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# Promotion of acid-induced membrane fusion by basic peptides. Amino acid and phospholipid specificities

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The ability of oligo- and polymers of the basic amino acids L-lysine, L-arginine, L-histidine and L-ornithine to induce lipid intermixing and membrane fusion among vesicles containing various anionic phospholipids has been investigated. Among vesicles consisting of either phosphatidylinositol or mixtures of phosphatidic acid and phosphatidylethanolamine rapid and extensive lipid intermixing, but not complete fusion, was induced at neutral pH by poly-L-ornithine or L-lysine peptides of five or more residues. When phosphatidylcholine was included in the vesicles, the lipid intermixing was severely inhibited. Such lipid intermixing was also much less pronounced among phosphatidylserine vesicles. Poly-L-arginine provoked considerable leakage from the various anionic vesicles and caused significantly less lipid intermixing than L-lysine peptides at neutral pH. When the addition of basic amino acid polymer was followed by acidification to pH 5–6, vesicle fusion was induced. Fusion was more pronounced among vesicles containing phosphatidylserine or phosphatidic acid than among those containing phosphatidylinositol, and occurred also with vesicles whose composition resembles that of cellular membranes (i.e., phosphatidylcholine / phosphatidylethanolamine / phosphatidylserine, 50:30:20, by mol). Liposomes with this composition are resistant to fusion by  $\text{Ca}^{2+}$  or by acidification after lectin-mediated contact. The tight interaction among vesicles at neutral pH, resulting in lipid intermixing, does not seem to be necessary for the fusion occurring after acidification, but the basic peptides nevertheless appear to play a more active role in the fusion process than simply bringing the vesicles in contact. However, protonation of the polymer side chains and transformation of the polymer into a polycation does not explain the need for acidification, since the pH-dependence was quite similar for poly(L-histidine)- and poly(L-lysine)-mediated fusion.

## Introduction

Membrane fusion constitutes a critical step in cellular processes such as secretion by exocytosis, endocytic uptake and degradation of macromolecules, particles and certain nutrients and also in the regulation of cell surface receptors and plasma membrane transport proteins. Membrane fusion is also utilized by enveloped viruses, toxin-producing bacteria and intracellular parasites during infection. The potential role of calcium ions and changes in pH as triggers of membrane fusion and the modulatory role of phospholipid head groups have been investigated in model systems [1–6]. It is very

likely, however, that in cellular systems membrane proteins take part not only in membrane-membrane recognition, but also in the actual fusion event.

We have undertaken studies of membrane vesicle interactions using synthetic oligo- and polypeptides to mimic basic peptide segments of membrane proteins. It is well known that many integral membrane proteins contain a cluster of basic residues on the cytosolic side of a membrane-penetrating hydrophobic peptide segment [7,8]. Such basic peptide segments might, potentially, play a role in membrane interactions and fusion, with or without demasking by proteolysis. We showed previously that homo-oligomers containing five or more L-lysine residues could induce extensive lipid intermixing at neutral pH among vesicles composed of phosphatidic acid and phosphatidylethanolamine, but not complete fusion-fission with intermixing also of the encapsulated aqueous spaces [9]. Furthermore, poly(L-histidine) has been found to cause fusion among vesicles containing various anionic phospholipids with or without zwitterionic phospholipids, in a process that

Abbreviations: *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine-Rhodamine B-sulfonyl)-dioleylethanolamine.

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depended on subsequent acidification [10,11]. It was proposed that the requirement for acidification resided in the need for polyhistidine to become protonated and polycationic. In the present paper we have compared the ability of homo-polymers of L-lysine, L-histidine, L-arginine and L-ornithine, with and without acidification, to induce lipid intermixing and fusion among phospholipid vesicles varying in composition.

## Materials and Methods

### Materials

Nonlabeled and fluorescent phospholipids were obtained [6] and phosphatidyl[Me-<sup>3</sup>H]choline was prepared [12] as described earlier. Calcein (fluorexon) was purchased from EGA-CHEMIE (Steinheim, F.R.G.) and was purified on Sephadex LH-20 [13]. Poly(L-lysine) (degree of polymerization 17 000), poly(L-arginine) ( $M_r$  44 000), poly(L-ornithine) ( $M_r$  30 000) and poly(L-histidine) ( $M_r$  11 000) were purchased from Sigma (St. Louis, MO). Di(L-lysine), tri(L-lysine), tetra(L-lysine), penta(L-lysine), poly(L-lysine) (2–5 kDa), di(L-arginine) and tri(L-arginine) were obtained from Bachem (Bubendorf, Switzerland). Penta(L-lysine) was found to be devoid of larger lysine peptides by gel chromatography on Sephadex G-25. Inositol hexaphosphoric acid was purchased from BDH (Poole, U.K.) and methyl  $\beta$ -D-galactopyranoside from Serva Feinbiochimica (Heidelberg).

### Preparation of vesicles

Large unilamellar phospholipid vesicles were prepared by reverse-phase evaporation [14]. The vesicles were extruded through a 0.2  $\mu$ m polycarbonate membrane (Nuclepore, Pleasanton, CA). Small unilamellar vesicles were prepared as described earlier [15]. The glycolipid phosphatidylethanol-*N*-lactobionamide (10 mol%) was included in most vesicle preparations to allow a direct comparison with earlier data where vesicles were brought in contact by a lectin [6,16].

### Determination of lipid intermixing

An assay based on fluorescence resonance energy transfer [17] was used to determine vesicle lipid intermixing essentially as described [5]. Then the vesicle preparations contained 5 mol% of either *N*-NBD-PE or *N*-Rh-PE. Control experiments showed that the kinetics of resonance energy transfer were the same when the vesicles contained 1 instead of 5 mol% of the fluorophores, excluding the possibility of interference from self-quenching. As shown elsewhere [9], lipid intermixing assessed by resonance energy transfer could be verified by an independent method that determines the intermixing of radiolabeled phosphatidylcholine and a glycopospholipid. In the assay, vesicles containing 60

nmol of lipid were added to 2 ml of a buffer containing 0.15 M NaCl/20 mM Hepes/1 mM EDTA (pH = 7.5). Half of the vesicles contained the energy donor (*N*-NBD-PE) and the other half the energy acceptor (*N*-Rh-PE). The sample was transferred to a cuvette equipped with an adjustable magnetic stirrer and was thermostated at 30 °C. An Aminco-Bowman spectrofluorimeter was used to measure fluorescence quenching due to resonance energy transfer. The fluorescence (excitation/emission at 470/532 nm) was recorded continuously, and values of relative fluorescence quenching were calculated as  $Q_i = (F_0 - F_i)/F_0$  expressed as a percentage. Here,  $F_i$  = fluorescence after  $i$  min and  $Q_i$  = relative quenching after  $i$  min.

### Assay for vesicle leakage

Calcein was encapsulated in vesicles at a self-quenching concentration (29 mM). Large unilamellar vesicles were used, and they were freed of nonencapsulated calcein on a column of Sephadex G-75 (Pharmacia). The experimental conditions have been described earlier [6]. The relative leakage was calculated as  $L_i = (F_i - F_0)/(F_t - F_0)$  expressed as a percentage, where  $L_i$  = relative leakage after  $i$  min,  $F_i$  = fluorescence after  $i$  min and  $F_t$  = fluorescence after disrupting the vesicles with Triton X-100.

### Assessment of vesicle content intermixing

The fluorimetric assay for intermixing of vesicle contents, based on formation of fluorescent complexes between terbium ions and dipicolinic acid [18,19] was used under the conditions earlier described [3] except that the mixing of contents was monitored at 30 °C instead of at 25 °C. Large unilamellar vesicles were used. In several experiments the retention of the terbium-dipicolinic acid signal that developed in response to peptide and acidification, was assessed by readjustment of pH (using 0.1 M NaOH) and de-aggregation of the vesicles with the sodium salt of inositol hexaphosphoric acid (0.1 M).

### Gel chromatography

To assess changes in the size of small unilamellar vesicles, gel chromatography was performed on a 1  $\times$  60 cm column of Sephacryl S-1000 (Pharmacia-LKB), pre-saturated with non-radiolabeled phospholipid vesicles. Before chromatography, vesicle samples were treated with basic peptide for 1 min and were then subjected to acidification as indicated. After 2 min pH was readjusted to 7.5 and inositol hexaphosphoric acid (sodium salt) was added to a final concentration of 0.1 M. Samples of 0.5 ml, containing 0.3  $\mu$ mol of vesicle lipid and a trace amount of [<sup>3</sup>H]phosphatidylcholine, were taken out and applied to the column. Fractions of 1 ml were collected at a flow rate of 0.2 ml/min.

## Results and Discussion

### Interactions among phosphatidic acid-containing vesicles

In agreement with previous results [9], the addition of poly(L-lysine) to large unilamellar vesicles composed largely of phosphatidic acid and phosphatidylethanolamine (1:2, by mol) induced rapid and extensive lipid intermixing with only limited release of vesicle contents (Fig. 1A). In contrast, corresponding amounts of poly(L-arginine) induced remarkable leakage of vesicle contents, but only limited lipid intermixing (Fig. 1A). Tri(L-arginine) was unable to induce either lipid intermixing or vesicle leakage (not shown). Quite small quantities (3–5  $\mu\text{g}$ ) of poly(L-ornithine) caused, like poly(L-lysine), extensive lipid intermixing at neutral pH (Fig. 1B), while increasing amounts lead to progressive release of vesicle contents and then a reduction of both lipid intermixing and vesicle leakage. This indicates that the basic poly(amino acid) at higher concentrations caused less cross-linking of vesicles and instead preferentially interacted with individual vesicles. Poly(L-ornithine)-induced lipid intermixing, like that induced by the L-lysine peptides [9], was reduced when part of the phosphatidylethanolamine was exchanged for phosphatidylcholine (Fig. 1B). The sensitivity to poly(L-ornithine) or L-lysine peptides was somewhat enhanced when the vesicles were previously brought in contact by a lectin (not shown), although lectin agglutination itself does not lead to lipid intermixing [6,20].

Any significant complete membrane fusion, defined as vesicle content intermixing, was not observed in response to L-lysine peptides or poly(L-histidine) at neutral pH (Fig. 2). However, when the incubation with basic peptide at neutral pH was followed by acidification, significant intermixing of vesicle contents occurred (Fig. 2), as well as some vesicle leakage (not shown). Poly(L-lysine) and poly(L-histidine) supported such acid-induced vesicle fusion equally well, while the extent of content intermixing was somewhat lower with penta(L-lysine). The terbium fluorescence that developed after acidification was rather well retained after readjustment of pH and de-aggregation of the vesicles (Fig. 2), indicating that it reflected the coalescence of vesicle-encapsulated aqueous spaces. In view of these results it was surprising that only a marginal increase in the size of these vesicles (less than that expected from one round of fusion) could be detected by gel chromatography (not shown). On the other hand, neither did the lectin-mediated, proton-induced vesicle interaction that resulted in content intermixing lead to any significant increase in vesicle size [6,16], nor was any considerable growth of similar vesicles reported after treatment with poly(L-histidine) followed by acidification [10]. Similar results were obtained also with vesicles consisting of phosphatidylethanol (not shown). One possible explanation for the apparent discrepancy between vesicle content intermixing and the size of the resulting vesicles is suggested by the finding that large

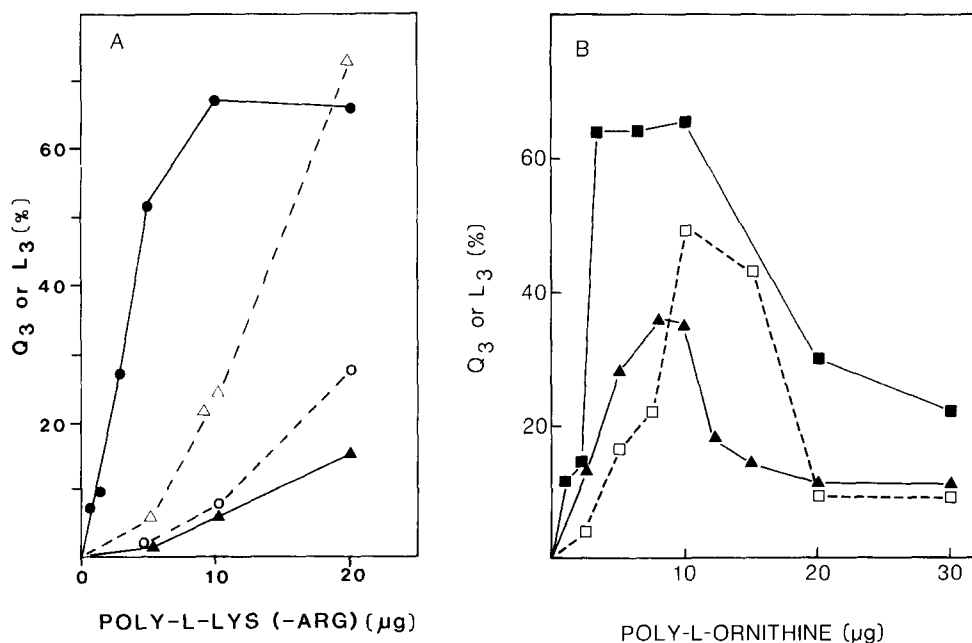


Fig. 1. (A) Resonance energy transfer (solid lines) and leakage (broken lines) seen when large unilamellar vesicles consisting of phosphatidylethanolamine, phosphatidic acid and phosphatidylethanol-*N*-lactobionamide (60:30:10, by mol), with and without fluorophore, were incubated with poly(L-lysine) (●, ○) or poly(L-arginine) (▲, △) for 3 min before measurement. (B) Resonance energy transfer (solid lines) and leakage (broken line) in response to poly(L-ornithine) using vesicles of the same composition (■, □), or with 35 (▲) of the total 60 mol% of phosphatidylethanolamine exchanged for phosphatidylcholine.  $Q_3$  and  $L_3$  are defined in Materials and Methods.

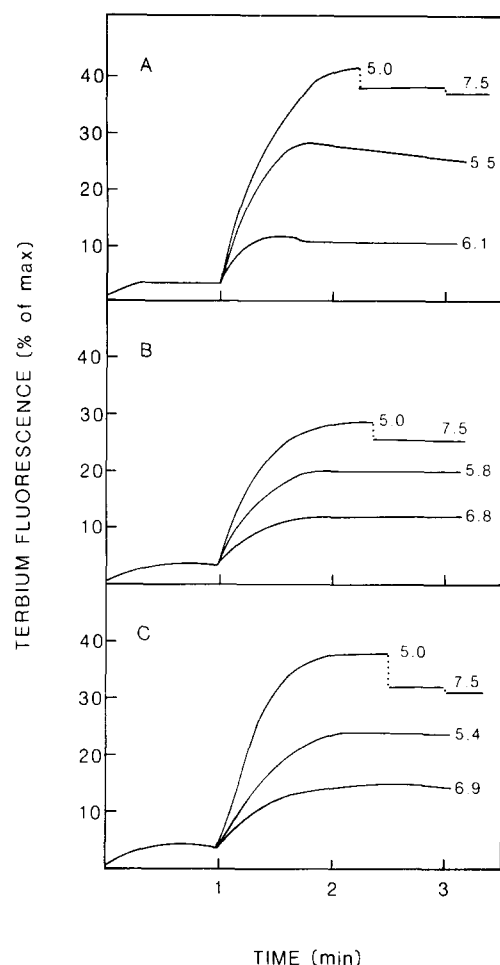


Fig. 2. Intermixing of contents among large unilamellar vesicles containing phosphatidylethanolamine, phosphatidic acid and phosphatidylethanol-*N*-lactobionamide (60:30:10, by mol) upon treatment with 10  $\mu\text{g}/\text{ml}$  of poly(L-lysine) (A), penta(L-lysine) (B) or poly(L-histidine) (C) followed by adjustment of pH 1 min later, as indicated. In samples adjusted to pH 5.0 the conditions causing fusion were reversed by readjustment of pH to 7.5, followed by the addition of inositol hexaphosphoric acid ( $\text{Na}^+$  salt; 0.1 M).

phosphatidic acid-containing vesicles may rearrange into smaller vesicles when the pH is raised [21,22], since pH was readjusted from acidic to neutral before gel chromatography.

When phosphatidylcholine replaced phosphatidylethanolamine in the phosphatidic acid-containing vesicles, peptide-induced lipid intermixing at neutral pH was virtually completely inhibited (Fig. 3). The response to subsequent acidification was maintained, however, with extensive lipid intermixing manifested as resonance energy transfer (Fig. 3), as well as intermixing of vesicle contents (Fig. 4) in the presence of both poly(L-lysine) and poly(L-histidine). The response in the presence of penta(L-lysine) was reduced, while poly(L-ornithine) supported acid-induced lipid intermixing to the same extent as poly(L-histidine) (Fig. 3). These results are strikingly different from those seen when

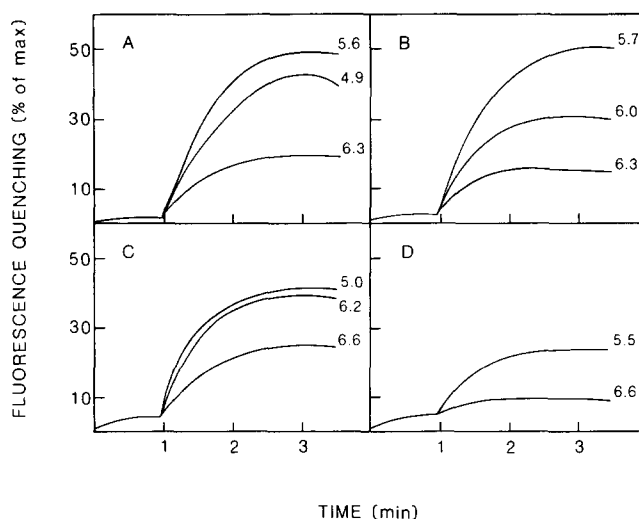


Fig. 3. Lipid intermixing among small unilamellar vesicles composed of phosphatidylcholine, phosphatidic acid and phosphatidylethanol-*N*-lactobionamide (60:30:10, by mol). The vesicles were treated with 10  $\mu\text{g}/\text{ml}$  of either poly(L-ornithine) (A), poly(L-histidine) (B), poly(L-lysine) (2–5 kDa; C), or penta(L-lysine) (D), in each case followed by adjustment of pH after 1 min as indicated.

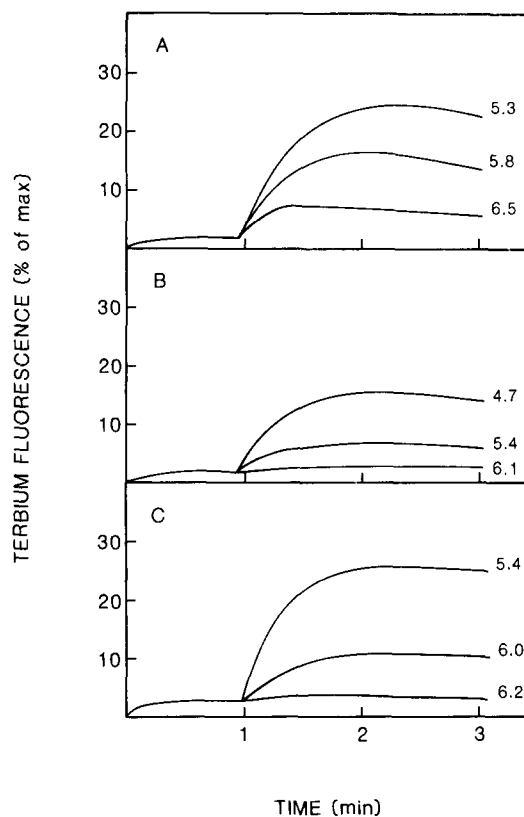


Fig. 4. Intermixing of contents among large unilamellar vesicles consisting of phosphatidylcholine, phosphatidic acid and phosphatidylethanol-*N*-lactobionamide (60:30:10, by mol), upon treatment with 10  $\mu\text{g}/\text{ml}$  of poly(L-lysine) (A), penta(L-lysine) (B) or poly(L-histidine) (C) and subsequent acidification (1 min later) to the final pH indicated.

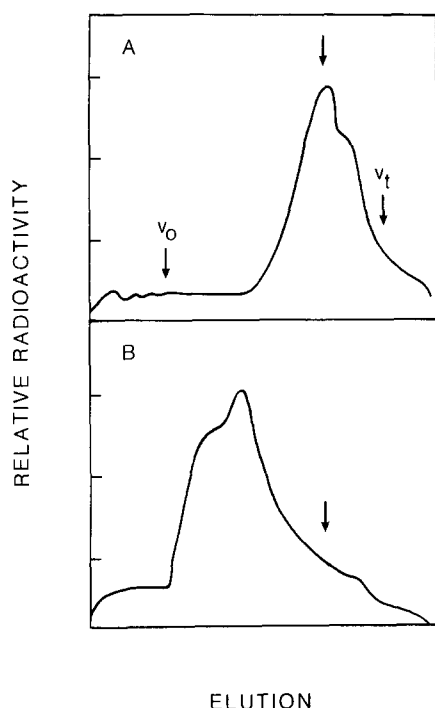


Fig. 5. Gel chromatography of small unilamellar vesicles consisting of phosphatidylcholine, phosphatidic acid and glycolipid (60:30:10, by mol). Treatments were: control (A) and poly(L-lysine) followed by acidification to pH 5.0 (B). Fusing conditions were reversed (see Materials and Methods) before chromatography. The void volume ( $V_0$ ) and total volume ( $V_t$ ) of the column, as well as the elution position of control vesicles, are marked by arrows.

intervescicle contact is established with a lectin. Then, neither lipid intermixing nor intermixing of vesicle contents could be induced by  $\text{Ca}^{2+}$  or by acidification among vesicles of this composition (not shown). The leakage induced by acidification to pH 4–6, in the

presence of any of the basic peptides, was quite small (< 20%) in the case of phosphatidic acid-phosphatidylcholine vesicles. As seen in Fig. 5, there was a clear increase in the size of small unilamellar vesicles with the same lipid composition, after pretreatment with poly(L-lysine) and acidification to pH 5.0. Similar results were obtained with poly(L-histidine) (not shown). Treatment with either of these peptides at neutral pH caused no increase in vesicle size. Neither could we detect any increase in the size of pure phosphatidylcholine vesicles after treatment with poly(L-lysine) or poly(L-histidine), followed by acidification (not shown).

#### *Vesicles containing phosphatidylinositol*

Large unilamellar vesicles consisting of phosphatidylinositol underwent surprisingly extensive lipid intermixing upon addition of L-lysine peptides at neutral pH (Fig. 6A). Inclusion of 60 mol percent phosphatidylcholine virtually abolished this vesicle interaction, while it was essentially unaffected by inclusion of the same amount of phosphatidylethanolamine. As with vesicles containing other anionic phospholipids, poly(L-arginine) induced less lipid mixing but more leakage (not shown). The basic peptides were unable to cause intermixing of vesicle contents among phosphatidylinositol vesicles at neutral pH (Fig. 7) despite extensive lipid intermixing, but subsequent acidification led to significant intermixing also of vesicle contents (Fig. 7). Poly(L-histidine), poly(L-lysine) and poly(L-arginine) were more efficient than penta(L-lysine) in this respect.

#### *Phosphatidylserine-containing vesicles*

Large unilamellar vesicles consisting of phosphatidylserine (Fig. 6B) or its mixtures with phosphatidyl-

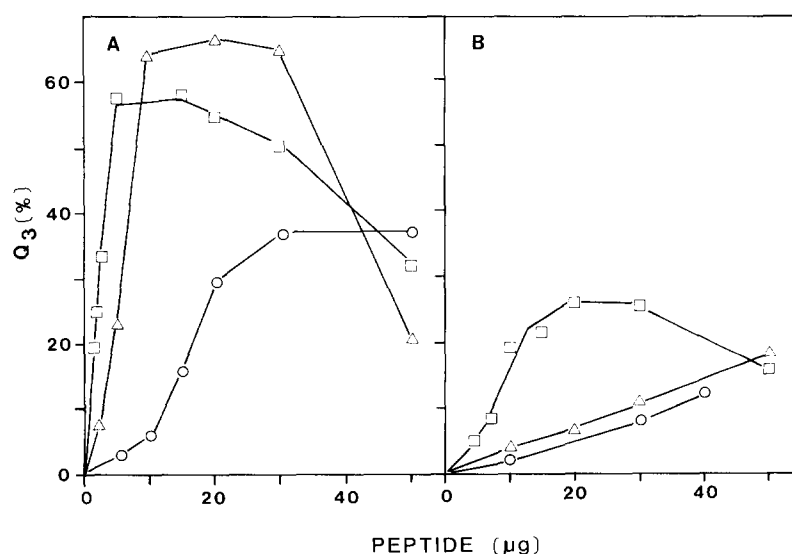


Fig. 6. Lipid intermixing among large unilamellar vesicles composed of phosphatidylinositol (A) or phosphatidylserine (B), with glycolipid (10 mol%) and fluorophore (5 mol%), upon treatment for 3 min with varying amounts of penta(L-lysine) (○), poly(L-lysine) (2–5 kDa) (Δ) or poly(L-lysine) (degree of polymerization 17000) (□).

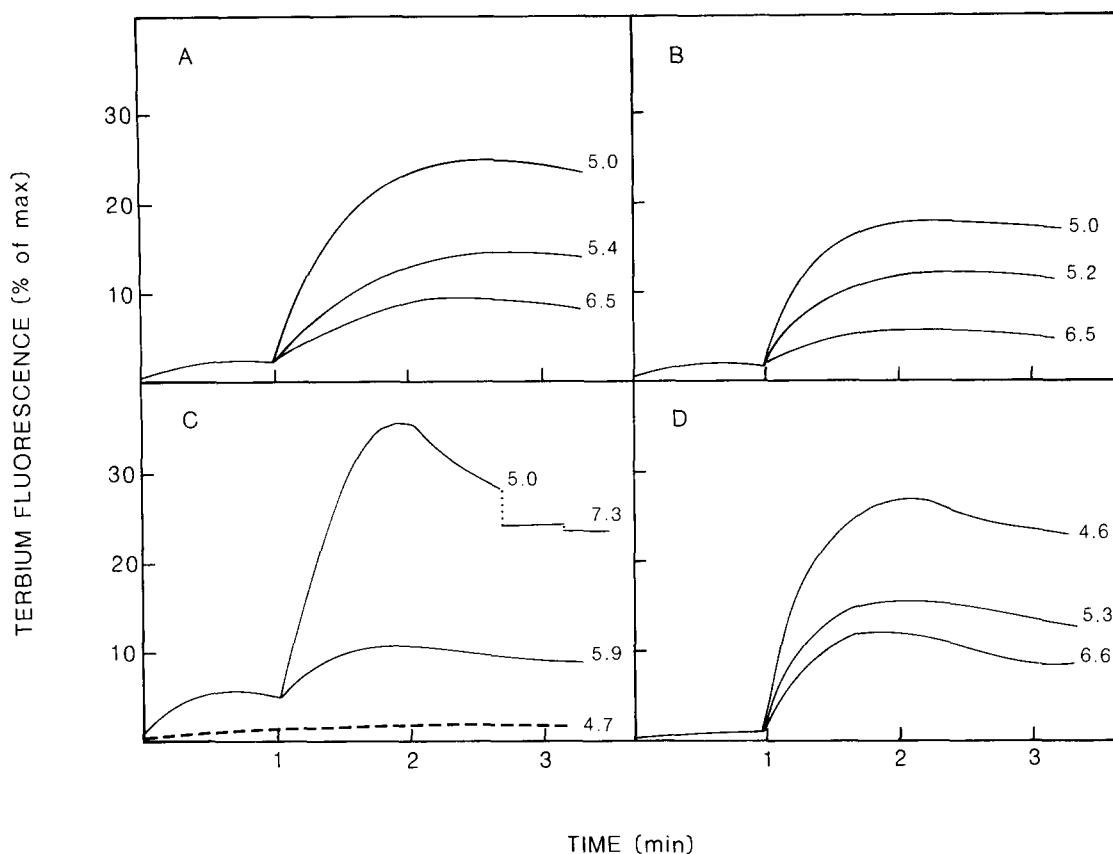


Fig. 7. Intermixing of contents among large unilamellar vesicles composed of phosphatidylinositol and glycolipid (90:10, by mol). Treatment with peptide (10  $\mu\text{g}/\text{ml}$ ) for 1 min was followed by acidification to the pH indicated. Peptides were: poly(L-histidine) (A), penta(L-lysine) (B), poly(L-arginine) (solid lines) or tri(L-arginine) (broken line) (C), or poly(L-lysine) (degree of polymerization 17000) (D). In (C; pH 5.0), the fusing conditions were reversed as described in the legend to Fig. 2.

ethanolamine (not shown) showed only limited lipid intermixing upon the addition of basic peptide at neutral pH, although a certain difference was seen between the response to poly(L-lysine) (2–5 kDa) and that to either penta(L-lysine) or poly(L-lysine) (degree of polymerization 17000) (Fig. 6B). Peptide-induced vesicle aggregation monitored by light-scattering was rapid and extensive when the longer peptides were added (not shown). This pronounced aggregation, coupled with only limited resonance energy transfer, argues against the possibility that the latter would be due to energy transfer between aggregated vesicles [23]. Furthermore, the lipid intermixing observed among phosphatidic acid-containing vesicles was confirmed by an independent assay for lipid intermixing [9]. As was the case with phosphatidic acid-containing vesicles, leakage from phosphatidylserine vesicles was quite small with L-lysine peptides and poly(L-histidine), but larger with poly(L-arginine). When pH was lowered after the addition of either poly(L-arginine), poly(L-histidine) or penta(L-lysine), rapid and extensive lipid intermixing occurred (Fig. 8). This response was seen with both small and large unilamellar vesicles and was, in contrast to the limited resonance energy transfer observed at neutral

pH, accompanied by intermixing of vesicle contents as determined by the terbium-dipicolinic acid method (not shown). There was good agreement between the assay for vesicle content intermixing and that for lipid intermixing with respect to both pH-dependence and peptide specificity. Some lipid intermixing was also supported by tri(L-lysine), in contrast to what was observed with vesicles containing other anionic phospholipids, but not by di(L-lysine) or tri(L-arginine) (Fig. 8). A comparison with acid-induced fusion among phosphatidylserine vesicles brought in contact by lectin–glycolipid interaction [6] shows that there is a clear difference in pH-dependence. Lectin-aggregated vesicles undergo fusion only at or below pH 4.5, while fusion is observed at significantly higher pH after vesicle aggregation by basic oligo- or polypeptides.

Among vesicles composed of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine in proportions similar to those occurring in mammalian cell membranes, there was again no or only limited lipid intermixing in response to the basic polypeptides at neutral pH, while significant lipid intermixing occurred after subsequent acidification (Fig. 9). The degree of lipid intermixing was somewhat lower than with vesicles

consisting largely of phosphatidylserine and a more extensive acidification was required, in particular in the presence of penta(L-lysine). Nevertheless, this lipid intermixing was, except in the case of penta(L-lysine), accompanied by an intermixing of vesicle contents (Fig. 10 D–F) that was similar to that seen with phosphatidylserine/phosphatidylethanolamine vesicles (Fig. 10A–C). As with vesicles containing only phosphatidylserine (not shown), a considerable increase in the size of small unilamellar vesicles, as determined by gel chromatography, was seen upon treatment of either of these vesicles with poly(L-histidine) and acidification to pH 5–5.5. After treatment with poly(L-lysine) or poly(L-ornithine), the acid-induced increase in vesicle size was rather less pronounced, and with penta(L-lysine), no

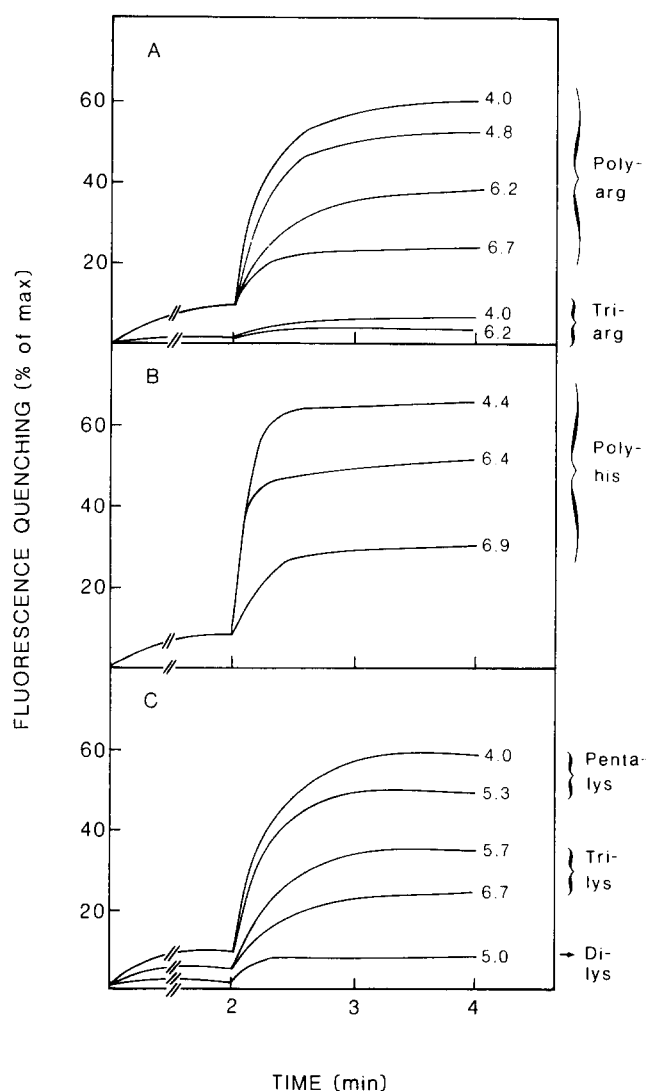


Fig. 8. Kinetics of lipid intermixing among large unilamellar vesicles consisting of phosphatidylserine and phosphatidylethanol-*N*-lactobionamide (10 mol%). The addition of peptide (10  $\mu\text{g/ml}$ ) was followed 2 min later by acidification to the final pH indicated. (A) Tri(L-arginine) or poly(L-arginine), (B) poly(L-histidine) and (C) di(L-lysine), tri(L-lysine) or penta(L-lysine).

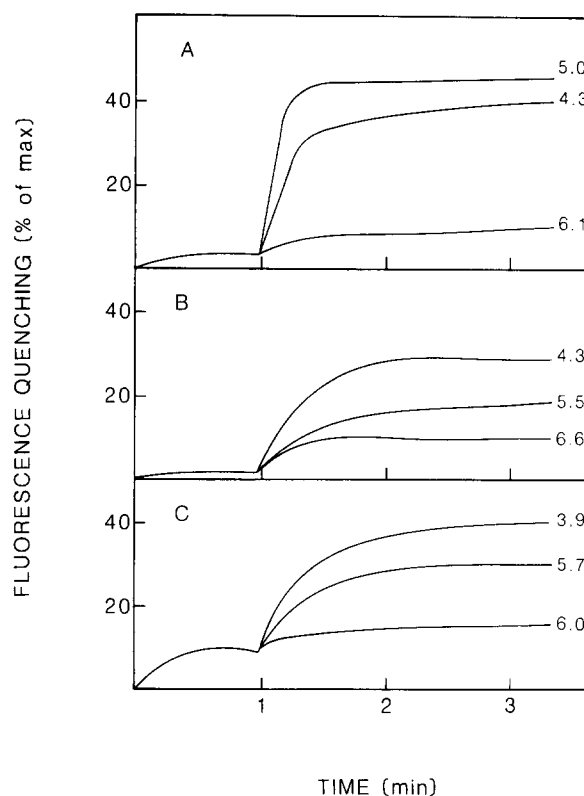


Fig. 9. Lipid intermixing among small unilamellar vesicles composed of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (20:30:50, by mol). In (A), poly(L-histidine) was added; in (B), poly(L-lysine) (2–5 kDa), and in (C) poly(L-arginine). In each case 10  $\mu\text{g/ml}$  of peptide was added at time 0 and the pH adjusted as indicated 1 min later.

such increase in vesicle size could be detected (not shown).

### Concluding remarks

The present paper extends previous studies of the interactions between basic oligo- and poly-(amino acids) and anionic liposomes [9–11,24–26] and identifies both amino acid and phospholipid specificities in these interactions. Thus, at neutral pH poly(L-arginine) interacts with the anionic liposomes in a more disruptive way than L-ornithine and L-lysine peptides that contain primary amino groups in their side chains. Furthermore, the latter induce significantly less lipid intermixing among phosphatidylserine- as compared to phosphatidylinositol- or phosphatidic acid-containing vesicles. Electrostatic interactions between adjacent phosphatidylserine molecules, involving the primary amino group of serine, might lead to a somewhat weaker interaction with L-lysine and L-ornithine peptides and thereby less lipid intermixing.

Acid-induced fusion among anionic phospholipid vesicles has been observed in earlier studies, where the vesicles were brought in contact by either a lectin [6] or

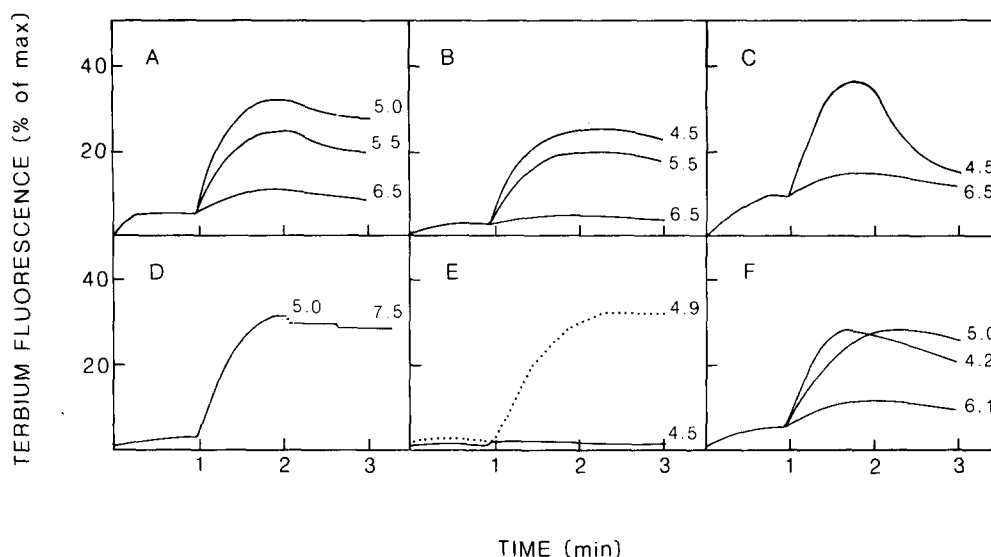


Fig. 10. Intermixing of contents among large unilamellar vesicles composed of phosphatidylserine, phosphatidylethanolamine and glycolipid (30:60:10, by mol) (A–C), or phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine (20:30:50, by mol) (D–F). Treatments were: poly(L-histidine) (A) and (D); penta(L-lysine) (B) and (E); poly(L-lysine) (E, broken line); and poly(L-arginine) (C) and (F). At time 0, 10  $\mu\text{g}/\text{ml}$  of peptide was added and 1 min later the pH was adjusted as indicated. In (D) the fusing conditions were reversed as described in the legend to Fig. 2.

poly(L-histidine) [10,11]. In the former case, protonation and dehydration of phospholipid head groups appears to be the cause of fusion. Accordingly, phosphatidylserine vesicles, in contrast to phosphatidic acid-containing vesicles, fused only at or below pH 4.5, the  $\text{pK}_a$  of the serine carboxyl group. In the latter case [10,11], fusion was triggered already at or below pH 6 and it was suggested that the pH dependence was due to protonation of the poly(L-histidine). Our present results, showing a similar pH dependence for the fusion of anionic vesicles with either poly(L-lysine) or poly(L-histidine), call for a different interpretation. Although other factors might contribute, we favour the idea that acidification also in this case triggers fusion primarily by causing protonation and dehydration of phospholipid head groups. The difference in pH dependence for peptide- and lectin-mediated fusion could reflect a difference in intermembrane distance, with a closer contact and even partial dehydration in the case of basic peptides. This interpretation is further discussed elsewhere [16]. Furthermore, the present results raise doubts regarding any direct relationship between the lipid intermixing induced at neutral pH and the fusion induced by subsequent acidification. Thus, phosphatidylcholine severely inhibits the former but much less so the latter and while the former is much more pronounced for phosphatidylinositol as compared to phosphatidylserine vesicles, the opposite holds for acid-induced fusion. Finally, it is remarkable that liposomes resembling mammalian cell membranes in composition undergo significant fusion at pH 5.0–5.5 in the presence of basic

polypeptide. However, it should be noted that five basic residues (i.e., penta(L-lysine)) is insufficient to support the acid-induced fusion of such vesicles.

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