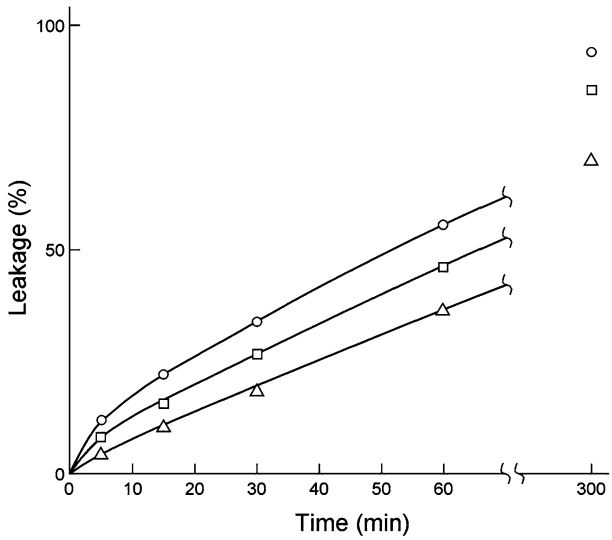

Fig. 4. Typical time courses of vesicle leakage measured by the ANTS/DPX vesicle leakage assay. The lipid concentration was 100 μM in NTB, pH 7.4 at 37°C and the concentrations of the mutated (E11V/E15V) fusion peptide used were 1.25 μM (Δ), 2.5 μM (□) and 5.0 μM (○), respectively.

vesicle-vesicle interaction that leads to both lipid mixing as well as membrane leakage. LPC inhibits both lipid mixing and content leakage as a result of inhibiting vesicle-vesicle interaction.

The aforementioned correlations can also be compared with the results of fluorescence studies on the interaction of fusion peptides with lipid

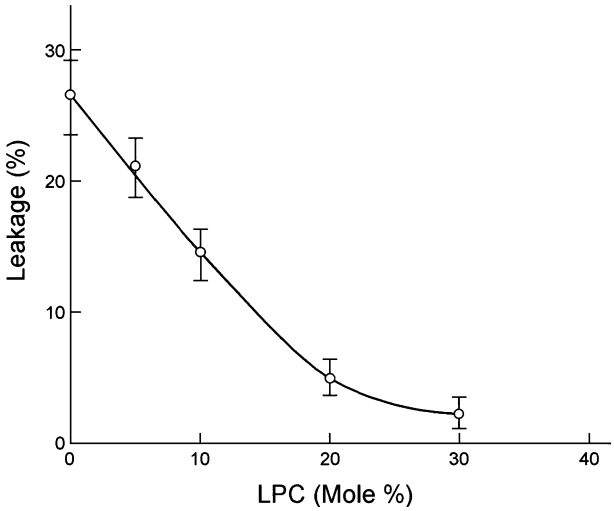

Fig. 5. The extent of vesicle leakage measured at 30 min by the same method as in Fig. 4 as a function of LPC concentration in the vesicle membranes. The lipid concentration was 100 μM in NTB, pH 7.4 at 37°C and the mutated (E11V/E15V) peptide concentration was 2.5 μM.

membranes, which are summarized in Table 1. The absorbance maximum of tryptophan of the fusion peptides in an aqueous solution was 280 ± 1 nm for both WT and the mutated peptides at pH 4.7 and 7.4 (or 7.3). The emission maximum was also approximately the same wavelength (351 ± 2 nm). In the presence of lipid vesicles in the aqueous solution, however, the emission maximum for the mutated peptide shifted to the blue by about 10 nm at both pH 4.7 and 7.4, while the wavelength of the absorption maxima of tryptophan was not altered. For the wild-type peptide, the fluorescence emission blue shift was about 15 nm at pH 7.4 and about 20 nm at pH 4.7, indicating that the interaction of the WT peptide with the membrane is pH dependent. This is consistent with the mentioned observation for the WT peptide-induced lipid mixing of vesicles, which was pH dependent.

For one set of our experiments, which was measured with a phase-modulated instrument [2], the mean excited-state lifetimes of tryptophan for these peptides were approximately the same, 2.5 ns, in aqueous solutions at pH 7.4 and pH 4.7 except for the lifetime of tryptophan fluorescence for the wild-type peptide at pH 4.7, which was 2.3 ns. The average excited-state lifetime was slightly shorter when the lipid vesicles were present in the aqueous solution (Table 1). For the other set of the experiments (Table 2), which was obtained by use of a time-resolved instrument [4], the mean excited-state lifetimes of tryptophan for the WT peptide was 2.6 ns at pH 4.7 and that in the presence of vesicle was longer. However, the rotational correlation time of tryptophan became much greater (five to ten times) in the presence of the lipid vesicle in aqueous solution and

Table 1. Fluorescence properties of intrinsic tryptophan of influenza virus fusion peptides.

Peptide (pH)	$\lambda_{Ex,\ Max}$ (nm)	$\lambda_{Em,\ Max}$ (nm)	Mean excited-state lifetime (ns)[a]	Steady-state anisotropy[b]	Rotational correlation time (ns)[c]
In NaCl buffer[d]:					
WT fusion peptide (4.7)	279	353	2.27	0.027	0.26
WT fusion peptide (7.4)	279	353	2.52	0.013	0.13
E11V/E15V (4.7)	279	350	2.59	0.030	0.34
E11V/E15V (7.4)	277	349	2.61	0.034	0.39
In NaCl buffer with lipid vesicles[e]					
WT fusion peptide (4.7)	282	331	2.06	0.087	1.04
WT fusion peptide (7.4)	281	338	2.26	0.072	0.87
E11V/E15V (4.7)	281	340	2.03	0.162	3.36
E11V/E15V (7.4)	281	342	1.80	0.141	2.13
In NaCl buffer with LPC-lipid vesicles[f]:					
E11V/E15V, 0 % LPC (7.3)	280	343	1.77	0.144	2.20
E11V/E15V, 12.5% LPC (7.3)	279	344	1.82	0.140	2.11
E11V/E15V, 25% LPC (7.3)	280	342	1.87	0.137	2.07

[a]The imprecision in the recovered excited-state life time is less than 5% RSD.
[b]The imprecision in the measured steady-state anisotropy is less than 3% RSD.
[c]The value of 0.26 for r_0 was used in calculating Trp rotational parameters from the Perrin expression (refs. 28, 33)
[d]10 μM peptide in 150 μM NaCl/10 mM sodium citrate (pH 4.7) or HEPES (pH 7.4)
[e]10 μM peptide in the presence of PC/PE (2:1) SUVs (100:1 lipid/peptide ratio)
[f]10 μM peptide in the presence of PC/PE (2:1) SUVs incorporated with different mole % LPC. Lipid/peptide ratio was 100:1.

Table 2. Fluorescence properties of intrinsic tryptophan of the WT influenza virus fusion peptide

Peptide (pH)	$\lambda_{Em.\ Max}$ (nm)	Steady-state anisotropy[a]	Mean excited-state lifetime (ns)[b]	Rotational correlation time (ns)[c]
In NaCl buffer[d] :				
WT fusion peptide (4.7)	352	0.026	2.60	0.29
In NaCl buffer with lipid vesicles[e]:				
WT fusion peptide (4.7)	335	0.096	3.82	2.23
In NaCl buffer with: LPC-lipid vesicles[f]:				
WT peptide, 0% LPC (4.7)	335	0.096	3.82	2.23
WT peptide, 12.5% LPC (4.7)	334	0.099	3.99	2.45
WT peptide, 25% LPC (4.7)	333	0.100	3.94	2.46

[a]λ_{Ex} = 275 nm; λ_{Em} = 290–450 nm; bandpass: Ex = 4 nm, Em = 8 nm.
[b]Mean excited-state lifetimes, $\tau_{av,}$ were obtained from the data given in Table 3 ($\tau_{av} = [\Sigma\alpha_i\ \tau_i^2 / \Sigma\alpha_i\ \tau_i\ ] = \Sigma f_i\ \tau_i$)
[c]Computed from Perrin equation assuming $r_0 = 0.26$ and τ_{av} is averaged excited-state life time from fit to a triple exponential decay model (Table 3)
[d]10 μM peptide in 150 μM NaCl/10 μM sodium citrate (pH 4.7)
[e]10 μM peptide in the presence of PC/PE (2:1) SUVs (100:1 lipid/peptide ratio)
[f]10 μM peptide in the preence of PC/PE (2:1) SUVs incorporated with different mole% LPC (mole% in the total lipids) (100:1 lipid/ peptide ratio).

also lower pH resulted in longer rotational correlation times than those at higher pH for both peptides, indicating clearly strong interaction of the peptides with membranes and more interaction at lower pH than at higher pH (*see* Table 1). In addition, the mutant peptide has a longer rotational correlation time in all conditions compared to the WT. This suggests that the greater hydrophobic nature of the mutant peptide allows it to interact more strongly with the membrane and to insert more deeply. For the LPC-containing vesicles, the rotational correlation time was virtually unchanged at different LPC concentrations, suggesting that the interaction of the peptide with lipid membranes was not greatly affected by the presence of LPC in the membranes up to 25 mole % LPC.

To provide some more information about the interaction of LPC incorporated into lipid membranes, the experiments of adsorption and desorption of LPC onto and from lipid monolayers and also the adsorption of the fusion peptide onto the lipid monolayers were performed. The first series of the experiments was the adsorption experiment of LPC from the aqueous subphase onto a lipid monolayer. A

Table 3. Summary of time-resolved intensity decay kinetics for tryptophan of the WT influenza virus peptide

Peptide (pH)	τ_1[b] (ns)	τ_2 (ns)	τ_3 (ns)	α_1[c]	α_2	α_3	f_1	f_2	f_3
In NaCl buffer[d] :									
WT fusion peptide (4.7)	5.29	2.13	0.40	0.040	0.303	0.307	0.219	0.653	0.128
In NaCl buffer with lipid vesicles[e]:									
WT fusion peptide (4.7)	8.04	3.45	0.81	0.019	0.210	0.143	0.153	0.727	0.120
In NaCl buffer with LPC-lipid vesicles[f]:									
WT peptide, 0% LPC (4.7)	8.04	3.45	0.81	0.019	0.210	0.143	0.153	0.727	0.120
WT peptide, 12.5% LPC (4.7)	7.97	3.51	0.81	0.021	0.208	0.119	0.170	0.731	0.099
WT peptide, 25% LPC (4.7)	7.93	3.55	0.89	0.020	0.205	0.121	0.159	0.729	0.112

[a]λ_{Ex} = 275 nm; λ_{Em} = 360, bandpass: Ex:32 nm, Em: 32 nm.
[b]τ_i is the *i*-th component of lifetime.
[c]α_i is the amplitude of the *i*-th component of lifetime.
[d]10 μM peptide in 150 μM NaCl/10 μM sodium citrate (pH 4.7) or HEPES (pH 7.4)
[e]10 μM peptide in the presence of PC/PE (2:1) SUVs (100:1 lipid/peptide ratio)
[f]10 μM peptide in the presence of PC/PE (2:1) SUVs incorporated with different mole % LPC. Lipid/peptide ratio was 100:1.

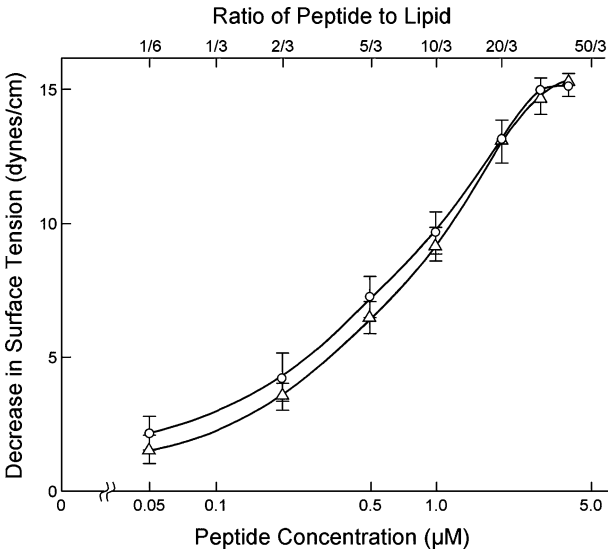

Fig. 6. The decrease in surface tension of DOPC/DOPE (2:1) monolayers containing 20 mole% lysolipid (Δ) and not containing lysolipid (○) as a function of the mutated (E11V/E15V) peptide concentration. The initial surface tension of the monolayer was 50 dynes/cm and after the monolayer formed and then, various amounts of the mutated peptide (in DMSO) were injected into the subphase of the monolayer. The experiment was performed at a room temperature of 24°C.

DOPC/DOPE (2:1) monolayer was first formed on the NHB surface at an area per molecule of 70 Å^2. Upon injection of an aliquot amount of LPC solution into the subphase and stirring the solution, a decrease in surface tension of the lipid monolayer occurred with time, which was considered to be due to the adsorption of lysolipid from the subphase solution onto the monolayer. For each experiment, a new lipid monolayer was prepared at the given area per molecule and thus it had approximately the same surface tension (~47 dynes/cm) initially. When the total number of LPC molecules in the subphase was greater than that of the lipid molecules of the monolayer, the monolayer surface tension decreased with different time courses but finally reached the same surface tension value (approximately 24.5 dynes/cm) at the stable state (at equilibrium). The time to reach half of the final surface tension value, $t_{1/2}$, varied with the concentration of LPC. For example, 10 μM LPC in the subphase is approximately 33 times more than that in the monolayer and its half time was approximately 40 s. In comparison, at 2 μM LPC in the subphase solution, the total number of LPC molecules corresponded to about 6.6 times the total number of lipid molecules in the monolayer, and $t_{1/2}$ was about 8 min. When the total number of LPC molecules injected into the subphase was less than that of lipid molecules of the monolayer, the rate of adsorption was very slow and the final equilibrium surface tension did not reach 24.5 dynes/cm but became stable at a value greater than 24.5 dynes/cm. In these cases, the final values were different at different LPC concentrations in the subphase (*results not shown*).

To observe LPC desorption from the membranes, the PC/PE monolayer-forming solutions containing LPC at different concentrations were used to form monolayers at a given area per molecule. After a transient change in surface tension, the surface tension stabilized within a minute or so. After that, there was no change in monolayer surface tension with time up to at least one hour. This result indicates that there was no desorption of LPC from the monolayer over at least an hour.

To determine whether LPC in the lipid monolayer influences the interaction of the peptides in the subphase with lipid monolayers, the adsorption of the peptides onto the monolayers containing different amounts of LPC was studied; the monolayer-forming

solution containing different concentrations of LPC was used to form monolayers at a given surface tension (i.e., 25 dynes/cm) on the NHB solution. Then, an aliquot of the peptide (E11V/E15V) solution in DMSO was injected into the subphase solution and the subphase was stirred well. The change in monolayer surface tension was then recorded. Typical experimental results are shown in Fig. 6. At 2 μM of peptide in the subphase, where the peptide and lipid ratio was approximately 20/3, the peptide adsorption to the monolayer was not affected by the presence of LPC (up to 25% mole concentration) in the lipid monolayer. As the ratio of peptide to lipid in the monolayer was reduced to less than 5/3, which corresponds to 0.5 μM peptide, the surface tension decrease was slightly less for the monolayer containing 20% LPC, which implies that the peptide adsorption was slightly reduced. However, these small differences in surface tension decreases between the monolayers with and without LPC were within the experimental error (1 dyne/cm) and we could not make a definite conclusion. Similar results were obtained for the monolayers at all concentrations of LPC incorporated. As a control, the effect of DMSO on the monolayer surface tension was measured. The addition of the DMSO only into the monolayer subphase, which corresponded to the maximum concentration of the peptide used in the experiments, caused only changes that were less than our experimental error.

Discussion

In this work, we have measured lipid mixing (vesicle fusion) between lipid vesicles and vesicle leakage induced by influenza fusion peptides (wild-type and mutated (E11V/E15V) peptides). The extent of lipid mixing between vesicles induced by the mutated peptide was several-fold greater in comparison to the wild-type peptide. While the vesicle lipid-mixing induced by the wild-type peptide showed a strong pH dependence, the mutated-peptide results showed only a slightly pH dependence; the extent of lipid mixing was greater at acidic pH (4.5–5.0) compared to neutral pH. A strong pH dependence of lipid mixing of lipid vesicles induced by the wild-type peptide has been reported by other workers [12, 34]. The extent of peptide-induced vesicle leakage was roughly parallel to that of lipid mixing between vesicles induced by the peptides. Thus, we think that both leakage and lipid mixing is caused by the interaction of the peptide with lipid membranes. The degree of interaction of peptides with lipid membranes was deduced from the rotational correlation time measured for tryptophan fluorescence of the fusion peptides with time-resolved fluorescence spectroscopy. When the peptides were in the solution containing lipid vesicles, the rotational correlation times were much longer (about 5–10 times longer) in comparison to those in the same aqueous solution in the absence of lipid vesicles. When the peptides were in the solution containing lipid vesicles, we assume that the peptides interacted with lipid vesicles and thus the peptide became less mobile. The mutated peptide showed a longer rotational correlation time with lipid membranes in comparison to the wild-type peptide, indicating that the mutated peptide has much stronger interaction or motional restriction with the lipid membranes in comparison to the wild-type peptide. The rotational correlation time data also indicated that both peptides interacted more strongly with membranes at pH 4.7 than at pH 7.4. These fluorescence results correlate well with those observed with vesicle fusion by the peptides at both pH values (5 and 7.4) and vesicle leakage. The rotational correlation times for the wild-type fusion peptides interacting with lipid vesicles at pH 5.0 and 7.4 were also measured by others [8], and their results show a similar trend with respect to pH as in this study. Peptide-induced vesicle fusion and leakage were inhibited by incorporation of LPC into the membrane in a concentration-dependent manner. Such inhibitions seem to be due to some alteration in the nature of the lipid membrane with incorporation of LPC, rather than the inhibition of the interaction between the peptide and lipid membrane by LPC in the membranes. This was concluded from the experiments of the peptide interaction with lipid membranes with and without LPC in the membrane by measuring the rotational correlation time of the tryptophan of the peptide and the adsorption of the peptide from the subphase onto lipid monolayers that do or don't contain lysolipid. Specifically, we found that the rotational correlation time was virtually unaffected regardless of the presence of LPC in the membrane and also the adsorption of the peptide onto monolayers was not altered with and without the incorporation of LPC into the monolayer. The fusion peptides are readily adsorbed onto lipid membranes with or without LPC. This result makes it unlikely that LPC inhibits by binding directly to the peptide, as suggested previously by Stegmann [16]. This conclusion agrees with that suggested by other workers using a different system [3]. Several other studies have also shown that LPC inhibits membrane fusion [1, 7, 22, 27]. One of the interpretations for inhibition of membrane fusion is that LPC in the outer membranes would inhibit the stalk formation between the interacting membranes as an intermediate stage for membrane fusion [7] and therefore inhibits membrane fusion. A second interpretation is that membrane fusion is initiated through a locally curved membrane area [24], which is the region of high surface energy or high surface tension, and LPC in membrane stabilizes a locally curved membrane, by lowering the surface energy, thus inhibiting

membrane fusion. In addition, it has been suggested that the presence of LPC in the target membrane may affect the conformation of the fusion peptide and thereby affect the rate of fusion [26].

Peptide adsorption onto the monolayer was not affected by the presence of LPC in the monolayer when the peptide concentration in the subphase was similar to that (i.e., 2 μM) for the peptide-induced vesicle fusion, while at the same peptide concentration range, LPC inhibited the peptide-induced vesicle fusion. These results suggest that the interaction of the peptide to the lipid membrane induced fusion of the membranes and lysolipid in the membrane prevented the fusion process. There may be an argument that at 2 μM peptide, for the monolayer system, the ratio of peptide to lipid was 20/3, while for the case of the vesicle fusion system, it was 1/30. However, the adsorption of peptide to membrane should be considered in terms of its chemical potential equilibrium between the aqueous and the membrane phases. The monolayer experimental system is not the same situation as that for the vesicle fusion experimental system; since in the vesicle fusion system, the concentration of lipid was much greater than that of the peptide, by the adsorption, the concentration of the peptide in the aqueous phase would reduce greatly, while in the monolayer system, the concentration of peptide does not change too much as long as the total amount of peptide is much larger than that of lipid. At these concentration ranges, the adsorption of peptide to the monolayer resulted in a trend of small reduction for the monolayer incorporated with LPC compared to that without LPC. However, the difference was within experimental error. Thus, the data from the rotational correlation time measurements would provide clearer evidence that LPC in lipid membranes does not affect the peptide interaction with lipid membranes.

In the present study, two more facts were obtained with regard to LPC in lipid membranes: 1) LPC is readily adsorbed onto lipid membranes from an aqueous solution and 2) LPC, once incorporated in the membrane, does not dissolve into the aqueous phase from the membrane. When the total amount of LPC in aqueous solution is greater than that of lipid molecules of the outer layer of the lipid membranes, the adsorption reached saturation and no further adsorption occurred. The surface tension at this saturation point for the air/water monolayer was approximately 24.5 dynes/cm. When the total amount of LPC was less than that of lipid molecules of the monolayer, the adsorption reached a certain value depending on the concentration of LPC in the aqueous solution and the system reached equilibrium. In such cases, the surface tension reached a value between 24.5 dynes/cm and that of the lipid monolayer without LPC, which was about 50 dynes /cm. To reach each equilibrium state, there was a specific time for each LPC concentration. From the force-area curve for the PC/PE (2:1) monolayer (*results not shown*), the surface tension of 24.5 dynes/cm is close to the monolayer collapse pressure (50 dynes/cm), which corresponds to the monolayer surface tension of 22 dynes/cm.

Another observation was that LPC virtually did not dissolve into the aqueous phase once it was incorporated into the monolayer membranes at least for one hour. These properties of LPC with regard to lipid membranes can provide relevant information for incorporation of LPC into membranes.

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