Lysine peptides induce lipid intermixing but not fusion between phosphatidic acid-containing vesicles

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Penta(L-lysine) and poly(L-lysine) (but not di- or tri(-L-lysine)) were found to induce rapid and extensive phospholipid intermixing between unilamellar vesicles consisting largely of phosphatidic acid and phosphatidylethanolamine, without causing significant intermixing of, nor leakage from, the encapsulated aqueous spaces. Neither did the size of such vesicles increase after treatment with lysine peptide, as determined by gel chromatography. The results suggest that lysine oligo- (or poly-) peptides may induce reversible semi-fusion between vesicles, but not complete membrane fusion.

Pentalysine *Polylysine* *Phosphatidic acid* *Lipid intermixing* *Membrane fusion*

1. INTRODUCTION

Membrane fusion is a ubiquitous cellular event, which is essential to activities such as secretion, uptake and degradation of macromolecules and particles, transport of certain nutrients and regulation of cell surface receptors and plasma membrane transport proteins. It is also utilized during infection by several enveloped viruses and intracellular parasites [1]. The potential role of Ca^{2+} and acidification as triggers of membrane fusion has been investigated in detail using model systems [2–6]. Major conclusions from this work are (i) that ionotropic fusion proceeds via reduction of the surface hydration of membranes and (ii) that phosphatidic acid is the only naturally occurring membrane phospholipid which is sensitive enough to physiologically relevant changes in Ca^{2+} or H^+ concentration to be involved in cellular membrane fusion.

However, there is reason to believe that membrane proteins may be directly involved, not only in membrane-membrane recognition but also in at least certain types of intracellular membrane fusion. The fact that certain viral proteins can induce membrane fusion [7] supports this suspicion, although the mechanisms involved here are not well understood. To investigate possible mechanisms of protein-induced membrane fusion we have undertaken studies of membrane vesicle interactions in a model system using synthetic lysine peptides to mimic basic peptide segments of membrane proteins.

2. MATERIALS AND METHODS

2.1. *Phospholipids*

Phosphatidylethanolamine (PE) was isolated from egg yolk [8], while phosphatidylcholine (PC) from egg yolk [9] and pure soybean PC obtained from Lucas Meyer (Hamburg) were used interchangeably. Soybean phosphatidic acid (PA) derived from soybean PC and phosphatidyl-[*Me*-^{3}H]choline were prepared as described [10]. In some experiments, pure PA purchased from Le Farm (Cologne), was used. *N*-(Lissamine-Rhodamine B-sulfonyl)dioleylphosphatidylethanolamine (N-Rh-PE) was purchased from Avanti Polar Lipids (Birmingham, AL), and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) was prepared essentially as in [11]. The purity of the fluorescent lipids was assessed by

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thin-layer chromatography in silica gel (solvent system: chloroform/methanol/water, 70:38:10).

2.2. *Other chemicals*

Phosphatidylethanol-*N*-lactobionamide (PELBA) was prepared as described [10]. *Ricinus communis* agglutinin (RCA) was obtained from Boehringer (Mannheim). Calcein (fluorexon) was purchased from EGA-Chemie (Steinheim) and was purified on Sephadex LH-20 [12]. Poly(L-lysine) (degree of polymerization 17000), was purchased from Sigma (St. Louis, MO). Di(L-lysine), tri(L-lysine), penta(L-lysine) and poly(L-lysine) (2–5 kDa) were obtained from Bachem (Bubendorf, Switzerland). Terbium chloride was purchased from Ventron (Karlsruhe), dipicolinic acid from Sigma and inositol hexaphosphoric acid from BDH (Poole, England). Penta(L-lysine) was found to be devoid of larger lysine peptides by gel chromatography on Sephadex G-25 (Pharmacia).

2.3. *Preparation of vesicles*

Large unilamellar phospholipid vesicles were prepared by reverse-phase evaporation [13]. The vesicles were extruded through a 0.2 μm polycarbonate membrane (Nucleopore, Pleasanton, CA). When small unilamellar vesicles were used, they were prepared as described in [14].

All vesicle preparations contained 10 mol% PELBA and, in the resonance energy transfer assay, 5 mol% of either N-NBD-PE or N-Rh-PE. The remaining phospholipid content of the vesicles was varied as described in the figure legends. Since the glycolipid PELBA was included in the vesicles, lectin-mediated intervesicle contact could be established as in [6].

2.4. *Assays for vesicle interactions*

2.4.1. Lipid intermixing

The resonance energy transfer assay for membrane lipid intermixing [15] was used as described [16]. The experimental details were as recently described [6]. In all assays, the peptide was injected as a 1 mg/ml solution. The values of relative fluorescence quenching were obtained as $Q_i = (F - F_i)/F$ expressed as percentage. Here, F_i = fluorescence after i min and Q_i = relative quenching after i min.

A new assay for lipid mixing was also applied, using PA-PE vesicles. Two preparations of sonicated vesicles were made, one with 30% PA, 50% PE and 20% PELBA; the other with 30% PA, 70% PE and a trace amount of [^{3}H]PC. Aliquots of each of the preparations, containing 90 nmol lipid, were mixed and incubated with basic peptide in 2 ml of the buffer used in [6], and were then agglutinated using RCA [10] and analyzed for radioactivity. Without lipid intermixing, the radiolabeled vesicles were not agglutinated, while complete intermixing of [^{3}H]PC and PELBA would render all vesicles radiolabeled and agglutinable.

2.4.2. Vesicle leakage

The calcein assay for assessment of vesicle leakage was used under the experimental conditions in [6]. The relative leakage was calculated by using $L_i = (F_i - F_0)/(F_t - F_0)$ expressed as percentage, where L_i = relative leakage after i min, F_i = fluorescence after i min and F_t = fluorescence after disrupting the vesicles.

2.4.3. Intermixing of aqueous contents

The fluorimetric assay described by Wilschut and Papahadjopoulos [17] was used under the experimental conditions in [18] except that 0.1 mM instead of 0.15 mM EDTA was used in the buffer and that the mixing of contents was monitored at 30°C instead of at 25°C. $M_i = (F_i - F_0)/(F_t - F_0)$ expressed as percent, where M_i = relative content intermixing and F_i and F_t are as defined above.

2.4.4. Gel chromatography

Gel chromatography was performed using a 1 × 60 cm column of Sephacryl S-1000 (Pharmacia) as in [19]. 0.5 ml of a suspension of small unilamellar vesicles (0.6 μmol lipid/ml) labeled with a trace amount of [^{3}H]PC was applied to the column, and fractions of 1 ml were collected. The flow rate was 0.2 ml/min.

3. RESULTS

3.1. *Effect of peptide size*

Oligo- and polymers of L-lysine were found to induce rapid and extensive lipid intermixing among vesicles containing PA and PE, as determined by fluorescence quenching between N-NBD-PE and N-Rh-PE. As shown in fig.1A, trilysine was ineffective, while pentalysine was almost as potent as

polylysine (2–5 kDa). We also observed that di- and trilysine were ineffective in vesicles brought in contact by the lectin RCA. The use of an independent assay for lipid intermixing (table 1) confirmed these results.

Interestingly, lipid intermixing was accompanied by no (pentalysine) or limited (polylysine) perturbation of vesicle integrity. The latter was determined from the release of calcein from the encapsulated aqueous space (fig.1B). A further increase in the lysine polymer size did not enhance lipid intermixing, but somewhat increased the perturbation of vesicle integrity.

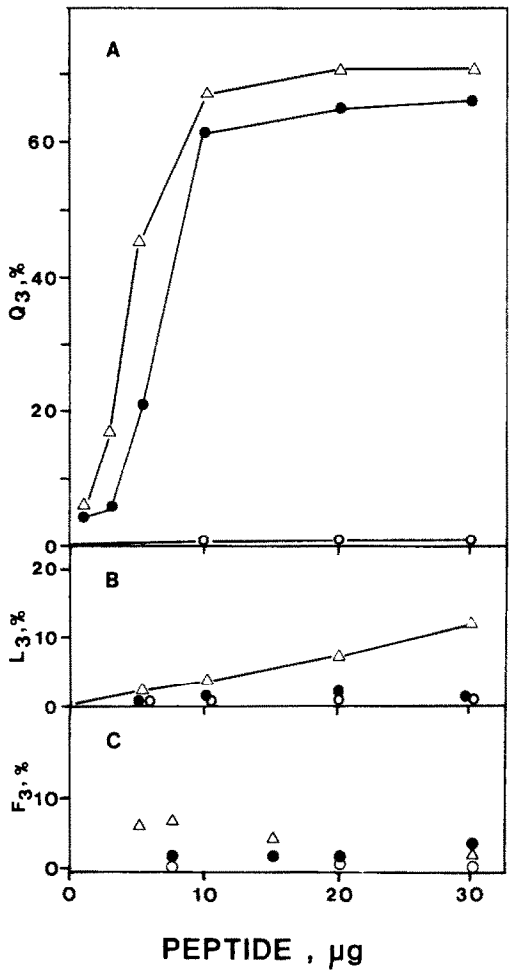

Fig.1. Lipid intermixing (A), vesicle leakage (B) and intermixing of vesicle contents (C) induced by lysine peptides differing in size. Lipid intermixing among vesicles containing 30 mol% PA and 55 mol% PE was monitored by fluorescence resonance energy transfer, leakage by the release of encapsulated calcein, and vesicle content intermixing by the formation of fluorescent Tb-DPA complexes. Trilysine (○), pentalysine (●) and polylysine (2–5 kDa) (△).

Table 1

Lipid intermixing assessed by the isotope mixing assay

Conditions	*A*	No.of experiments
Control	6.3	4
Pentalysine (15 μg), 3 min	46.8	2
Pentalysine (30 μg), 3 min	45.4	3
Trilysine (30 μg), 3 min	1.1	3
Pentalysine (30 μg), without RCA	−1.3	2

The intermixing of [^{3}H]PC and/or the glycophospholipid PELBA among small unilamellar PA-PE (30:60) vesicles was determined as described in section 2.4. It is expressed as A = (activity in agglutinated vesicles)/(total activity), and given as a percentage. Controls were agglutinated with RCA after incubation without lysine peptide

3.2. *Effect of vesicle composition*

An electrostatic interaction between peptide and lipid vesicles is clearly involved in the responses described above. Vesicles lacking an anionic phospholipid did not show any lipid intermixing when pentalysine was added (not shown). On the other hand, the response was also significantly modulated by replacement of PE with PC. The latter lipid caused inhibition of lipid intermixing among vesicles also containing PA (fig.2). This inhibition was not due to impaired intervesicle contact, since it also occurred in the presence of lectin.

3.3. *Lack of complete vesicle fusion*

The lipid intermixing described above appears to be far too rapid to be caused by molecular exchange of lipid between the vesicles (fig.3), but could conceivably be due to peptide-induced vesicle fusion, as has been suggested regarding polylysine [20]. However, we have been unable to demonstrate true fusion, defined as intermixing also of the interior aqueous spaces of the vesicles (fig.1C) although the fluorimetric assay used is highly sensitive under the present conditions of no, or very limited vesicle leakage. Furthermore, the

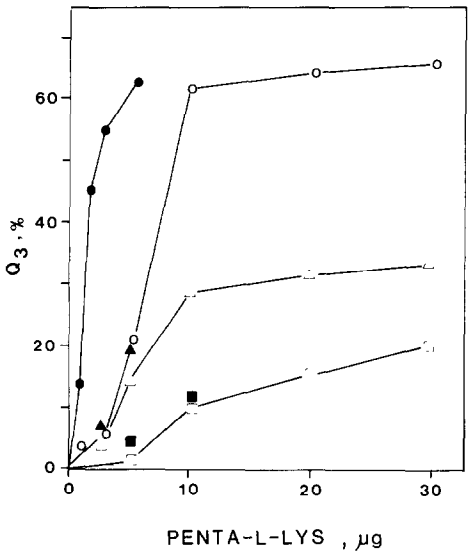

Fig.2. Pentalysine-induced lipid intermixing among PA-PE-PELBA-fluorophore (30:55:10:5) vesicles in which PE was exchanged for PC to the extent of 0 (○, ●), 20 (△, ▲) or 50 (□, ■) mol% of the total vesicle lipids. Filled symbols denote the presence of lectin (RCA).

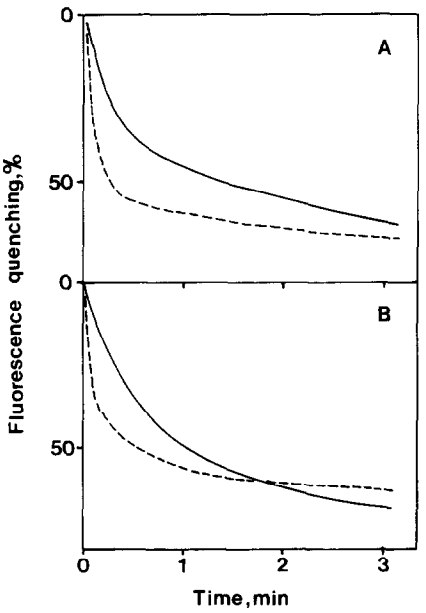

Fig.3. Kinetics of fluorescence quenching due to resonance energy transfer for PA-PE-PELBA-fluorophore (30:55:10:5) vesicles. In (A), the vesicles were incubated with 10 μg pentalysine (——) or with RCA (120 μg) followed by 6 μg pentalysine (– – –); in (B), with 10 μg polylysine (2–5 kDa) (——) or with RCA (120 μg) followed by 5 μg polylysine (2–5 kDa) (– – –).

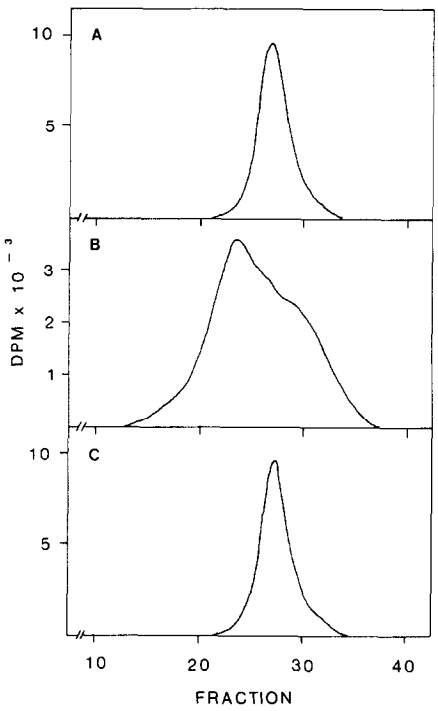

Fig.4. Gel filtration of radiolabeled small unilamellar vesicles containing 30 mol% PA, 60 mol% PE and 10 mol% PELBA. The vesicles were chromatographed on a Sephacryl S-1000 column without further treatment (A), after treatment with 4 mM Ca^{2+} followed 3 min later by 6 mM EDTA (B), or pentalysine (40 μg/ml, broken line or 80 μg/ml, solid line) followed 3 min later by 0.1 mM inositol hexaphosphoric acid (C).

size of vesicles has been determined by gel chromatography on high-porosity polyacrylamide, after reversal of the treatment with peptide by the addition of inositol hexaphosphoric acid. The results indicated that pentalysine, in contrast to Ca^{2+}, induced a reversible vesicle interaction, which was not accompanied by an increase in vesicle size (fig.4).

4. DISCUSSION

It has previously been concluded that polylysine induces fusion between anionic lipid vesicles containing PE [20,21]. This conclusion was mainly based on the ability of polylysine to induce vesicle aggregation, vesicle lipid intermixing and leakage of vesicle contents.

Our results show that lysine peptides, including pentalysine, do indeed induce rapid and extensive lipid intermixing among vesicles containing phosphatidic acid and PE. However, this interaction is accompanied neither by a growth in vesicle size, as determined by gel chromatography, nor by an intermixing of intravesicular aqueous spaces. In the case of pentalysine the leakage from vesicles is also negligible. True vesicle fusion can therefore, apparently, be ruled out as the mechanism responsible for lipid intermixing. These findings also point out that fusion assays based on assessment of lipid intermixing may give falsely positive results.

It has been shown by De Kruijff and Cullis [22] that polylysine triggers a bilayer-to-hexagonal H_{II} transition in cardiolipin-PE systems and intramembrane particles, or inverted micellar structures, are likely intermediates in this transition [23,24]. We propose that the lysine peptides induce bilayer joining at sites of vesicle contact, possibly involving the formation of inverted micellar structures. In the present system these structures would then be readily reversible upon removal of the peptide and not proceed further; neither to complete fusion nor to a macroscopic hexagonal H_{II} phase.

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