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Polylysine induces pH-dependent fusion of acidic phospholipid vesicles: a model for polycation-induced fusion

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Polylysine induced aggregation and fusion of negatively charged small unilamellar phosphatidylcholine vesicles containing at least 10% anionic lipid. Aggregation was followed by absorbance changes and fusion was assayed both by electron microscopy and by fluorescence energy transfer between lipid probes. A method for preparing asymmetric vesicles, where the fluorescent probes were present only in the inner monolayer of the vesicle membrane, was developed. These vesicles were used to distinguish the inner and outer monolayer when measuring lipid mixing between vesicles. Since polylysine induced lipid mixing of both monolayers equally, fusion of these vesicles did occur. The extent of fusion was dependent on the charge ratio between bound polylysine and phosphatidylserine (PS) in the outer monolayer and was optimal at a ratio of about 1:1. Excess polylysine inhibited fusion. At a given concentration of polypeptide, fusion increased as the pH was lowered toward 3 with an apparent pK_a near 4. Since this value is close to the pK_a of the PS-carboxyl groups and far from the pK_a of the lysine ε-amino groups, the pH dependence observed for fusion resides in the lipids rather than in the peptide. Fusion was dependent on the available lysine and not the size or molarity of the polypeptide. The data indicate that there must be sufficient sites on the vesicles and sufficient polypeptide to achieve effective aggregation. For fusion to occur after aggregation, charges on the vesicles must be neutralized either by polypeptide–PS interaction or by protonation of the PS carboxyl groups. Optimal conditions for fusion occur when charge neutralization is possible without completely covering the vesicles with polypeptide. The results are consistent with the notion that the polypeptide is necessary for fusion because of requirements for crosslinking, but limits fusion by steric inhibition.

Introduction

Although it has been shown that charged bilayers spontaneously fuse in the presence of millimolar concentrations of divalent cations, it is apparent that physiological fusion processes are more complicated. The lipid and ion concentration requirements for divalent cation-induced fusion of

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; Rho-PE, lissamine rhodamine sulfonyl phosphatidylethanolamine; PE, phosphatidylethanolamine; DPPG, dipalmitoylphosphatidylglycerol; SUV, small unilamellar vesicles; REV, reverse-phase ether-evaporated vesicles; E_{ET}, energy transfer efficiency; A, absorbance.

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lipid bilayers have been extensively studied. Bilayer fusion will occur at unphysiologically high concentrations of calcium and with high amounts of fusogenic lipids (e.g., phosphatidylethanolamine, cardiolipin, phosphatidylserine) in the bilayer [1,2]. A number of proteins are also capable of mediating bilayer fusion. They include proteins which have evolved to fuse membranes such as viral spike proteins (e.g., Refs. 3, 4) as well as some that are not likely to participate in biological fusions (e.g., bovine serum albumin [5]). Protein-mediated fusion can involve both hydrophobic and electrostatic interactions between the protein and the participating membranes.

In this paper, we focus on the electrostatic portion of the interaction by considering the polycation polylysine, which we show induces pH-dependent fusion of PC vesicles containing at least 10% PS. We examine the basis for the pH dependence and describe the effects of varying parameters related to polycation-induced fusion: (a) the mol% of anionic lipid, (b) the amount of lysine available for binding and charge neutralization, and (c) the pH.

Polylysine-induced vesicle fusions have been reported previously [6–10]. In contrast to most of these reports on polylysine-induced fusion, our vesicle lipid composition is resistant to divalent cation induced fusion. Moreover, by combining fusion measurements with binding and aggregation determinations, we distinguished various parameters that are important when soluble proteins induce fusion.

We measured fusion in terms of lipid mixing followed by energy transfer between two fluorescent lipid probes. To demonstrate that the observed lipid mixing is due to vesicle fusion rather than some other lipid mixing process is generally difficult. We used a nonspecific phospholipid exchange protein to prepare asymmetrically labeled vesicles that allowed us to distinguish dilution of the inner and outer monolayers. This assay makes it possible to clearly establish that ordered mixing of both monolayers occurs as expected for true fusion events, as opposed to other lipid mixing processes (monomer exchange, inverted micellar intermediates) that will disproportionately involve the outer monolayer or result in random mixing of both monolayers (detergent effects).

Materials and Methods

Materials. Polylysines (M_r 4000, 16000, 20000, 90000) were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were prepared in distilled water and stored frozen. The lipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and stored at −70°C until used. All experiments were done at room temperature (22–25°C).

Electron microscopy. Lipid samples were made 30% w/w in glycerol and frozen in gold cups in Freon-22. Freeze fractures were performed in a Balzars BA 360-M apparatus and replicas were floated on water. The replicas were washed for 60 min in Clorox, twice with water, and mounted on bare 75 × 300 mesh copper grids. Residual lipid was removed from replicas by chloroform evaporation. The samples were examined in a Siemens IA electron microscope.

Aggregation. Vesicle aggregation was followed by absorbance (A) changes at 400 nm under conditions similar to those used for fusion measurements [11]. These vesicles did not contain the fluorescent probes so all the A changes were due to light scattering.

Lipid mixing. Sonicated unilamellar vesicles (SUVs) were prepared from phosphatidylcholine (PC) and phosphatidylserine (PS) as described previously [12]. Apparent fusion was monitored by following the energy transfer efficiency between two fluorescent probes incorporated in one population of the vesicles (1 mol% each of *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) and lissamine rhodamine sulfonyl phosphatidylethanolamine (Rho-PE)) [12–16]. Energy transfer efficiency (E_{ET}) is a function of the probe surface density and the assay monitors the dilution of the fluorescent lipids by following the decrease in energy transfer from NBD-PE to Rho-PE. The fluorescence intensity of Rho-PE was virtually independent of pH from pH 3 to 7.4 and that of NBD-PE decreased slightly at the lowest pH values. All experiments were done at constant pH. An apparent E_{ET} was determined by dispersing the membranes with detergent ($C_{12}E_9$) which was assumed to be equivalent to infinite dilution of the probes with no energy transfer so that:

$$E_{ET} = 1 - \frac{F_x}{F_d} \quad (1)$$

where F_x and F_d are the fluorescence at 530 nm at the time of observation and in the presence of detergent, respectively. We mixed labeled and unlabeled vesicles at a ratio of 1 : 20 (unless noted otherwise) and report the change in energy transfer efficiency (ΔE_{ET}) from the initial value to that observed at 10 min. ΔE_{ET} of about 1 is equivalent to complete dispersal of the probes. The extent of fusion is indicative of the fraction of vesicles able to fuse after the initial aggregation induced by polylysine.

Asymmetric vesicle preparation. SUVs containing the two fluorescent probes on the inner monolayer only were prepared by using the nonspecific phospholipid exchange protein isolated as described by Crain and Zilversmit [17]. The isolated phospholipid exchange protein ran as a single band on SDS-polyacrylamide gels. The concept for preparing the vesicles is similar to that of Pagano et al. [18], who used lipid capable of spontaneous exchange to form asymmetric vesicles. Fluorescently labeled SUVs prepared as described above were mixed with a 10-fold excess of unlabeled vesicles prepared by ether evaporation (REVs) [19] composed of PS : PC (1 : 1). The REVs served as an acceptor for the fluorescently labeled lipids on the outer monolayer of the SUVs and a source of replacement lipid for PS and PC. Identical mixtures were incubated in the absence of phospholipid exchange protein to serve as a control for spontaneous lipid exchange. Initial samples were taken to determine E_{ET} and lipid concentration. The mixtures were incubated for 24 h on a rotating shaker at 37°C and samples were taken periodically to follow the progress of the exchange. The two populations of vesicles were separated by centrifugation in an airfuge (120 000 $\times g_{av}$) for 5 min, causing the REVs to pellet and leaving the SUVs in the supernatant. The E_{ET} in the SUV portion was determined as described by Eqn. 1 and the concentrations of the two probes were determined fluorescently by direct excitation in detergent-dispersed aliquots. The E_{ET} and amount of fluorescent lipid remaining in the SUVs after 24 h are given in Table I as the percentage of the initial values for control vesicles and for those incubated in the presence of phospholipid exchange protein. The amount of NBD-PE and Rho-PE decreased by about 10% in the vesicles incubated in the absence of phospholipid exchange protein, presumably due to spontaneous exchange. However, the E_{ET} of vesicles incubated with phospholipid exchange protein remained identical to the initial values but the amount of NBD-PE and Rho-PE was reduced to about 50% of the initial values, as would be expected if the outer monolayer lipids were replaced and the inner monolayer remained unperturbed. It is important to note that the initial values were determined immediately after the phospholipid exchange protein addition so that the decreases in the amounts of NBD-PE and Rho-PE after incubation with phospholipid exchange protein are not due to direct phospholipid exchange effects on fluorescence quantum yield.

TABLE I

CHARACTERISTICS OF ASYMMETRIC VESICLES

Amounts of NBD-PE and Rho-PE (F_{NBD}, F_{Rho}, respectively) and E_{ET} of fluorescently labeled SUV before and after incubation (24 h, 37°C) with unlabeled REV in the absence (control) or presence of nonspecific phospholipid exchange protein are given as percent of the original values. F_{NBD} and F_{Rho} were measured after dissolving the vesicles with detergent.

	F_{NBD}	F_{Rho}	E_{ET}
Control	91	91	96
+ exchange protein	55	54	97

Binding. Polylysine binding to PS : PC (1 : 1) REVs was measured as a function of pH and peptide molecular weight. Binding was determined from the amount of polylysine remaining in the supernatant after centrifuging a mixture of polypeptide and vesicles at 120 000 $\times g_{av}$ in an airfuge (Beckman). The polylysines were ^{14}C-labeled by reductive addition of formaldehyde to the ϵ-amino groups. Approximately 5% of the groups were labeled, giving specific activities of 2.02 mCi/mmol lysine, 5.3 mCi/mmol lysine and 5.5 mCi/mmol lysine for the 4000, 22 000 and 90 000 M_r polylysines, respectively. Vesicles (97 μM total lipid) and polylysine were mixed and samples were taken for liquid scintillation counting before and after centrifugation. REVs were chosen for these experiments because they pellet in the absence of aggregation, ensuring that any peptide

associated with the vesicles will be in the pellet regardless of the extent of aggregation.

Results

Polylysine-induced fusion

The electron micrographs (Fig. 1) show that incubation of PS : PC vesicles with polylysine (M_r 20 000) produced aggregation. In addition, the size of the vesicles increased (Fig. 1B and C) compared to control vesicles (Fig. 1A), which had not been exposed to polylysine. Examination of five random fields for each treatment indicated that the average size increased at least 3-fold upon exposure to the polypeptide. The very large vesicles observed in Fig. 1B after exposure to pH 3.5 were not observed in the preparations at pH 7.4.

In order to quantify this aggregation and fusion, we measured A and fluorescence energy transfer between lipid probes incorporated into the vesicles. Fig. 2A shows typical traces of the absorbance changes at 400 nm due to aggregation induced by the addition of polylysine as a func-

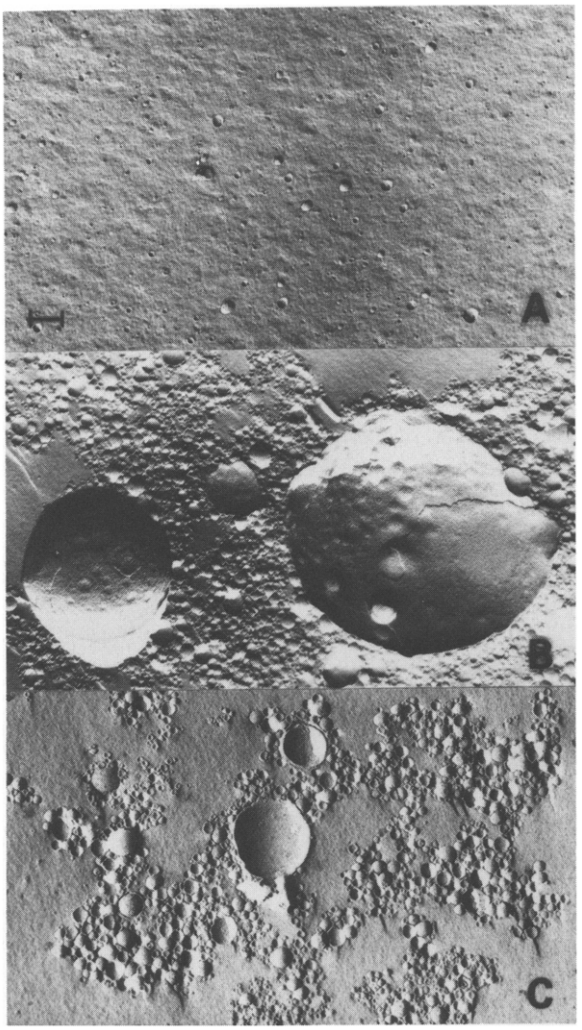

Fig. 1. Freeze cleavage electron micrographs of SUVs before (A) and after the addition of polylysine at pH 3.5 (B) and 7.4 (C). The bar represents 100 nm.

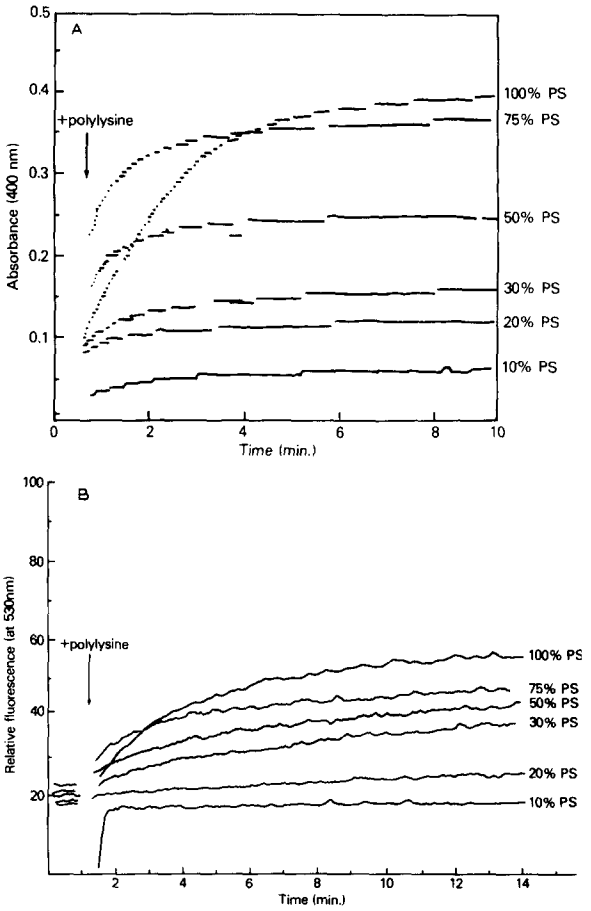

Fig. 2. Increases in absorbance (aggregation) (A) and relief of rhodamine-quenching of NBD-PE (B) as a function of time after the addition of polylysine (M_r 16 000) to SUVs (PS : PC, 50 μg/ml) at pH 7.4. The mole ratio of lysine : serine was constant (1 : 1). No fluorescent probes were present for the absorbance measurements. For the lipid mixing determinations, 5% of the SUVs were labeled with 1 mol% each of Rho-PE and NBD-PE. Fluorescence data are expressed relative to the value after addition of detergent ($C_{12}E_9$) to totally disperse the lipids.

tion of time. In the experiment shown in Fig. 2B, NBD-PE and Rho-PE labeled vesicles were mixed with unlabeled vesicles in a 1 : 20 ratio. The fluorescence at 530 nm (F_{530}) was monitored after addition of polylysine. We observed a fluorescence increase at 530 nm due to reduced energy transfer between the probes as they were diluted over a larger surface area upon fusion: similar kinetics were reported by Uster and Deamer [10]. (Changes in light scattering associated with aggregation contribute less than 5% to F_{530}.)

Increases in F_{530} could be due to direct effects of the polycation binding on E_{ET} affecting the quantum yield or due to lipid mixing resulting from mechanisms other than fusion such as an increase in the exchange rate among lipids in the outer monolayers. That the energy transfer changes were actually reporting fusion is indicated in several ways. We know that the F_{530} increase is not simply caused by interaction of polylysine with the probe, since we observed no change when polylysine was added to vesicles labeled with NBD-PE only. Second, increases in NBD-PE fluorescence were always matched by concomitant decreases in Rho-PE fluorescence. Finally, we observed that an increase in E_{ET} (a decrease in F_{530}) occurred when vesicles containing NBD-PE were mixed with vesicles containing Rho-PE in the presence of polylysine (data not shown).

To exclude the possibility that polylysine induced lipid exchange, we prepared vesicles with fluorescent probes only on the inner monolayer (see Methods, Table I). If polylysine were simply enhancing lipid monomer exchange or joining only the outer monolayers via a non-bilayer structure such as an inverted micelle, one would expect no dilution of the fluorescent probes with the asymmetric vesicles. Our observations are given in Table II, where the actual changes in fluorescence are compared with the predicted changes if only the outer monolayers mix or if all the lipids mix. As seen with both symmetric and asymmetric vesicles, the observed change in fluorescence closely matches the predicted change for both monolayers mixing with the respective monolayer of the unlabeled vesicles. The amount of probe dilution in the asymmetric vesicles is nearly identical to that observed for symmetric vesicles (Table II). This indicates that the lipid mixing is an

TABLE II

PREDICTED AND OBSERVED RESULTS FOR LIPID MIXING WITH SYMMETRICAL AND ASYMMETRICAL PROBE DISTRIBUTION WHEN LABELED AND UNLABELED PROBES ARE MIXED IN A RATIO OF 1:10 FOR 10 min AT pH 4

Predicted results are calculated from Fung and Stryer [13] where E_{ET} is related to probe surface density: $(\Delta A/A_0) = \ln(F_i/F_{max})/\ln(F_x/F_{max})$ where F_i and F_x are the initial and observed fluorescence at 530 nm, respectively. F_{max} is the NBD-PE fluorescence when the probes are at infinite dilution. ΔA = change in area/probe. A_0 = initial area/probe.

	$(\Delta A/A_0)_{predicted}$	$(\Delta A/A_0)_{observed}$
Symmetric vesicles		
exchange	6.7 [a]	
fusion	10	9.5
complete mixing	10	
Asymmetric vesicles		
exchange	0	
fusion	10	9.6
complete mixing	32 [a]	

[a] Based on outer : inner surface area of SUV being 2 : 1 [34].

ordered event where the two monolayers maintain their integrity, as expected for fusion (as opposed to dissolution due to detergent-like effects). (If the fusion products of SUVs swell to form spheres, the amount of lipid on the inner monolayer must increase and the inner monolayer $\Delta A/A$ for 10 fused vesicles would be 14 instead of 10. Similarly, $\Delta A/A$ for asymmetric vesicles fusing among themselves should be 0.4 instead of 0 if flip-flop occurs. Our data suggest this does not happen. In practice, however, our results are not sufficiently accurate for a firm conclusion, especially if only a fraction of the population fuses or becomes spherical or the swelling is not complete.) Moreover, we observed very minimal release of liposome-encapsulated lucifer yellow (measured by the relief of self-quenching) on the addition of polylysine under low pH conditions where virtually 100% lipid mixing occurs (data not shown).

Larger unilamellar vesicles (REVs) did not fuse perceptibly at neutral pH and fused slightly (about 10% of the observed signal with SUVs) at pH 3, although aggregation was apparent under similar conditions. (Vesicles of intermediate size do fuse and fusion appears to be graded with size (A. Walter, M. Kolber, and R. Blumenthal, unpublished results).)

The possibility that fusion resulted from a synergistic effect of polylysine and calcium was considered. Fusion was not inhibited by the addition of EDTA (up to 10 mM) or citrate (10 mM) nor enhanced by the addition of calcium (up to 15 mM). A very small amount of Ca^{2+}-induced fluorescence change was observed at low pH in both the absence and presence of polylysine.

Effect of the mol% acidic phospholipids

Aggregation and fusion induced by polylysine (M_r 16000) increased as the mol% of PS increased (Fig. 2). These experiments were done at constant lysine : serine ratio (1 : 1 mol ratio). The extents of aggregation and fusion as reported by the plateau values in Fig. 2 were plotted as a function of mol% PS in Fig. 3. Aggregation increased linearly with the fraction of PS, but fusion increased more rapidly between 10 and 30 mol% than between 30 and 100 mol% PS. Maximal fusion per aggregate was observed at 30 mol% PS but maximal fusion was observed at 100% PS. However, we note that the rates of fusion and aggregation are significantly slower at 100% PS than at lower PS mol fractions (Fig. 2). No fusion was observed in the absence of PS (100% PC) at any polylysine concentration, even after long incubation times.

PC is considered an inhibitor of fusion so the increased fusion at higher PS might be due to the decrease in the fraction of 'inhibiting lipid' rather than an increase in surface charge density. However, vesicles composed of PE : PS : PC (4 : 1 : 5) did not fuse significantly at neutral pH even though PE is considered a fusogenic lipid (data not shown).

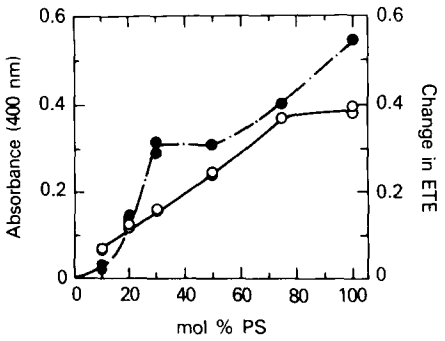

Fig. 3. ΔE_{ET} (closed circles) and absorbance (400 nm) (open circles) as a function of mol% PS in PC vesicles (50 μg/ml). Measurements were done at pH 7.4 at a constant lysine : serine ratio (1 : 1) using M_r 16000 polylysine. ETE, energy transfer efficiency.

pH dependence of fusion

We noted in our electron micrographs (Fig. 1) that very large fusion products were only observed at pH 3. In order to determine whether aggregation and fusion are pH-dependent, we measured absorbance and ΔE_{ET} as a function of pH for PS : PC (1 : 1 mol ratio) vesicles in the presence of polylysine (M_r 20000 (Fig. 4). Fusion increased as the pH was lowered, with maximal ΔE_{ET} at pH 3, the greatest change being between pH 5 and pH 3. Absorbance, or aggregation, also showed a pH dependence in the same range, but was much less marked than the pH dependence of fusion.

Polypeptide concentration dependence

The effect of polylysine concentration on both aggregation and fusion was examined. The lysine-to-serine charge ratio was varied by increasing the polylysine concentration while the amount of lipid was fixed. Aggregation (absorbance at 400 nm) at 10 min after mixing with polylysine (M_r 20000) of PS : PC (1 : 1) vesicles is shown as a function of the lysine-to-serine ratio at pH 7.4, 4.5 and 3.0 in Fig. 5A. Absorbance increased to a plateau value with increasing polylysine concentration. Maxi-

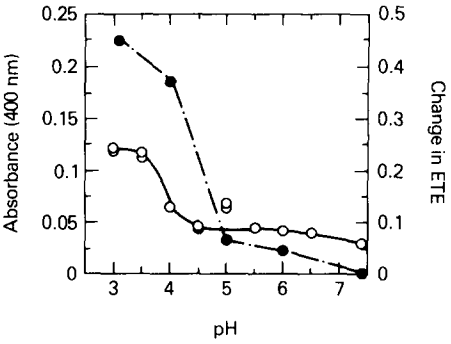

Fig. 4. The effect of pH on polylysine-induced fusion (closed circles) and aggregation (open circles). ΔE_{ET} of PS : PC (1 : 1) vesicles (44 μM lipid) was measured 10 min after the addition of polylysine (0.22 μg/ml of M_r 20000 polymer). Absorbance at 400 nm was measured 10 min after polylysine (4 μM in lysine or 0.8 μg/ml) and PS : PC vesicles (56 μM total lipid) were mixed. Lines are drawn between points for clarity. ETE, energy transfer efficiency.

mum absorbance or aggregation was identical for all three conditions. However, the lysine : serine ratios required to achieve half-maximal absorbance values were 0.1, 0.2 and 0.25 for pH 3, 4.5 and 7.4, respectively.

Fig. 5B is a plot of fusion as a function of increasing amounts of polylysine (M_r 20000) at pH 7.4, 4.5 and 3.0. The extent of fusion increased with increasing relative positive (lysine) charge concentration and then began to decrease somewhat, in contrast to the plateau observed for aggregation at high lysine concentrations. The maximum extent of fusion was greatest at pH 3 and the lysine : serine ratios that yielded optimal fusion were 0.1, 0.7 and 1.0 for pH 3, 4.5 and 7.4, respectively. Approximately 10% of the PS is estimated to be charged at pH 3.0 (calculated from the pK_a and ionic strength following McDonald et al. [20]; no corrections were attempted

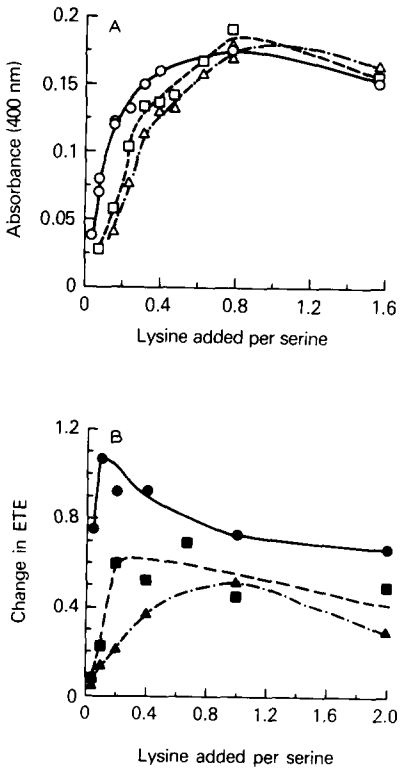

Fig. 5. Absorbance at 400 nm (A) and ΔE_{ET} (B) as a function of the lysine : serine ratio at pH 3.0 (circles), 4.5 (squares) and 7.4 (triangles). The vesicle concentration was constant at 56 μM total lipid. Vesicles were PS : PC (1 : 1). ETE, energy transfer efficiency.

for the effects of polylysine binding.) Thus it is reasonable to surmise that protons are affecting fusion, in part, due to their ability to reduce the net charge on the vesicle-polypeptide complex.

Effect of polypeptide size on binding, aggregation and fusion

To determine the effect of polypeptide size on the fusion phenomenon, we measured polylysine binding to PS : PC (1 : 1) vesicles and polylysine-induced aggregation and fusion at pH 7.4 and 3 with a small (M_r 4000, 20 residue), medium (M_r 20000, 100 residue) and large (M_r 90000, 450 residue) polylysine species. These measurements were chosen in order to distinguish size effects on binding and aggregation from those on membrane fusion.

The amount of polylysine bound per available serine is shown as a function of the amount of lysine added (given as added lysine : serine ratio) for the three different-sized polylysines (Fig. 6A). Almost all of the added lysine bound until the amount of lysine added was in excess of the outer monolayer PS. This suggests that almost all of the lysine moieties on a bound peptide were associated with the vesicles. The extents of aggregation (Fig. 6B) and particularly fusion (Fig. 6C) were also similar for all three molecular weight polypeptides. Thus, the extent of fusion at pH 7.4 appears to be directly correlated to the amount of lysine bound to the vesicles. Several more subtle points may be derived from Fig. 6. First, aggregation induced by the 450-residue peptide was slightly less than that observed with the 100-residue peptide when compared in terms of equal lysine, as shown. This slight decrease may be due to a lower number of attachment sites suitable for the larger peptide. Second, aggregation induced by the M_r 4000 peptide at high lysine : serine was lower but the extent of fusion was greater when compared with the other molecular weight polylysines.

The results of the binding, aggregation and fusion measurements done at pH 3 are shown in Fig. 7. In contrast to the measurements at pH 7.4, the extents of binding, aggregation and fusion were clearly lowest with the 20-residue polylysine. However, neither aggregation nor fusion were lowered as much as would be predicted by direct

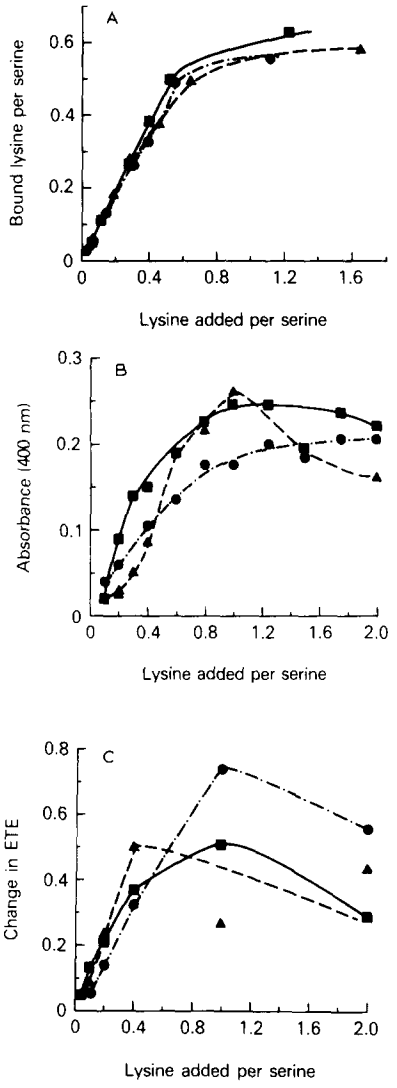

Fig. 6. Interactions of 20-residue (circles), 100-residue (squares) and 450-residue (triangles) polylysines with PS:PC (1:1) vesicles at pH 7.4. (A) Binding to REVs (96 μM total lipid). (B) Aggregation of SUVs (56 μM total lipid). (C) Fusion between SUVs (56 μM total lipid). Lines are drawn for clarity. ETE, energy transfer efficiency.

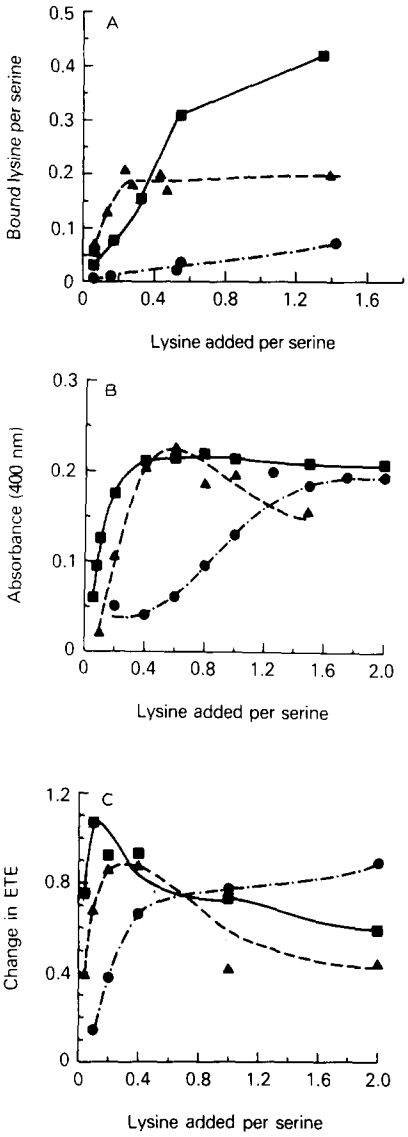

Fig. 7. Interactions of different chain-length polylysines at pH 3. Concentrations are identical to those in Fig. 6. (A) Polylysine binding to REVs. (B) Polylysine-induced aggregation of SUVs. (C) Polylysine-induced fusion of SUVs. Circles, squares and triangles represent the 20-residue, 100-residue and 450-residue polylysines, respectively. ETE, energy transfer efficiency.

correlation between binding and vesicle–vesicle interaction. In fact, at high lysine:serine ratios, the 20-residue polylysine is a better fusagen than the larger molecules (Fig. 7C). Again, as observed at pH 7.4, we note that aggregation induced by the 450-residue polylysine was somewhat less than that induced by the 100-residue molecule when compared on the basis of lysine moieties in spite of greater binding (lysine : serine < 0.5). Extents of binding, aggregation and fusion induced by the 450-residue polylysine at higher total lysine are all less than those observed with the 100-residue molecule. Only 1 molecule of 450-residue polylysine is needed to neutralize all the PS per SUV that remains charged at pH 3. Thus, it is easy to

envision serious 'parking problems' for such a large polymer on these vesicles.

Discussion

Our results indicate that polylysine induces massive aggregation and some fusion of negatively charged vesicles. Membrane fusion was demonstrated by electron microscopy and by a new assay in which we observed mixing of the inner monolayer upon addition of polylysine as predicted by an ordered fusion event. The extents of aggregation and fusion were dependent on the fraction of negatively charged lipid in the vesicles, the relative amount of polylysine, and the pH.

We find that for fusion to occur, it is necessary to have (a) enough charged binding sites and lysine residues to crosslink the vesicles, and (b) significant charge neutralization achieved either by polylysine or by protons. The synergistic effect of protons on polylysine-induced fusion suggests that once sufficient vesicle aggregation occurs due to crosslinking vesicles with polylysine, fusion is enhanced if charge neutralization can be achieved by protons. Polylysine, then, can be considered a steric inhibitor for fusion. We have summarized the factors enhancing and inhibiting fusion in Table III and will discuss the evidence for each below. A similar balance between barriers to fusion and ways to overcome those barriers exists in biological membrane fusion [21].

Optimizing conditions for aggregation and fusion

The extents of aggregation and fusion are increased when the number of potential polylysine binding sites (mol% PS) or the number of binding events (amount of lysine) is increased. Specifically, it is clear from Figs. 2 and 3 that increasing the mol% PS in the vesicles increases aggregation and fusion induced by polylysine even when the lysine : serine ratio is held constant. As the ratio of lysine : serine increases with vesicles of fixed mol% PS, the extent of fusion increases until excess lysine is added to the vesicle mixture (Figs. 5, 6 and 7).

Polylysine is required for aggregating PS : PC vesicles and can, but is not required to, neutralize the charge on the PS as needed for the very close membrane apposition thought to be necessary for fusion. The importance of charge neutralization is indicated in three ways. First, at neutral pH, the extent of fusion is greatest when the lysine : serine ratio is near 1. Second, at low pH where protons can compete with lysine in titrating the serine carboxyl group, fusion is again greatest when the lysine + proton : serine ratio is estimated to be 1 : 1 (Fig. 5). Third, the extent of fusion correlates with the number of lysine residues and not the moles of polypeptide at pH 7 (Fig. 6), indicating that net charge rather than the number or distribution of bound patches determines fusion.

However, once the vesicles are aggregated, excess polylysine appears actually to inhibit fusion. Steric inhibition of fusion is indicated by the optimal mol% PS (Fig. 3), the pH dependence (Figs. 1 and 4) and the decreased fusion at high lysine : serine (Figs. 5, 6 and 7). In Fig. 3, we show optimal fusion per aggregate at about 30 mol% PS at constant lysine : serine. Thus, as a greater percentage of the vesicle surface is coated with lysine, there are fewer regions where the apposing bilayers can be in sufficiently close contact for fusion. This interpretation implies that regions of bare bilayer are required for fusion.

That steric inhibition is important is also suggested by the steep pH dependence. By lowering the pH in the region of the pK_a for the PS carboxyls, protons can effectively compete with the lysine ϵ-amino groups to bind and neutralize the charges. Once aggregation is initiated, protons are more effective than the polypeptide in providing the necessary conditions for fusion (charge neutralization and bilayer-bilayer apposition), since fusion at low pH can be greater than fusion at neutral pH with optimal amounts of polylysine.

Similarly, Figs. 3, 5, 6 and 7 all indicate that

TABLE III

FACTORS WHICH ENHANCE OR INHIBIT POLYLYSINE-INDUCED FUSION OF PS : PC VESICLES

	Enhance	Inhibit
mol% PS	binding sites	electrostatic repulsion
Polylysine	crosslinking, charge neutralization	steric hindrance
pH	charge neutralization	H^+ competition with polycation binding

very high amounts of polylysine, while important for aggregation, are not necessarily optimal for fusion. Electrostatic or steric inhibition would explain the plateau and decrease in fusion observed at lysine : serine ratios greater than 1 in Figs. 5, 6 and 7. At ratios above 1, all the vesicles are coated with polylysine, which can, at best, be bound so that half the lysine groups are oriented toward the vesicle surface and half are facing the aqueous phase [22]. Thus, the vesicles present a net positive charge and repel each other, and there are no remaining binding sites for further vesicle–lysine interactions. Gad et al. [23] reported a similar effect of excess polylysine when polylysine plus calcium are used to fuse large phosphatidylcholine : cardiolipin vesicles.

In general, fusion is independent of polylysine chain length or the molar concentration of the polymer, but is dependent on the lysine : serine ratio. Gad et al. [7] reported slight polylysine chain-length dependencies for PC : PE : cardiolipin (1 : 6 : 3) vesicle aggregation at neutral pH, but the differences were very small. The size dependencies observed at low pH indicated that both 'parking' and electrostatic inhibitions must be considered (450-residue polylysine). Also, these low-pH data suggest that an optimal fusagen might be one with a significant binding constant which is nevertheless lower than that of the larger peptides (compare the 20-residue with the 100- and 450-residue molecules).

pH dependence of fusion

Other protein-induced fusions have followed the pH dependence of the protein structure. For example, myelin basic protein [24], bovine serum albumin [5] and clathrin [12] all induce fusion with a pH dependence that appears to correlate with a structural change in the protein. Another polycation known to induce fusion between vesicles containing negative charges is the model polypeptide polyhistidine [24,10]. The pH dependence in the latter case is due to titration of the imidazole groups on the histidine (pK_a about 6) converting polyhistidine from the uncharged to fully charged peptide. In other cases, such as fusion induced by the glycoprotein from vesicular stomatitis virus [3] or apocytochrome *c* [16], it is not clear whether changes in the lipids, in the protein, or in both are responsible for the pH dependence of fusion. Our observations with polylysine-induced fusion clearly demonstrate that fusion occurs to a much greater extent at pH values near the pK_a of the lipids (about 4.5 under these conditions) and not the pK_a of the peptide (about 9). A precedent for this result is the spontaneous fusion of PE vesicles containing anionic lipid when the pH is dropped below the pK_a of the latter. (We note that for PE-containing vesicles lipid mixing may occur by means other than fusion. Experiments such as ours with the asymmetrically labeled vesicles allow quantitative descriptions of different types of lipid mixing.) Examples include PS : PE (1 : 1) (A. Walter, unpublished observation), PS : PE (4 : 1) and PA : PE (4 : 1) vesicles which fuse spontaneously at pH 3.0 [26]. PC-containing vesicles do not fuse spontaneously at these pH values, probably due to the relatively stable bilayer structure and large hydrostatic repulsion of PC compared to PE (cf. Ref. 27).

Nature of polylysine binding and membrane destabilization

These polylysines bind to PS-containing vesicles in a stoichiometric manner, i.e., approximately 1 lysine per outer monolayer PS molecule. The binding constant is high, probably due to cooperation among the many possible salt bridges. Hartman and Galla [22] have shown that polylysine becomes structured when binding to acidic vesicles and the most likely configuration is a modified β structure, with every other lysine pointing to the membrane surface. This is consistent with our observations.

Polylysine binding to vesicles is reported to have a hydrophobic component as well as the electrostatic interactions. The evidence includes perturbations in lipid packing [28] and careful analysis of the electrostatic component by varying ionic strength [29]. Raman studies of polylysine effects on DPPG bilayers indicate that at high (e.g. stoichiometric) concentrations of peptide, the acyl chains interdigitate [30]. Finally, an early report shows that polylysine penetrates the lipid bilayer to the *trans* aqueous solution [31].

Cation-induced fusion

Polylysine-induced fusion is different from fu-

sion induced by divalent cations. First, most of these studies of polylysine-induced fusion were done on vesicles containing 50 mol% or less PS in egg PC which do not fuse (or fuse very little) in the presence of Ca^{2+} (e.g. Ref. 33) even at high divalent ion concentrations. Furthermore, in contrast with calcium-induced fusion, we observe very little leakage of vesicle contents. Polylysine interactions with 100% PS vesicles have little effect on sodium and glucose permeabilities [28,32]. Gad et al. [23] also observed much more fusion of PE- and cardiolipin-containing vesicles when comparing polylysine and calcium at equal charge ratios, but, in contrast to our results, they observed significant leakage. The leakage may be explained by the tendency of PE and cardiolipin to form nonbilayer phases, especially since by changing the lipid composition Gad [8] inhibited polylysine-induced leakage but not fusion.

Two smaller polyamines, spermine and spermidine (40–400 μM), will aggregate but not fuse phosphatidate : PS : PE : cholesterol (1 : 2 : 3 : 1) vesicles [34]. These polyamines do lower the calcium threshold needed for fusion to occur among these vesicles. Polylysine, however, does not appear to work in conjunction with calcium, since neither the addition of excess amounts of EDTA or citrate buffer nor the addition of extra calcium affected fusion.

Conclusions

Polylysine has been reported to induce fusion of PE-vesicles [6] and a variety of vesicle types [8,9] which can also be induced to fuse with divalent cations at neutral pH. In this report, we have shown that polylysine is also able to induce fusion of SUVs that are not susceptible to fusion by divalent cations. Differences in the distribution of charge and the types of membrane perturbations may explain the difference between this polycation and the divalent cations. Aggregation and fusion are pH-dependent and, in contrast to other cation-induced fusion events, the pH dependence clearly resides in the lipids. Fusion is optimal when the number of sites for vesicle–peptide–vesicle interaction are maximized and when the net charge on the aggregated system is near zero. This behavior is expected for any polycation-induced fusion and will result in a pH dependence near the pK_a of either component of the system. Electrostatic interactions such as this one described for a model polypeptide may play a role in physiological fusions such as the pH-dependent, anionic-lipid-requiring fusion of the vesicular-stomatitis-virus G protein [3].

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