Biochemistry

 Volume 24, Number 1 January 1, 1985

Articles

pH-Dependent Fusion of Liposomes Using Titratable Polycations†

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Received February 14, 1984

ABSTRACT: Polylysine promoted extensive membrane mixing of liposomes only if the buffer pH was below the pK_a of the lysyl residues. This observation suggested that fusion could be regulated in a physiological pH range if the homopolymer of L-histidine was substituted as fusogen. Microgram quantities of polyhistidine were added to liposomes composed of soybean phospholipids, or to defined phospholipid–cholesterol mixtures which simulate the lipid composition of plasma membranes. A quantitative resonance energy transfer assay determined the extent of lipid phase mixing related to fusion. No fusion was detected at pH 7.4, but when the pH was lowered to 6.5 or below, fusion was rapid and substantial. The extent of membrane mixing increased with progressive acidification of the vesicle–fusogen suspension. The charge density of each polyhistidine molecule, not the total cationic charge per vesicle, influenced the extent of fusion. The kinetics of the fusion reaction were rapid, as membrane mixing was completed within 1 min. If the vesicle suspension was acidified before fusogen addition, the rate of membrane mixing slowed 4-fold. This, as well as a slight increase in light scattering noted whenever polyhistidine was added at pH 7.4, suggests an enhancement of fusion kinetics by preaggregation of vesicles at neutral pH. The lipid composition, regulation of membrane mixing by pH in a physiological range, and rapid kinetics suggest that this model of liposome fusion may be pertinent to understanding some biological fusion events.

The complexities of isolating and manipulating the elements of biomembrane fusion have prompted the use of simple model membrane systems for investigating this complex phenomenon. The most widely studied model system has been the fusion of phosphatidylserine (PS)[1] vesicles in the presence of calcium ion (Papahadjopoulos et al., 1974, 1976). The direct interaction of calcium ion with acidic phospholipid leads to vesicle aggregation and phase separation; the membrane discontinuities at phase boundaries generated by calcium ion have been proposed as the sites at which membrane coalescence is initiated (Papahadjopoulos, 1978). Ca^{2+}–PS interactions have been studied most extensively with respect to lipid composition and divalent cation concentrations. From these studies, it is known that the presence of other phospholipids will affect the likelihood of a successful fusion event (Vanderwerf & Ullman, 1980; Düzgünes et al., 1981; Uster & Deamer, 1981). Increased molar content of PC is inhibitory to fusion, while substituting PE in its place restores fusion competence. Cholesterol also plays an influential role as it can increase the potential membrane coalescence of complex lipid mixtures (Uster & Deamer, 1985). Despite such manipulations of lipid composition, it has not been possible to lower the fusion threshold for PS-containing vesicles below millimolar Ca^{2+} concentrations to the micromolar concentrations used by isolated biological preparations such as synaptosomes (Blaustein, 1974). Altstiel & Branton (1983) have shown in a cell-free system how the maximal in vitro rate of lysosome fusion with reconstituted, clathrin-stripped, coated vesicles is

† Supported by National Science Foundation Grant PCM 80-01953 (to D.W.D.) and by a Jastro-Shields Research Award (to P.S.U.).

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[1] Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Chol, cholesterol; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CA9C, cholesteryl anthracene-9-carboxylate; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; kDa, kilodalton.

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enhanced 5-fold between 1 and 10 μM $CaCl_2$. The disparity between Ca^{2+} concentrations required by organelle fusion and by liposome fusion suggests the involvement of other components and a different site of calcium ion activity. Nevertheless, some progress in lowering the Ca^{2+} threshold of liposome fusion has been made by using synexin (Hong et al., 1981) or polyamines (Hong et al., 1983) to aggregate the vesicles.

With the site of Ca^{2+} action in situ open to some question, attention has also focused on the involvement of peptides in model membrane fusion events. Purified HN and F membrane proteins of Sendai virus will fuse liposomes reconstituted with glycophorin (Oku et al., 1982), and some other proteins which can associate with membranes, such as clathrin (Steer et al., 1982), will initiate PC vesicle fusion under particular conditions. While calcium ion does not fuse PC vesicles by itself, tubulin and millimolar Ca^{2+} levels promote PC liposome fusion (Kumar et al., 1982). Highly basic peptides like human myelin protein (Stollery & Vail, 1979; Lampe et al., 1983), polylysine (Gad et al., 1982; Lampe & Nelsestuen, 1982), and protamine (Uster & Deamer, 1985) also fuse liposomes, and at cationic charge concentrations well below those of divalent cations.

Liposome studies have been primarily concerned with determining the nature of fusogens which initiate membrane coalescence. Model systems should also identify mechanisms which can regulate fusogenic activity, as biological events such as stimulus secretion are responsive to cellular control (Henson et al., 1978). Ca^{2+} is one possible source of regulation; its release from intracellular storage sites is concomitant with such phenomena as pancreatic acinar cell exocytosis (Chandler & Williams, 1978)) and will initiate the osmotically driven lysis of isolated platelet α-granules (Van der Meulen & Grinstein, 1983). Cohen et al. (1984) have devised a model system in which Ca^{2+} promotes liposome adsorption to a planar membrane, thereby permitting subsequent vesicle–planar membrane fusion events to be driven by an osmotic potential.

The activity of numerous cellular functions is known to be sensitive to changes in intracellular pH (Nuccitelli & Heiple, 1982). This suggests another possible means of regulating membrane fusion promoted by model peptides. Recently, a model of pH-triggered liposome fusion using the purified, reconstituted G protein of vesicular stomatitis has been reported which is dependent on pH and target membrane composition (Eidelman et al., 1983). In this paper, we describe a new model of liposome fusion in which a titratable polycationic peptide fuses vesicles of composition similar to that of biological membranes. Relatively small changes of pH in a physiological range initiate membrane mixing by altering the charge density of the fusogen.

Experimental Procedures

Materials. Bovine brain phosphatidylserine, soy phosphatidylethanolamine, 1-palmitoyl-2-oleylphosphatidylcholine, and NBD-PE were purchased from Avanti/Polar Lipids, Inc. (Birmingham, AL), and stored under nitrogen at −20 °C. Asolectin was prepared from soybeans according to Kates (1972), and thin-layer chromatography of the phospholipid preparation showed three spots with a relative abundance of PE > PC > PS. Phospholipid concentrations were determined by the organic phosphate analysis of Lowry et al. (1954). The fluorescent probe cholesteryl anthracene-9-carboxylate was obtained from Molecular Probes, Inc. (Junction City, OR). Cholesterol, polyhistidine, polylysine, and miscellaneous materials were purchased from Sigma (St. Louis, MO).

Liposome Preparation. Lipid mixtures were prepared from stock lipid solutions in chloroform. CA9C (2 mol %) (fluorescence donor) or NBD-PE (energy acceptor) was added to separate aliquots of 3 μmol of total phospholipid. Twenty percent of the lipid from each labeled aliquot was removed and recombined to simulate the signal from "mock-fused" (100% probe intermixing) vesicles. Each of the three samples was dried under nitrogen, put under vacuum for at least 0.5 h in order to remove residual organic solvent, and reconstituted with standard buffer (100 mM NaCl, 5 mM TES, and 0.1 mM EDTA, pH 7.4). SUVs were prepared by bath sonicating each vesicle suspension to optical clarity and were diluted to 0.2 mM lipid. LUVs were prepared according to Pick (1981) at 20 mM lipid and diluted to a final concentration of 0.2 mM.

Fusion Assay. Liposome fusion was determined by using a quantitative resonance energy transfer assay as described (Uster & Deamer, 1981). Briefly, liposomes of a given composition are labeled with either fluorescent donor or energy acceptor, and equimolar amounts of the vesicle suspensions are mixed. The quenching of donor fluorescence upon membrane fusion is proportional to the extent of lipid phase intermixing. Homotypic fusion events (donor–donor or acceptor–acceptor vesicles) are silent; therefore, all liposomes have participated in at least one fusion event at 50% quenching of donor fluorescence. This is determined by comparing quenching induced by the fusion protocol with donor fluorescence of the mock-fused standard (100% probe intermixing).

Fluorescent measurement of probe intermixing was recorded by using an Aminco-Bowman spectrophotofluorometer at appropriate monochromator settings for CA9C (370-nm excitation, 450-nm emission). The extent of membrane mixing was determined by comparing the degree of donor fluorescence quenching induced in the "premixed" liposome sample with the signal from mock-fused liposomes subjected to the identical fusion protocol. The pH of vesicle suspension was determined by using an Orion Research Model 5704 pH meter with a combination electrode. Fusogens, EDTA, or heparin at the concentrations used did not change the pH of liposome samples. pH determinations were taken after aliquots of stock 0.5 N HCl or NaOH were added, as well as after the addition of fusogen binding agents.

Vesicle aggregation was monitored turbidimetrically. Relative changes in light scattering at 90° were determined with both monochromators set at 500 nm, and all other conditions were as described above.

Results

Fluorometric Determination of Membrane Mixing. Liposome fusion has been studied with fluorescence assays that measure either the mixing of entrapped aqueous contents (Wilschut & Papahadjopoulos, 1979; Kendall & MacDonald, 1982) or the mixing of membrane bilayers (Vanderwerf & Ullman, 1979; Struck et al., 1981; Uster & Deamer, 1981). These complementary methods have inherent advantages and limitations. The aqueous mixing assays have been used to follow liposome fusion kinetics, but at least for some experimental systems, leakiness during fusion events may lead to an underestimation of the rate (Kendall & MacDonald, 1982). Membrane mixing assays are not susceptible to this possible source of error but also have particular limitations. Like lipids of biological origin (Roseman & Thompson, 1980), it is possible that the fluorescent lipophilic probes may diffuse as monomers from one bilayer to another (Nichols & Pagano, 1982).

However, for the resonance energy transfer assay used in this work, we have determined that the donor, a cholesteryl ester analogue, shows no detectable equilibration between labeled and unlabeled membranes over the course of hours.

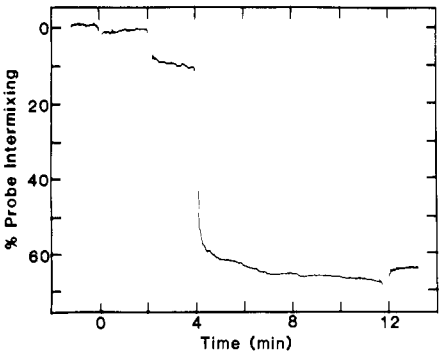

FIGURE 1: Neutral pH initiates polylysine-induced fusion. A suspension of asolectin LUVs was alkalinized to pH 10 at the start of the experiment, and 4 μg/mL polylysine (16 kDa) was added 2 min later. At this time, a small increase in probe intermixing was seen. After an additional 2 min, the pH was readjusted to neutral pH which initiated at least one round of vesicle fusion. Heparin (100 μg/mL) was added to remove the considerable aggregation, but with little resonance energy signal change, indicating that the assay followed the kinetics of fusion accurately.

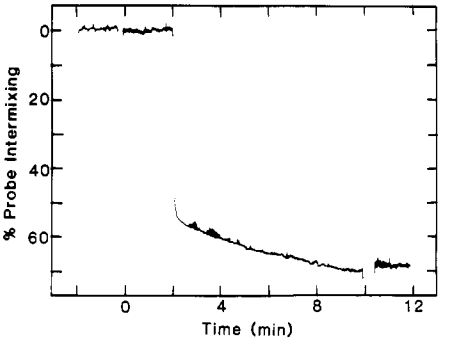

FIGURE 2: Acidic pH initiates polyhistidine-induced liposome fusion. Asolectin LUVs at pH 7.4 had 4 μg/mL polyhistidine (8 kDa) added to the suspension, but no membrane mixing occurred until the buffer was acidified to pH 5.8. The addition of 100 μg/mL heparin produced little change in the fluorescence signal, but vesicle aggregation was dispersed.

There is no appreciable exchange of lipid probes in aggregated but unfused vesicles (Uster & Deamer, 1981). Similarly, the rate of NBD-PE monomeric diffusion is so slow as to be negligible over a similar time course (Nichols & Pagano, 1981), thereby ensuring that the assay is specific for liposome fusion. The spectral characteristics of the assay are appropriate for monitoring pH-triggered liposome fusion; control experiments indicate that CA9C fluorescence is pH insensitive (data not shown). Small but significant changes in the quantum yield of NBD-PE were noted at different pHs, but the absorption spectrum was not altered, indicating that NBD-PE is a suitable energy acceptor.

Lipid mixtures which do not show spurious changes in donor fluorescence during the aggregated state were selected in order to compare the kinetics of different fusion protocols. In general, liposomes which have a relatively small molar fraction of acidic phospholipid (25% or less) are not prone to erroneous signals caused by aggregation. We have limited our present inquiry on pH-regulated fusion either to liposomes composed of asolectin phospholipid (PE, PC, and PS), a soybean extract that displayed virtually no artifactual quenching, or to liposomes composed of a complex lipid mixture with only 8 mol % PS. It is important to develop models in which fusogen must interact successfully with liposomes that reflect the lipid composition of biological sources. While these vesicles cannot duplicate the bilayer leaflet asymmetry of native membranes, they should be more germane to understanding how fusion may proceed in situ than liposomes which contain exceptionally high molar fractions of acidic lipid.

pH-Dependent, Polycation-Induced Fusion. The contribution of electrostatic interactions to the fusogenic activity of polylysine was determined by titrating the ε-amino residues. A suspension of asolectin LUVs (Figure 1) was adjusted from pH 7.4 to pH 10 with no significant change of probe fluorescence. When 4 μg/mL polylysine was added to the vesicle preparation 2 min following alkalinization, there was immediate donor quenching equivalent to 10% probe intermixing. This could be due to a residual charge on polylysine, for at pH 10 the 16-kDa peptide still has residual charged groups. Alternatively, penetration of side-chain residues or vesicle cross-linking through hydrogen bonding may also contribute to liposome fusion. Two minutes after the addition of polylysine, the liposome suspension was adjusted to pH 6.9 The restoration of original charge density caused immediate liposome fusion, such that the first round of fusion (50% probe intermixing) was complete within 6 s after the pH was lowered. The kinetics of membrane mixing were biphasic, with the initial rate (>7% probe intermixing/s) completed within 15 s, and a second slower phase (approximately 0.016% probe intermixing/s) continued indefinitely.

Heparin, a polyanion which disperses aggregation induced by polycations, was then added to the vesicle suspension to determine whether polycation-induced aggregation is responsible for some fraction of fluorescence quenching. The addition of a 25-fold excess of heparin had little effect on the energy transfer signal, suggesting that for asolectin vesicles probe intermixing monitors fusion kinetics with minimal contribution from aggregation. From the titration of polylysine, one may conclude that electrostatic interactions are predominantly responsible for initiating liposome fusion.

The pH shifts that were able to trigger polylysine-induced fusion were outside physiological range, and we therefore attempted to repeat the experiments at neutral pH by substituting polyhistidine as the fusogen (Figure 2). If 4 μg/mL polyhistidine was added to asolectin LUVs at pH 7.4 (time = 0 min), no liposome fusion was detected. When the suspension was acidified to pH 5.8, the first round of fusion was completed within 6 s after the addition of acid. The slow phase of membrane mixing was more noticeable (0.03% probe intermixing/s) but ceased when 100 μg/mL heparin was added. Control experiments verified that there was less than 6% probe intermixing after asolectin LUVs were incubated for 10 min at pH 5.5 in the absence of polyhistidine and that the percentage of probe intermixing did not change when the pH was readjusted to 7.9.

Probe Exchange during Aggregation at Acidic pH. Probe exchange without true bilayer coalescence might also occur between highly aggregated bilayers under mildly acidic conditions. This was tested by incubating 16:84 PS:PC SUVs with polylysine at neutral and acidic pH. Saturating concentrations of fusogen (lysyl amino residue:lipid phosphate, 1:1) ensured that aggregation was so extensive as to be visibly turbid. SUVs have two-thirds of their lipid on the outer surface leaflet, thereby making as much as 67% of the lipid probe available for exchange under acidic, aggregated conditions. PS–PC (16:84) vesicles displayed relatively little probe intermixing in the presence of polycations and were an appropriate choice because extensive fusion at neutral pH will obscure the signal due to probe exchange. The Henderson–Hasselbach equation suggests that nearly all of polylysine's residues are protonated at neutral and acidic pH. Therefore, by comparing probe intermixing at neutral and acidic pH, it was possible to determine what fraction of the energy transfer signal is due to

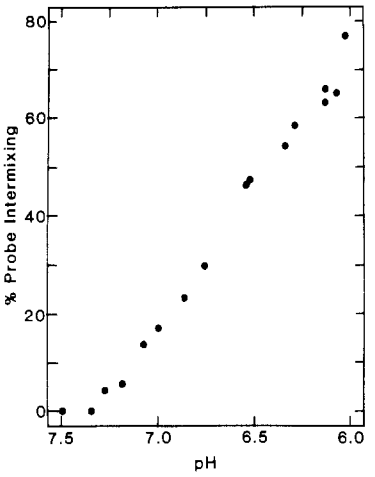

FIGURE 3: pH regulates the extent of polyhistidine-induced membrane mixing. The extent of probe intermixing was determined 30 s after asolectin SUVs preincubated with 4 μg/mL polyhistidine were acidified with aliquots of 0.5 N HCl. The final pH of each sample was determined about 3–6 min after acidification. Replicate samples acidified below pH 6 exhibited no more than 75% probe intermixing.

nonfusogenic, pH-mediated processes.

At pH 7.0, 6.0, or 5.0, the subsequent addition of polylysine induced only 16 ± 1% probe intermixing. In this pH range, acidification does not promote probe diffusion through the aqueous phase. Also, PS–PC liposomes were first aggregated at pH 7.0 (16% probe intermixing), and aliquots of 1.0 N HCl were added to the buffer. When the vesicle–fusogen suspension was lowered to pH 6, there was no significant increase in probe intermixing. However, if the pH of the aggregated vesicle suspension was acidified from pH 7 to pH 5, an increase of approximately 9% probe intermixing was seen (25% total probe intermixing). We conclude that corrections for pH-mediated, nonfusogenic probe exchange are not required in the pH range of 7.4–6. Mechanisms other than true bilayer coalescence may lead to a slight, but no major, distortion of the energy transfer signal below pH 6.

pH and Extent of Membrane Mixing. In a different set of experiments using asolectin SUVs, the extent of probe intermixing as a function of final buffer pH was investigated (Figure 3). The SUV preparation, like LUVs, displayed biphasic kinetics. There was always a rapid component which was essentially complete within 12 s followed by a much slower rate (~0.1% probe intermixing/s) or a complete cessation of fusion. The values plotted in Figure 3 were determined for the extent of probe intermixing 30 s after an aliquot of HCl was added to a sample of liposomes preincubated with polyhistidine at pH 7.4. Acidification of the vesicle–fusogen mixture promoted membrane mixing such that 50% probe intermixing of SUVs was achieved at a final pH of approximately 6.5. The extent of probe intermixing did not go beyond 70–75% even if this vesicle suspension was acidified to pH 5. In the range of pH 7.3–6.0, the increase in asolectin SUV fashion can be correlated with an increase in the relative charged fraction of histidyl residues. The Henderson–Hasselbach equation suggests that 5–50% of L-histidine imidazole in bulk solution bears a net charge at this pH range, given the pK_a of 5.97. However, the true pK_a of imidazole in polyhistidine adsorbed to a vesicle surface is likely to be substantially different due to counterion concentrations, electrostatic interactions, and peptide conformation. The actual fraction of charged residues at a given pH cannot be reasonably estimated under these conditions.

Comparison of Three Fusion Protocols. These initial studies indicated that pH would regulate fusion in liposomes

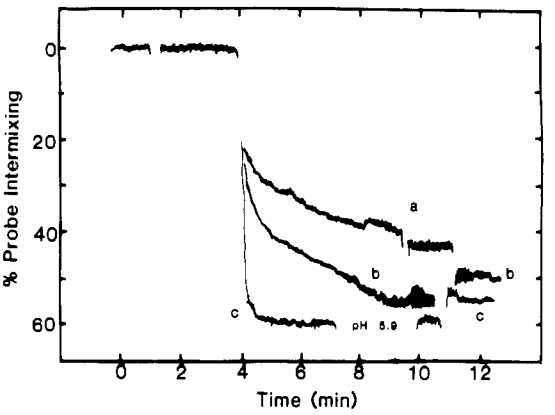

FIGURE 4: Kinetics of three fusion protocols are compared. PS–PE–PC–Chol (8:18:26:48) LUVs were treated as follows. (a) Fourteen millimolar $CaCl_2$ was incubated with the vesicles for approximately 6 min and chelated with 60 mM EDTA. (b) Another sample of liposomes was incubated with 4 μg/mL protamine, a histone-like basic protein. The aggregation was dispersed 6 min later with 100 μg/mL heparin. (c) The liposomes had 4 μg/mL polyhistidine added at pH 7.4 but did not begin membrane mixing until the buffer was titrated to 5.9. Again, 100 μg/mL heparin dispersed aggregation but had little effect on resonance energy transfer.

derived from a plant source enriched in PE and PS. One critical test of any model system is its ability to promote membrane mixing of bilayers which reflect the relative abundance of particular lipid classes in situ; therefore, experimentation continued using liposomes composed of PS–PE–PC–Chol (8:18:26:48).

The kinetics of this pH-triggered fusion reaction are contrasted with Ca^{2+} and another basic polypeptide, protamine, which is fully charged at pH 7.4 (Figure 4). These LUVs were incubated with 14 mM $CaCl_2$ (scan a) or 4 μg/mL protamine (scan b), the histone-like, DNA binding protein of vertebrate sperm. The minimum fusogen concentrations required to achieve at least 50% probe intermixing of this complex lipid mixture were 14 mM $CaCl_2$ and 4 μg/mL polycation. Increasing the fusogen concentration would increase the extent of membrane mixing slightly, but these concentrations were selected in order to compare the time required to promote one complete round of vesicle fusion. It is evident that protamine increases the rate and extent of fusion well over that elicited by the divalent cations, even though the bulk phase concentration of calcium ion's total cationic charge was more than 100-fold greater than the polycation bulk charge. Polyhistidine (4 μg/mL; scan c of Figure 4) was added at the first break on the fluorescence trace. While no change in probe intermixing was seen, light scattering (Figure 5B) revealed a slight but noticeable turbidity indicative of aggregation. When the pH was acidified to 5.9, fusion was immediate, complete, and noticeably faster than protamine-induced fusion.

Figure 5 contrasts the light-scattering kinetics, a relative measure of vesicle size and state of aggregation, of this liposome preparation in the presence of the three fusogens. At a 90° scattering angle, Ca^{2+}-induced fusion of these liposomes showed a slight but constant gain which was abolished upon EDTA addition (Figure 5A, lower trace). Protamine caused a rapid increase in light scattering as indicated by the upper trace (Figure 5A). This aggregation was visible to the naked eye and was completely dispersed when heparin was added. Light scattering indicative of aggregation was also seen when polyhistidine was incubated with liposomes (Figure 5B) at neutral pH, although no membrane mixing was recorded. When liposome fusion was triggered by acidification, there was another transient increase in light scattering which rapidly decreased. Finally, when fusion was terminated by heparin

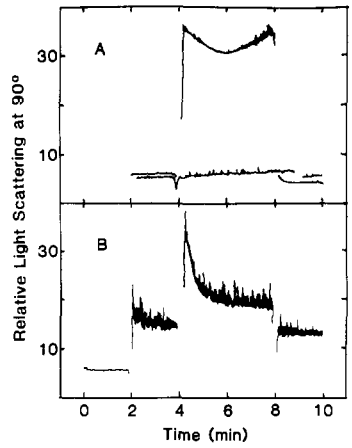

FIGURE 5: 90° light-scattering changes are induced by the three fusion protocols. (A) Fusogen was added at time = 4 min. The lower trace is the relative light scattering produced by adding 14 mM $CaCl_2$ to PS–PE–PC–Chol (8:18:26:48) LUVs. Instrument sensitivity has been reduced in order to keep polycation light-scattering traces on scale. The upper trace is vesicle aggregation produced by 4 μg/mL protamine. One hundred micrograms per milliliter heparin (time = 8 min) dispersed the aggregation. (B) The light-scattering changes of polyhistidine are more complex. When 4 μg/mL polyhistidine was added to the vesicles, there was an immediate but moderate increase in light scattering. This was increased even further when the buffer pH was acidified but eventually decreased to a plateau. Heparin decreased light scattering greatly, but not entirely. Phase microscopy revealed the presence of very small residual aggregates.

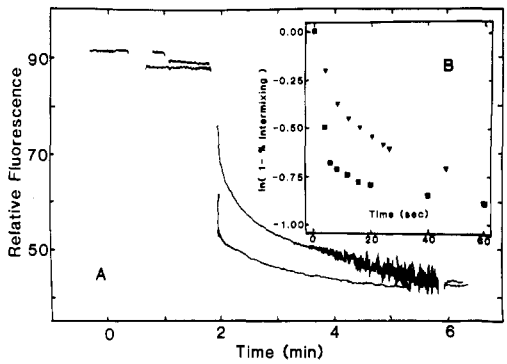

FIGURE 6: Rate of membrane mixing is reduced when vesicles are not preaggregated at neutral pH. (A) The lower trace follows the time course of membrane mixing when PS–PE–PC–Chol (8:18:26:48) LUVs were incubated with 4 μg/mL polyhistidine and then acidified to pH 6.1 90 s later. After 6 min, the vesicle solution was readjusted to pH 8.4 but with little change in apparent membrane mixing. Other liposomes were first acidified to pH 6.0 (upper trace), and then polyhistidine was added. The kinetics of membrane mixing were about 4-fold slower. (B) This difference between initial rates was determined by plotting the change in membrane mixing during the first 60 s according to Altstiel & Branton (1983). The kinetics of polycation-induced fusion do not follow a single first-order rate constant during the first minute of fusion events. (■) Polyhistidine added prior to acidification; (▼) polyhistidine added after the vesicle suspension was acidified.

addition, or as in this example by alkalinization, there was a further reduction in light scattering.

The increase in the initial rate of probe intermixing may be due to preaggregation of liposomes by polyhistidine, as suggested by the increase of light scattering at neutral pH. This was tested by adding polyhistidine to samples of liposomes preadjusted to pH 6.1 (Figure 6A). If aggregation was rate limiting, the kinetics of membrane mixing should be slower. Liposome fusion during the first 60 s (Figure 6B) was replotted by using the method of Altstiel & Branton (1983) to clarify the difference between the pseudo-first-order rate constants. Initial fusion kinetics were substantially slower (about 4-fold) when fusogen was added following acidification, instead of

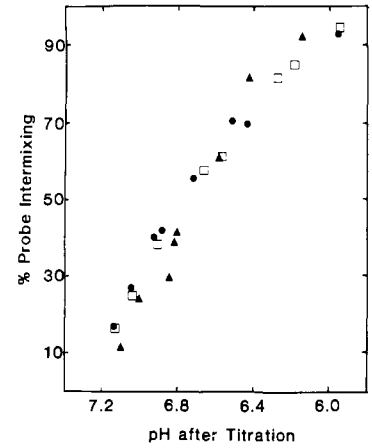

FIGURE 7: Polyhistidine charge density regulates the extent of membrane mixing. PS–PE–PC–Chol (8:18:26:48) SUVs were incubated with (●) 4, (▲) 8, or (□) 16 μg/mL polyhistidine for 2 min and then adjusted to final pH by an aliquot of HCl. The extent of probe intermixing was determined 60 s after acidification. Increasing amounts of polycation did not compensate for decreasing the number of charged residues at a given pH, suggesting that the charge density, and not the number of cationic charges associated per vesicle, influences the likelihood of successful fusion events.

prior to lowering the pH. These data are consistent with the interpretation that the rate-limiting step of polycation-induced fusion is aggregation of vesicles.

Fusogen Charge Density Affects Membrane Mixing. Our results indicate that electrostatic interactions play a dominant role in the initiation of fusion events between liposomes. We may ask whether the extent of probe mixing could be related to the actual charge density of the fusogen or to shielding of the net lipid charge such that the total cationic charge per vesicle would determine the likelihood of a successful fusion event. These alternatives were examined by incubating another PS–PE–PC–Chol (8:18:26:48) SUV preparation with three concentrations of polyhistidine and comparing the percent probe intermixing with the relative fraction of charged histidyl residues as surmised from the final pH (Figure 7). Acidification increases the average number of protonated residues per fusogen molecule; therefore, the ionic fraction is a function of the final pH. The actual fraction of charged residues cannot be calculated precisely, because attempts to measure the pK_a of polyhistidine adsorbed to these liposomes were not successful. For all three polycation concentrations, the histidyl:total phospholipid ratio was 0.4 or less, ensuring that histidyl residues were not saturating the total lipid phosphate. If the total cationic charge per liposome was the critical factor, then increasing the concentrations of polycation should elicit more probe intermixing at a given buffer pH. However, the data points are superimposed for all three fusogen concentrations over the entire range of pHs examined, suggesting that increased charge density of the fusogen determines the likelihood of a successful fusion event being initiated.

DISCUSSION

In this report, polyhistidine-induced fusion of model membranes has been followed by using a resonance energy transfer assay developed in our laboratory. The complex lipid mixtures used in this study were selected in order that the kinetics of membrane coalescence would be accurately followed. Liposomes which simulate the relative abundance of the three major phospholipid classes and cholesterol as found in eukaryotic plasma membranes were able to meet this requirement. It is significant that this model system does not require vesicles with large molar fractions of acidic phospholipids. No ex-

traordinary lipid requirements or self-segregation of the membrane into large, preexisting acidic phospholipid-rich domains need to be postulated.

Titratable polycations also initiated fusion of small or large unilamellar vesicles, but it would appear the extent of membrane mixing was greater for SUVs than for LUVs at a given pH. This tendency was noted for both lipid mixtures. Asolectin LUVs acidified to pH 5.8 underwent one complete round of fusion (55%) as shown in Figure 2, but SUVs composed of the same lipid extract underwent 70–75% probe intermixing at this same pH (Figure 3). In like manner, LUVs of PS–PE–PC–Chol (8:18:26:48) underwent 55–65% probe intermixing (Figures 4c and 6) at pH 6.1–5.8, while SUVs of similar composition displayed nearly complete membrane mixing (Figure 7). Futher experimentation is needed to verify the effect of vesicle size, but it does suggest the radius of vesicle curvature affects polyhistidine's fusogenic ability like that of divalent cation fusogens. For instance, magnesium ion will fuse phosphatidylserine SUVs (Nir et al., 1983) but not LUVs (Düzgünes et al., 1981), and the rate of Ca^{2+}-induced PS liposome fusion decreases sharply as the vesicle diameter increases (Nir et al., 1982).

One consequence of the polycationic nature of polyhistidine is its enhancement of the overall rate of liposome fusion events. The rapdity with which model membrane coalescence occurs using pH to titrate polycations is at least 30-fold faster than Ca^{2+}-induced fusion and is due, in part, to polyhistidine's aggregation of liposomes without membrane mixing at neutral pH. Hydrophobic interactions between uncharged imidazole residues of the polyamino acid and the hydrophobic phase lead to surface pressure increases of lipid monolayers at pH 7.4 (Shafer, 1974). These hydrophobic interactions may be responsible for vesicle aggregation in the model system that we have described. A detailed mathematical analysis has been used to separate the relative contribution of aggregation and membrane coalescence to Ca^{2+}-induced fusion kinetics and determined that aggregation is the rate-limiting step (Nir et al., 1980, 1983). We find that vesicle–vesicle aggregation with polyhistidine is also rate limiting, because preacidification of the liposome suspension before fusogen addition slows the overall kinetics of membrane mixing.

In other respects, polyhistidine's fusogenic activity is substantially different from that of divalent cations. Considerably less polycation is required to initiate fusion events. Four micrograms per milliliter polyhistidine or polylysine will promote the first round of fusion, with several hundredfold less cationic charge than the 14 mM Ca^{2+} needed to induce equivalent membrane mixing (Figure 4). This could be the result of concentrated polycation at the liposome surface. Hammoudah et al. (1981) have shown that the Gouy–Chapman equation predicts the counterion concentration of La^{3+} will be greater than that for divalent cations because of increased valency. This is sufficient for La^{3+} to displace Ca^{2+} from membranes and promote La^{3+}-induced liposome fusion at submillimolar concentrations.

Unlike inorganic cations, the charge density of cationic peptides can be titrated. Our results suggest that greater charge density increases the extent of membrane mixing, which is interpreted as the enhanced probability of one or more fusion events occurring per original liposome. Increased charge density changes the interactions between fusogen and vesicle surface. While Ca^{2+} can be displaced from bilayer surfaces (Hammoudah et al., 1981), the binding of polylysine is irreversible at neutral pH (Gad, 1983). Electrostatic interactions between polyhistidine and lipid appear to be responsible for initiating vesicle fusion. Previous studies have shown that divalent and polycation interactions with acidic phospholipid provoke a similar response with respect to at least one surface property. The presence of Ca^{2+} induces a net decrease in the surface pressure of PS monolayers, which is indicative of phospholipid surface area condensation (Papahadjopoulos, 1968). This evidence, as well as data obtained from differential scanning calorimetry and X-ray diffraction, led to the hypothesis that Ca^{2+} binding promotes acyl chain crystallization, initiates lateral phase separation, and leads to fusion events at microdomain discontinuities (Papahadjopoulos, 1978). Shafer (1974) noted similar decreases in surface pressure when acidic lipid monolayers were incubated with polyarginine, -lysine, or -ornithine at pH 7.4. Polyhistidine, in contrast, caused surface pressure increases of these monolayers at neutral pH. Shafer did not investigate the surface effects of polyhistidine near or below its pK_a; however, one would predict that a similar surface pressure decrease would occur. Thus, increasing the charge density of polycations would appear to change acyl chain packing, which could lead to organization changes analogous to those described for Ca^{2+}–PS interactions.

pH-triggered liposome fusion provides a model in which fusogen can be activated by an intracellular regulatory mechanism. The cycling of membrane-bound receptors is known to be pH sensitive. The Na^+/H^+ ionophore monensin and the weak organic amine chloroquine elevate the pH of a prelysosomal compartment (Maxfield, 1982) and block the cycling of receptor-bound ligands (Van Leuven et al., 1980; Basu et al., 1981; Marshall et al., 1981; Ciechanover et al., 1983) at an internal compartment. The acidification of this compartment is necessary for receptor–ligand dissociation (Tietze et al., 1982; Harford et al., 1983), but as Harford et al. (1983) note, these studies do not exclude the possibility that vesicle movement or fusion is also impaired by elevated intravesicular pH.

Monensin also reversibly halts the intracellular movement of secretory and membrane proteins through the Golgi stack [see Tartakoff (1983) for a review], and recently the same effect and site of arrest have been demonstrated for glycosphingolipids (Saito et al., 1984). Complex glycosylation of proteins and lipid does not occur in monensin-treated cells, but this is not attributed to a general inhibition of oligosaccharide-processing enzymes such as galactosyl transferase [Quinn et al., 1983; Tartakoff, 1983; Saito et al., 1984; see Wilcox et al. (1982)]. Cells become intensely vacuolated after ionophore treatment (Wilcox et al., 1982), and the Golgi cisternae are greatly dilated (Tartakoff & Vasselli, 1978). Griffiths et al. (1983) have demonstrated that stomatitis virus G protein accumulates within swollen *medial* elements of the Golgi stack in BHK cells, suggesting that movement through the Golgi is blocked prior to the *trans* elements.

Evidence suggesting the direct involvement of pH comes from studies using enveloped virus infection of cultured cells. White et al. (1981) have shown with several enveloped viral classes that acidifying the medium initiates virus–cell membrane fusion. The viral spike glycoproteins are the source of this pH-sensitive fusogenic activity. Marsh et al. (1983) reconstituted liposomes with Semiliki Forest virus spike glycoproteins and observed that these "virosomes" retained about one-fourth of the native virus' capacity to fuse with baby hamster kidney (BHK-21) cells. Virosome–membrane coalescence requires a brief (30–60 s) exposure to pH 5.5. Madin–Darby canine kidney (MDCK) cells can be infected with influenza virua such that viral glycoprotein transport to the cell surface will initiate plasma membrane fusion with gang-

lioside-containing liposomes when incubated at low pH (Van Meer & Simons, 1984). Polyhistidine-induced fusion may be a useful model of enveloped virus infection.

At the present time, it is not known whether cationic peptides or cationic domains of membrane-associated proteins are involved with in situ fusion events. However, the examples cited above may well be pH-sensitive events that can be modeled by the simple polyhistidine–liposome system which has been described. Liposomes are a potential vehicle by which macromolecules could be transferred to eukaryotic cells (Ostro & Giacomoni, 1983), but, thus far, it has not been possible to design delivery protocols in which liposome–cell membrane fusion occurs with useful frequency. In general, liposomes are readily endocytosed, and it may be possible to promote the simultaneous uptake of vesicles and polyhistidine fusogen into endosomes. An enhanced delivery of liposome contents to the cytosol would occur if acidification of this compartment leads to liposome–endosome fusion. pH-triggered, polyhistidine-mediated fusion merits further study, both as a model system from which useful observations concerning general fusion mechanisms may be obtained and as a useful tool in cell biology.

ACKNOWLEDGMENTS

We thank Drs. L. Huang, M. G. MacNamee, and D. Papahadjopoulos for helpful discussions. While the manuscript was in preparation, we learned that the investigations of Wang & Huang (1984) in a similar model membrane system agree with our conclusions.

Registry No. Chol, 57-88-5; polylysine, 25104-18-1; polylysine, SRU, 38000-06-5; polyhistidine, 26062-48-6; polyhistidine, SRU, 26854-81-9; 1-palmitoyl-2-oleylphosphatidylcholine, 6753-55-5.

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Modulation of Membrane Fusion by Membrane Fluidity: Temperature Dependence of Divalent Cation Induced Fusion of Phosphatidylserine Vesicles†

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Received March 1, 1984; Revised Manuscript Received August 24, 1984

ABSTRACT: We have investigated the temperature dependence of the fusion of phospholipid vesicles composed of pure bovine brain phosphatidylserine (PS) induced by Ca^{2+} or Mg^{2+}. Aggregation of the vesicles was monitored by 90° light-scattering measurements, fusion by the terbium/dipicolinic acid assay for mixing of internal aqueous volumes, and release of vesicle contents by carboxyfluorescein fluorescence. Membrane fluidity was determined by diphenylhexatriene fluorescence polarization measurements. Small unilamellar vesicles (SUV, diameter 250 Å) or large unilamellar vesicles (LUV, diameter 1000 Å) were used, and the measurements were done in 0.1 M NaCl at pH 7.4. The following results were obtained: (1) At temperatures (0–5 °C) below the phase transition temperature (T_c) of the lipid, LUV (PS) show very little fusion in the presence of Ca^{2+}, although vesicle aggregation is rapid and extensive. With increasing temperature, the initial rate of fusion increases dramatically. Leakage of contents at the higher temperatures remains limited initially, but subsequently complete release occurs as a result of collapse of the internal aqueous space of the fusion products. (2) SUV (PS) are still in the fluid state down to 0 °C, due to the effect of bilayer curvature, and fuse rapidly in the entire temperature range from 0 to 35 °C in the presence of Ca^{2+}. The initial rate of leakage is low relative to the rate of fusion. At higher temperatures (15 °C and above), subsequent collapse of the vesicles' internal space causes complete release. At 0–5 °C, collapse does not occur, and the extent of fusion is limited; as the vesicles grow in size and start to resemble LUV, the fusion process slows down. (3) With Mg^{2+}, LUV (PS) aggregate massively in the entire temperature range from 0 to 35 °C, but fusion and release of contents do not occur. (4) SUV (PS) do fuse in the presence of Mg^{2+}. The initial rate of leakage relative to fusion is severalfold higher than during Ca^{2+}-induced fusion of SUV. At low temperatures, this results in an early and complete release of vesicle contents. At 20 °C and above, complete release does not occur. Fusion stops spontaneously as the vesicles grow in size, and part of the aqueous contents is retained in the vesicles for a prolonged period of time. The results point out that a cation-induced isothermal phase transition of the bilayer lipids is not a prerequisite for the fusion process. Prerequisite for fusion appears to be that the vesicle is in an overall fluid state when the cation has bound to its outer surface.

The development of the fluorescent Tb/dipicolinic acid (Tb/DPA)[1] method registering the mixing of the aqueous contents of phospholipid vesicles (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1981) has considerably facilitated the study of membrane fusion in model systems. Particularly, the divalent cation induced interaction between vesicles containing acidic phospholipids such as phosphatidylserine (PS), cardiolipin, phosphatidic acid or phosphatidylinositol has been investigated extensively (Wilschut et al., 1980, 1981, 1982, 1983; Düzgüneş et al., 1981a,b, 1984a; Bentz

† This investigation was supported by the National Institutes of Health (GM-26369 and GM-28117), by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.), by the American Heart Association (grant-in-aid, with funds contributed by the California affiliate), and by NATO (RG 151.81).

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[1] Abbreviations: CF, carboxyfluorescein; DPA, dipicolinic acid; DPH, diphenylhexatriene; PS, phosphatidylserine; PC, phosphatidylcholine; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/85/0424-0008$01.50/0