Proton-Induced Fusion of Oleic Acid–Phosphatidylethanolamine Liposomes†

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Received March 1, 1984; Revised Manuscript Received November 29, 1984

ABSTRACT: Liposomes composed of oleic acid and phosphatidylethanolamine (3:7 mole ratio) aggregate, become destabilized, and fuse below pH 6.5 in 150 mM NaCl. Fusion is monitored by (i) the intermixing of internal aqueous contents of liposomes, utilizing the quenching of aminonaphthalene-3,6,8-trisulfonic acid (ANTS) by *N,N'-p*-xylylenebis(pyridinium bromide) (DPX) encapsulated in two separate populations of vesicles, (ii) a resonance energy transfer assay for the dilution of fluorescent phospholipids from labeled to unlabeled liposomes, (iii) irreversible changes in turbidity, and (iv) quick-freezing freeze–fracture electron microscopy. Destabilization is followed by the fluorescence increase caused by the leakage of coencapsulated ANTS/DPX or of calcein. Ca^{2+} and Mg^{2+} also induce fusion of these vesicles at 3 and 4 mM, respectively. The threshold for fusion is at a higher pH in the presence of low (subfusogenic) concentrations of these divalent cations. Vesicles composed of phosphatidylserine/phosphatidylethanolamine or of oleic acid/phosphatidylcholine (3:7 mole ratio) do not aggregate, destabilize, or fuse in the pH range 7–4, indicating that phosphatidylserine and phosphatidylcholine cannot be substituted for oleic acid and phosphatidylethanolamine, respectively, for proton-induced membrane fusion. Freeze–fracture replicas of oleic acid/phosphatidylethanolamine liposomes frozen within 1 s of stimulation with pH 5.3 display larger vesicles and vesicles undergoing fusion, with membrane ridges and areas of bilayer continuity between them. The construction of pH-sensitive liposomes is useful as a model for studying the molecular requirements for proton-induced membrane fusion in biological systems and for the cytoplasmic delivery of macromolecules [Straubinger, R. M., Düzgüneş, N., & Papahadjopoulos, D. (1985) *FEBS Lett. 179*, 148–154].

The cell fusion activity of several lipid-enveloped viruses, such as the Semliki Forest, vesicular stomatitis, and fowl plague viruses, is induced by mildly acidic pH (6.1–5.5; White et al., 1981). Entry of the viral genome into the cytoplasm is thought to result from the fusion of the viral membrane with the membrane of the endosome (Marsh et al., 1983) or lysosome (Helenius et al., 1980) after endocytosis of the virus in coated vesicles and acidification of the vesicle lumen. Fusion of the viral membranes with liposomes has also been shown to depend on low pH (White & Helenius, 1980; White et al., 1982). In these systems, fusion is mediated by membrane proteins (White et al., 1983). Proton-induced membrane fusion may also be important in various aspects of intracellular membrane sorting, particularly those related to receptor-mediated endocytosis. Budding of vesicles from intracellular components of the endocytotic pathway which results in the redistribution of receptor and ligand in the recently identified CURL (compartment of uncoupling of receptor and ligand; Geuze et al., 1983), as well as the differential localization of a variety of molecules following endocytosis (Pastan & Willingham, 1981), is likely to involve proton-mediated fusion between the luminal leaflets of the endocytotic vesicle membrane, since these vesicles appear to be acidified soon after pinching off from the plasma membrane (Tycko & Maxfield, 1982). The molecular mechanism of these fusion events is not known.

The endocytotic pathway has also been shown to be the primary route by which negatively charged liposomes are internalized by cultured mammalian cells (Straubinger et al., 1983a, 1985a). Acidification of endosomes containing liposomes appears to enhance the membrane permeability of encapsulated fluorescent markers that are weakly acidic and to enable such molecules to gain access to the cytoplasm (Straubinger et al., 1983a). It may also be possible to improve the cytoplasmic delivery of membrane-impermeable and pH-insensitive molecules by the use of appropriately designed (pH-sensitive) liposomes which may fuse with or destabilize transiently the endosome membrane, much as the viruses mentioned above inject their genome into the cytoplasm, but without the necessity of viral membrane proteins.

In this paper we demonstrate that liposomes composed of a 3:7 (mole ratio) mixture of oleic acid and phosphatidylethanolamine exhibit a high sensitivity to pH changes, aggregating, destabilizing, and fusing below pH 6.5. A preliminary report of our findings has been published (Düzgüneş et al., 1983a).

Materials and Methods

Phosphatidylethanolamine (PE),[1] prepared by transphosphatidylation of egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE), and *N*-(lissamine Rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). Egg PC was purified as described

† This work was supported by National Institutes of Health Grants GM-28117, CA-35340 (to D.P.), and HD-10445 (to D.S.F.), a grant-in-aid from the American Heart Association with funds contributed by the California affiliate (to N.D.), and a postdoctoral fellowship (PF-2331) from the American Cancer Society (to P.A.B.).

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; OA, oleic acid; ANTS, aminonaphthalene-3,6,8-trisulfonic acid; DPX, *N,N'-p*-xylylenebis(pyridinium bromide); MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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by Papahadjopoulos & Miller (1967). Oleic acid (OA), 2-(*N*-morpholino)ethanesulfonic acid (MES), and *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) were obtained from Sigma. NaCl and citric acid were from Mallinckrodt; $CaCl_2$, $MgCl_2$, and EDTA were from Fisher. 1-Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N*,*N*'-*p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Junction City, OR). Water was twice distilled, the second time in an all-glass apparatus, and further purified in a Barnstead Nanopure apparatus.

Liposomes were prepared by drying the lipid mixtures from a chloroform solution under vacuum (<1 mmHg). The resulting thin film was hydrated in 150 mM NaCl, 10 mM TES, pH 7.4, and 0.1 mM EDTA at 30 °C and vortexed vigorously for 10 min under argon. An aqueous solution of calcein (60 mM adjusted to 300 mOsm/L with NaCl, pH 7.4) was substituted in experiments to determine the leakage of liposome-encapsulated molecules. The resulting multilamellar vesicles were extruded through polycarbonate membranes of 0.2-μm pore diameter (Unipore, Bio-Rad, Richmond, CA) to form oligolamellar vesicles of a uniform size distribution (Olson et al., 1979; Düzgüneş et al., 1983b) and vortexed again. Freeze–fracture electron micrographs of these vesicle preparations revealed that they were of approximately 0.2-μm diameter. No small vesicles were observed in the preparations. Reverse-phase evaporation was not utilized for these experiments because of the difficulty in producing uniform dispersions of vesicles composed of OA and PE at pH 7.4 and 150 mM NaCl. Where appropriate, liposomes were separated from free calcein by gel filtration on a Sephadex G-75 column (Pharmacia) (1 × 18 cm). Vesicles were stored on ice, under argon and in the dark. Lipid concentrations were determined by phosphate analysis (Bartlett, 1959).

Calcein leakage from liposomes was observed as the increase in fluorescence upon dilution of the initially self-quenched dye into the external medium. An SLM-4000 fluorometer was used for continuous monitoring of fluorescence, using an excitation wavelength of 430 nm and an emission wavelength of >530 nm (Corning 3-68 cutoff filter). Maximum fluorescence was determined by lysing the vesicles with 0.1% Triton X-100 at the pH used in the experiment.

Membrane fusion was monitored by an assay for the dilution of nonexchangeable fluorescent lipid probes from labeled vesicles to unlabeled vesicles (Struck et al., 1981; Rosenberg et al., 1983). NBD-PE and Rh-PE were incorporated into one population of vesicles ("labeled vesicles") at 1 mol % each, a probe concentration at which there is significant NBD quenching by Rh. The residual fluorescence was taken as 0% of maximum fluorescence. Vesicles to be used for the calibration of the fluorescence to 100% maximum fluorescence contained 0.1 mol % of each of these lipids. The labeled vesicles were mixed with vesicles containing no fluorescent phospholipids ("unlabeled vesicles") at a ratio of 1:9 and a total lipid concentration of 0.05 μmol/mL. Complete intermixing of all the bilayers upon fusion would be expected to result in a membrane containing 0.1 mol % each of the two fluorescent phospholipids, which was taken as the theoretical maximum fluorescence. In some experiments, vesicles containing 0.3 mol % each of NBD-PE and Rh-PE were mixed with unlabeled vesicles at a 1:1 ratio, and the maximum fluorescence was set by using vesicles containing 0.15 mol % probe molecules. The vesicles were suspended in 1 mL of 150 mM NaCl, 10 mM TES, pH 7.4, and 0.1 mM EDTA at 20 °C in a quartz fluorometer cell and were stirred constantly by means of a magnetic stirrer. NBD fluorescence measurements were made continuously by using an excitation wavelength of 450 nm and an emission wavelength of 530 nm. Fusion was monitored as the relief of NBD fluorescence quenching by Rh, as dilution of the fluorescent lipids into unlabeled vesicles resulted in a decrease in the efficiency of resonance energy transfer between NBD and Rh.

Fusion was also monitored by the ANTS/DPX fluorescence assay for the intermixing of internal aqueous contents of vesicles (Ellens et al., 1985a). Two populations of vesicles were prepared containing either 25 mM ANTS/89 mM NaCl or 90 mM DPX/47 mM NaCl in addition to 10 mM TES, pH 7.4. After gel filtration on a Sephadex G-75 column (1 × 18 cm) to eliminate nonencapsulated material, the vesicles were suspended in 150 mM NaCl, 10 mM TES, pH 7.4, and 0.1 mM EDTA, at equal concentrations (0.025 μmol of lipid/mL for each). ANTS fluorescence (>530 nm) was followed in an SLM 4000 fluorometer by means of a Corning 3-68 cutoff filter, with the excitation wavelength at 360 nm. The quenching of ANTS fluorescence by DPX (Smolarsky et al., 1977) is a measure of the intermixing of aqueous contents. Release of contents from the liposomes and their dilution into the medium do not result in the decrese of fluorescence, since quenching by DPX is highly concentration dependent (Ellens et al., 1984).

Release of contents was measured independently by encapsulating 12.5 mM ANTS/45 mM DPX/68 mM NaCl/10 mM TES, pH 7.4, as described above and following the increase in ANTS fluorescence as the contents are diluted into the medium (Ellens et al., 1984).

Aggregation of the vesicles was followed by the increase in turbidity (Düzgüneş & Ohki, 1977) at 450 nm in a Beckman 34 spectrophotometer, or by 90° light scattering (Wilschut et al., 1980) at 430 nm in the fluorometer.

The pH of the liposome suspension was lowered accurately and reproducibly by injecting 10 μL of 1 M MES, acetate, or citrate buffers, which had been adjusted to different pHs. The final pH of the solution was measured by a Corning pH electrode. The effect of pH on NBD fluorescence was minimal, with no more than 10% quenching of fluorescence at pH 3.3. $CaCl_2$ or $MgCl_2$ was introduced into the fluorometer cell by injecting aliquots of 0.2 M stock solutions.

Rapid-freezing freeze–fracture electron microscopy was performed as described by Bearer et al. (1982). An 8-μL aliquot of a liposome suspension (approximately 10 μmol of lipid/mL) was mixed with 2 μL of 50 mM citrate, 150 mM NaCl, and 0.1 mM EDTA, pH 4.0 (final pH 5.3), at approximately 20 °C and frozen within 1 s or after 10 s, using a Polaron quick-freeze apparatus with a copper block cooled with liquid He. The frozen samples were fractured in a Balzers freeze–fracture apparatus, replicated by platinum/carbon rotary shadowing, and observed in a Siemens 101 electron microscope.

Results

Fusion of phospholipid vesicles involves the intermixing of internal aqueous contents and of membrane components and results in an increase of the average vesicle diameter. We have followed the intermixing of contents by the ANTS/DPX fluorescence assay (Ellens et al., 1985a), the intermixing of membrane components by the dilution of NBD-PE and Rh-PE from labeled to unlabeled vesicles (Struck et al., 1981), and the morphological changes associated with fusion by rapid-freezing freeze–fracture electron microscopy (Bearer et al., 1982). We have also monitored the destabilization of the vesicles by the release of encapsulated ANTS/DPX complex (Ellens et al., 1984) or calcein (Allen & Cleland, 1980).

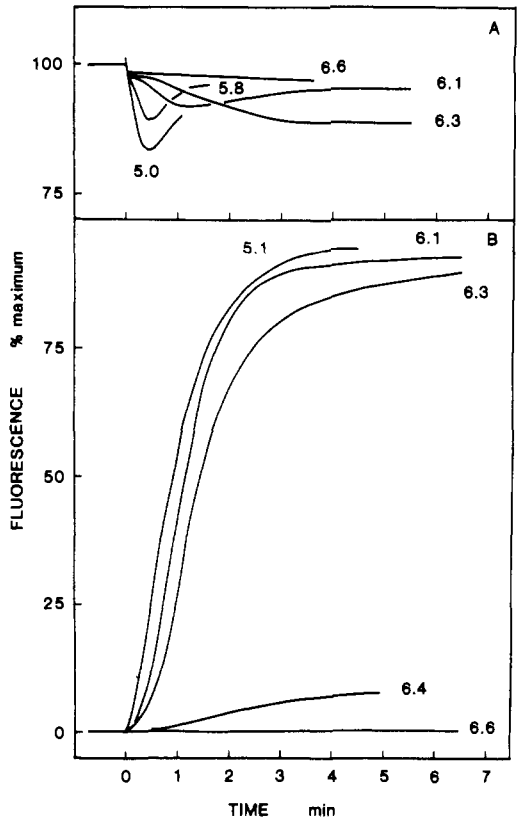

FIGURE 1: Time course of fusion of oleic acid/PE (3:7) liposomes (A) and the release of internal aqueous contents (B) induced by lowering the pH to the indicated values. Fusion was monitored by the ANTS/DPX assay. The fluorescence of the initial suspension of ANTS/vesicles and DPX/vesicles (0.025 μmol of lipid/mL each) was set to 100%. Release was followed by the dequenching of ANTS/DPX initially encapsulated in the liposomes (0.05 μmol/mL). The pH was lowered at $t = 0$; temperature = 20 °C.

The time course of the intermixing of aqueous contents of OA/PE (3:7) liposomes is shown in Figure 1A. The decrease in fluorescence is the result of the quenching of ANTS fluorescence by DPX, initially encapsulated in separate populations of vesicles and now intermixing via fusion of the liposomes. Fusion was observed when the pH was lowered to 6.3 and lower. The time course of intermixing was biphasic at pH 6.3–6.1, with an initial lag phase after which the fluorescence decreased. At lower pH (5.8–5.0), the decrease in fluorescence was immediate, and the initial rate of intermixing of contents increased with decreasing pH. The aqueous contents of the liposomes also leaked rapidly at the threshold pH for fusion, as shown by the dilution of the ANTS/DPX complex into the medium and the relief of quenching of ANTS fluorescence, indicating the immediate destabilization of the liposome membrane (Figure 1B). Similar results were obtained in experiments utilizing calcein-loaded liposomes (data not shown). The leakage of contents was also reflected in the fusion curves by the increase of fluorescence subsequent to the intial decrease, due to the dilution of ANTS and DPX into the medium. The intermixing of aqueous contents appeared to proceed at a rate considerably slower than that of the release of contents (see, for example, the traces for pH 6.3).

The accurate determination of the initial rate or maximal extent of fusion is rendered difficult by this rapid leakage of contents. Thus, it appeared that a fusion assay based on the intermixing of membrane components would provide a more reliable measure of the initial rate and extent of fusion. We have therefore studied the fusion of OA/PE liposomes by means of a resonance energy transfer assay for the dilution

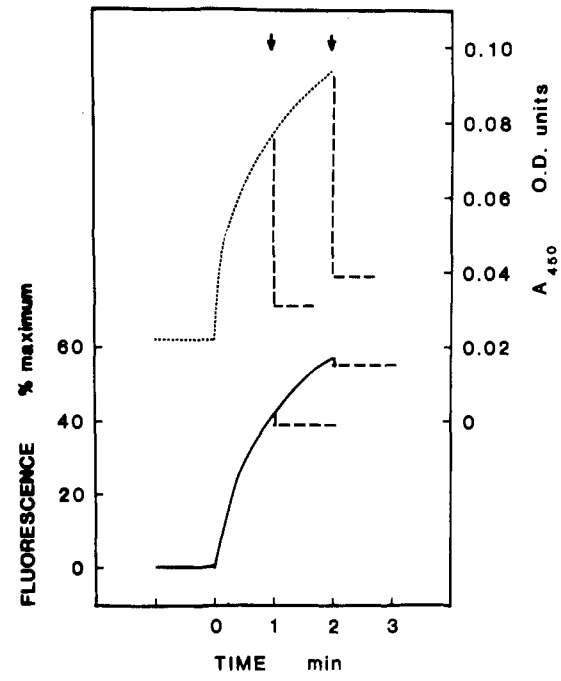

FIGURE 2: Time course of aggregation as measured by the change in turbidity at 450 nm (dotted line) and the fusion (solid line) of oleic acid/PE (3:7) liposomes upon acidification of the medium from pH 7.4 to 5.3 by the addition of citrate buffer at $t = 0$. Fusion was monitored by the lipid mixing assay using a 1:9 mixture of labeled and unlabeled vesicles. The pH was returned to 7.4 at times indicated by the arrows by the addition of a 12-μL aliquot of 1 N NaOH (dashed lines). The fluorescence is given as the percent of maximal fluorescence, obtained as described under Materials and Methods (left-hand ordinate). The absorbance is given on the right-hand ordinate.

of labeled lipids into the membranes of unlabeled vesicles (Struck et al., 1981). In this assay, NBD-PE and Rh-PE are incorporated in the same bilayer, and the fluorescence of NBD is quenched by the proximity of Rh in the plane of the membrane. When fusion takes place between labeled and unlabeled vesicles, the surface density of the fluorescent molecules decreases, resulting in enhanced NBD fluorescence. The vesicle concentration dependence of the initial rate of fusion detected by this assay and the Tb/DPA assay has been compared in two phospholipid vesicle systems and was found to be similar (Rosenberg et al., 1983). The possibility of lipid transfer among aggregated but nonfusing vesicles has also been investigated. No lipid transfer has been detected among large unilamellar PS vesicles which aggregate but do not fuse in the presence of Mg^{2+} (Rosenberg et al., 1983). However, it is conceivable that under certain conditions lipid transfer may occur in the absence of fusion, or the membranes of liposomes may intermix without leading to communication between the internal aqueous compartments (Rosenberg et al., 1983; Ellens et al., 1985a,b).

Figure 2 shows the time course of the dilution of labeled lipids (solid line) and aggregation and increase in size of the vesicles as determined by changes in turbidity (dotted line) induced by lowering the pH from 7.4 to 5.3. The fluorescence was stable at pH 7.4, indicating that there was no transfer of fluorescent lipids from labeled to unlabeled vesicles. Rapid intermixing of membrane components occurred when the pH was lowered, and fusion could be arrested by returning the pH to 7.4 (dashed lines). If the vesicles aggregate reversibly, the absorbance of the vesicle suspension is expected to return to the original level (Nir et al., 1981; Ohki et al., 1982) after the pH is returned to neutral. However, if an irreversible change has occurred, such as fusion, the absorbance is expected to

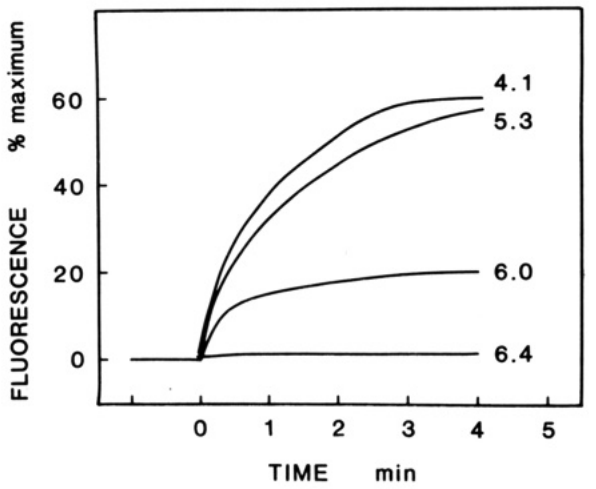

FIGURE 3: Time course of fusion of oleic acid/PE liposomes in media of various pH monitored by the fluorescent lipid mixing assay. The pH was changed to the values indicated in the figure at $t = 0$. Fusion is expressed as the percent of maximum fluorescence obtained as explained under Materials and Methods. Labeled (1 mol % of each probe) and unlabeled liposomes were mixed at a 1:9 ratio at a final lipid concentration of 0.05 μmol/mL.

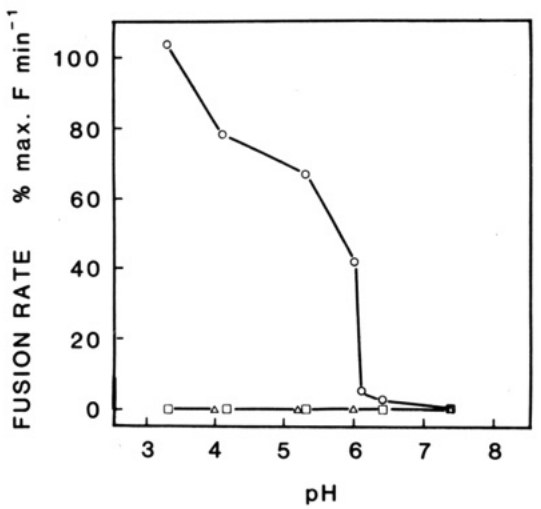

FIGURE 4: pH dependence of the initial rate of fusion of oleic acid/PE (circles), oleic acid/PC (squares), and PS/PE (triangles) liposomes, determined by the fluorescent lipid mixing assay using a 1:9 mixture of labeled and unlabeled vesicles. The initial rate is given as the percent of maximum fluorescence (F) per minute.

remain at a higher value than that of the original suspension (Nir et al., 1980; Ohki et al., 1982). The dashed lines in the upper curve of Figure 2 show increased residual absorbance after the pH is returned to 7.4 following incubation at pH 5.3, corroborating the evidence for fusion obtained by the fluorescence assay. The time course of lipid intermixing induced by various pH levels is shown in Figure 3. The threshold of fusion was just below pH 6.4. The initial rate of fusion is plotted as a function of pH in Figure 4 and shows a rapid increase below pH 6.0. In contrast, no fusion of OA/PC or PS/PE liposomes was observed at pH values as low as 4. Absorbance and light-scattering measurements indicated that these vesicles did not aggregate (data not shown).

In the experiments utilizing the lipid dilution assay reported above, the labeled lipids were present at 1 mol % each within the membrane of the labeled liposomes, and these liposomes were mixed with unlabeled liposomes at a 1:9 ratio. We have also performed experiments using labeled vesicles, containing 0.3 mol % each of the fluorescent lipids, mixed with unlabeled vesicles at a 1:1 ratio. In the latter case, one round of fusion

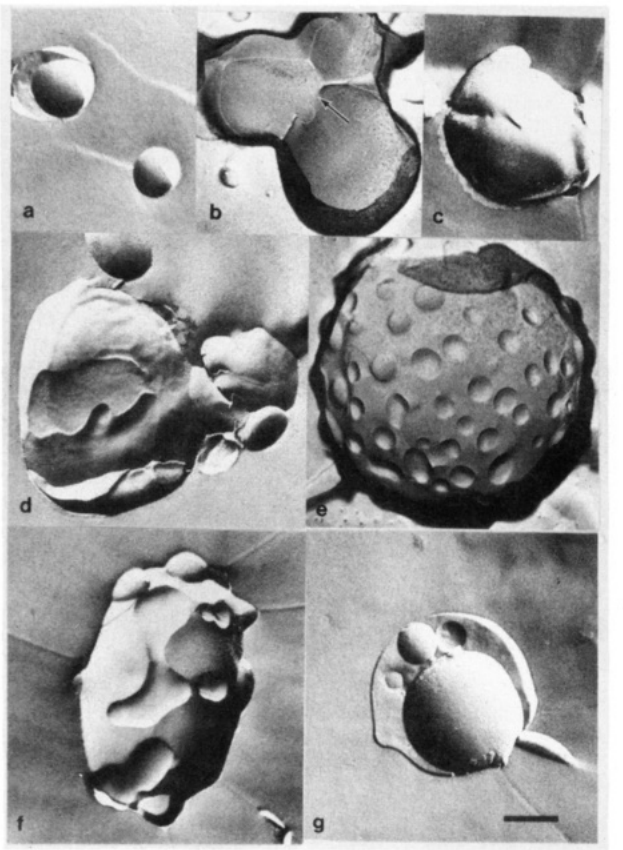

FIGURE 5: Quick-freezing freeze–fracture electron micrographs of oleic acid/PE liposomes within 1 s of lowering the pH to 5.3. Panel a, control liposomes; panels b–g, liposomes within 1 s after initiating fusion. Bar, 0.2 μm.

is expected to yield 50% maximum fluorescence (determined by vesicles containing 0.15 mol % fluorescent lipids), whereas in the former case one round of fusion is expected to give approximately 25% maximum fluorescence (T. M. Allen and N. Düzgüneş, unpublished results). However, the 1:9 case is more sensitive to multiple rounds of fusion. The assay with the 1:1 mixture showed a pH dependence of the initial rate of fusion similar to the 1:9 mixture (data not shown). The fusion vs. time curves leveled off after 1 min following the reduction of pH, in contrast to the curves shown in Figure 3. This behavior is expected because multiple rounds of fusion would not cause as much an increase in NBD-PE fluorescence as would the first round of fusion.

Comparison of Figures 1 and 3 indicates that the rate of lipid mixing (as detected by the dilution of labeled phospholipids) is considerably faster than the rate of intermixing of aqueous contents. This observation suggests that substantial lipid mixing occurs while the vesicles are in the aggregated state but before their aqueous interiors have communicated; this could be due to monomer transfer between vesicles or to intermixing of the outer monolayers of the adhering vesicles (Rosenberg et al., 1983; Ellens et al., 1985a; Allen & Düzgüneş, 1985).

We have examined the morphology of OA/PE liposomes after exposure to low pH by means of rapid-freezing freeze–fracture electron microscopy (Bearer et al., 1982). Figure 5 shows freeze–fracture replicas of liposomes within 1 s of lowering the pH from 7.4 to 5.3. The average size of the liposomes was already severalfold larger (panels b–g) than that of the unstimulated preparation (panel a). The region of adhesion as well as areas of bilayer continuity between aggregated liposomes (or their fusion products) could be dis-

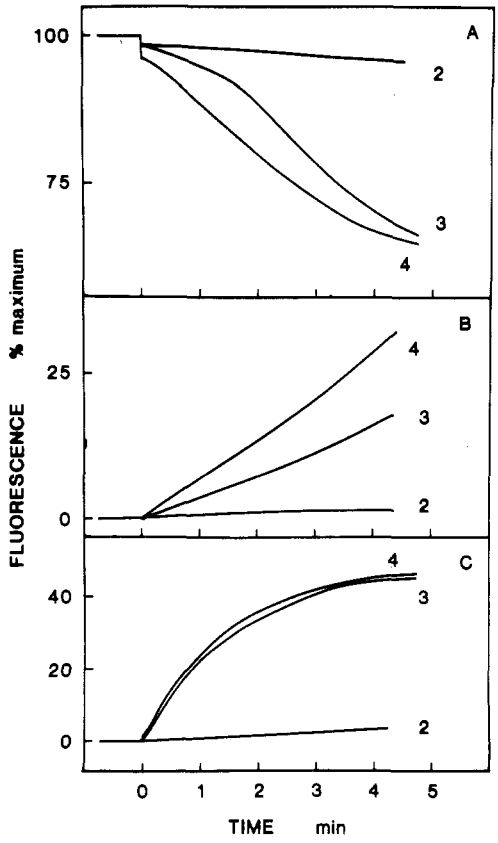

FIGURE 6: Time course of the intermixing of aqueous contents (A), release of contents (B), and mixing of lipids (C) during Ca^{2+}-induced fusion of oleic acid/PE (3:7) liposomes. Lipid mixing was measured by the lipid dilution assay using a 1:9 mixture of labeled (1 mol % of each probe) and unlabeled liposomes. The other assays were as described in Figure 1. Ca^{2+} at the indicated concentrations (in millimolar) was added at $t = 0$.

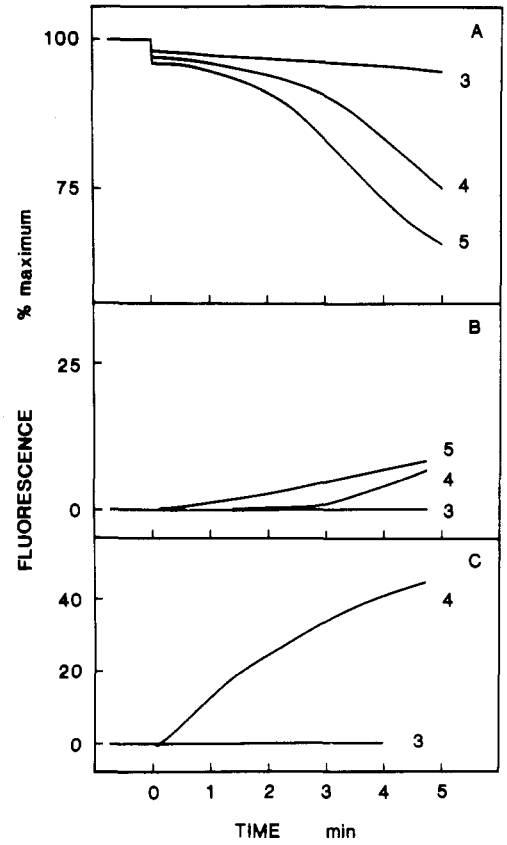

FIGURE 7: Time course of the intermixing of aqueous contents (A), release of contents (B), and mixing of lipids (C) during Mg^{2+}-induced fusion of OA/PE liposomes. Experimental conditions were as described for Figure 6. Mg^{2+} at the indicated concentrations (in millimolar) was added at $t = 0$.

cerned [panels b (arrow) and c]. Ridges, and complementary grooves on the opposite side of the membrane, were a common feature in all images of fusing liposomes (panels b–d). The growth of vesicle size appeared to have been completed within 1 s after reduction of the pH to 5.3, since no further growth was observed in replicas of liposomes stimulated for 10 s (not shown). Since the rate of fusion increases with vesicle concentration (Wilschut et al., 1980, 1981), fusion would be expected to be much faster at the vesicle concentration used in electron microscopy (10 μmol of lipid/mL) than in the kinetic measurements (0.05 μmol/mL). Among the fused liposomes, there were some with unusual morphologies. These liposomes (about 1 in 100) displayed large blisters and indentations (panel e). Panel f in Figure 5 shows the indentations partially fused with each other. The structure displayed in panel g is illuminating for the interpretation of these indentations and blisters; small vesicles appear to be trapped between the bilayers of a multilamellar liposome, which itself may be the product of several fusion events. Since the liposomes used in this study are oligolamellar liposomes extruded to an average size of 0.2-μm diameter, it is likely that fusion proceeds via the coalescence of the outermost bilayers, leaving the inner bilayers as intact vesicles. In time, these vesicles could also fuse with each other and the adjacent bilayers of the larger liposomes.

Fusion of OA/PE liposomes could also be induced by Ca^{2+} or Mg^{2+}. The time course of the intermixing of contents (A), the release of contents (B), and the intermixing of membrane components (C) of OA/PE liposomes in the presence of Ca^{2+} and Mg^{2+} are shown in Figures 6 and 7, respectively. The

Table I: Modulation of Low pH Induced Fusion of OA/PE (3:7) Liposomes by Divalent Cations[a]

	initial rate of fluorescence decrease (% max/min)		
pH	no divalent cation	2 mM Mg^{2+}	2 mM Ca^{2+}
7.4	0.6	0.6	1.9
6.2	5.3	13.1	240.0
5.1	30.0	28.8	375.0

[a] Fusion was monitored by the ANTS/DPX assay for the intermixing of aqueous contents. Divalent cations were introduced 2 min before the reduction of pH from 7.4.

threshold concentrations were 3 mM for Ca^{2+} and 4 mM for Mg^{2+}. The release of contents proceeded at a slower rate than fusion with both cations, but its onset was immediate for Ca^{2+} and delayed for Mg^{2+}. In the ANTS/DPX assay, the extent of fluorescence decrease in the presence of either cation was considerably higher (Figures 6A and 7A) than that obtained by lowering the pH (Figure 1A), which may be explained by the immediate and extensive leakage of contents observed in the latter case. As with low pH, the intermixing of lipids also proceeded at a faster rate than the intermixing of contents in the presence of Ca^{2+} and Mg^{2+} (Figures 6A,C and 7A,C).

The low pH induced fusion of OA/PE liposomes could be modulated by the presence of Ca^{2+} or Mg^{2+} in the incubation medium. OA/PC (3:7) liposomes, however, could not be induced to fuse even in the presence of 10 mM Ca^{2+} at pH 6.1 (data not shown). Table I shows the initial rate of intermixing of aqueous contents of OA/PE liposomes in the presence of Ca^{2+} or Mg^{2+} at three different pHs. In these experiments, the divalent cations were added 2 min prior to the reduction of pH from 7.4. The fusion-enhancing effect of Ca^{2+} was obtained at pH values both near the threshold

of fusion (pH approximately 6) and below it (pH approximately 5). The effect of Mg^{2+} was apparent only at pH 6. In these experiments, the pH was lowered to 5 by injecting an aliquot of 1 M acetate buffer into the incubation medium. Citrate was not utilized because of its metal chelation property. It was observed that the rate of fusion was faster with acetate than with citrate at pH approximately 5. This observation is most likely due to the difference in the permeation of the buffer anions through the membrane and is being investigated further.

It was also of interest to ascertain whether the OA/PE liposomes could fuse with the OA/PC liposomes at low pH. When labeled OA/PE vesicles were mixed with unlabeled OA/PC liposomes (1:9 ratio) and the pH was lowered to 4.1, no intermixing of phospholipids could be detected, indicating that the presence of PE in both membranes is necessary for membrane fusion (data not shown). Although at this pH the OA/PE vesicles would be expected to fuse with each other, the OA/PC liposomes are in excess and would clearly have the opportunity to interact or collide with OA/PE vesicles. It is not known whether the absence of fusion relates merely to the lack of aggregation of the appropriate vesicles or to inhibition of the membrane fusion reaction by PC (Düzgüneş et al., 1981a).

Discussion

The results presented in this paper indicate that liposomes composed of a mixture of OA and PE can undergo fusion when the pH is lowered from 7.4 to 6.0 and below. This process does not require divalent cations, as does the fusion of liposomes composed of a variety of acidic phospholipids and their mixtures with zwitterionic phospholipids (Nir et al., 1983; Düzgüneş, 1985). Pure PS vesicles and vesicles composed of PS/PE or phosphatidate/PE have been shown to fuse when the pH is lowered to 3–3.5 (Papahadjopoulos et al., 1977; Hope et al., 1983), but this pH is much lower than that achieved intracellularly. The range of pHs required to induce fusion in the present system is similar to the range obtained in endocytotic vesicles and lysosomes (Tycko & Maxfield, 1982; Ohkuma & Poole, 1978; de Duve et al., 1974). The pH sensitivity is increased in the presence of Ca^{2+} or Mg^{2+}, which are likely to be constituents of the lumen of the endosome.

PE is a necessary component of the liposomes in conferring pH sensitivity, since its replacement by PC completely abolishes the aggregation and fusion induced by low pH. The physicochemical properties differentiating PE and PC have been reviewed recently (Hauser et al., 1981). The lower hydration of PE compared to PC (Jendrasiak & Hasty, 1974) and its propensity to form nonbilayer structures (Reiss-Husson, 1967; Cullis & de Kruijff, 1979) have been proposed to be important factors which determine the difference in fusion susceptibility of vesicles composed of acidic phospholipids and PE or PC (Düzgüneş et al., 1981a). The ability of PE head groups to form hydrogen bonds between each other (Hauser et al., 1981; Boggs, 1980) may also be a contributing factor. The OA component of the membrane appears to provide a negative charge with a pK within the physiologically relevant pH range, which allows PE to form vesicles at pH 7.4. Von Tscharner & Radda (1981) have reported a pK_a of 7.0 for 12-(9-anthroyloxy)stearic acid in dimyristoylphosphatidylcholine membranes. Kantor & Prestegard (1978) have shown the pK_a of [1-^{13}C]myristic acid in egg PC to be 8.4. Schullery et al. (1981) have estimated the intrinsic pK_a of palmitic acid in dipalmitoylphosphatidylcholine to be about 8.5. Hauser et al. (1979) have shown a gradual decrease in electrophoretic mobility of stearic acid/egg PC liposomes as the pH is lowered from 9 to about 6.5, at which point the mobility diminishes. These observations suggest that, in the vesicle system used in our study, lowering the pH to 6 neutralizes the surface charge and allows the close approach of vesicles. The PC-containing vesicles do not aggregate, most likely because of the repulsive hydration force between PC bilayers (Le Neveu et al., 1976), whereas the PE-containing vesicles can approach to closer distances (Lis et al., 1982). It is known that vesicles made of pure PE aggregate when the pH is lowered from alkaline to neutral (Kolber & Haynes, 1979). Fusion of pure PE vesicles induced by lowering the pH from 9 to 5 has been demonstrated by monitoring the intermixing of aqueous contents (Baldwin et al., 1985; Ellens et al., 1985b). Thus, it appears that when OA is protonated at low pH, it can no longer provide the electrostatic repulsion to keep the vesicles in a dispersed state. When the vesicles' surfaces approach each other at low pH, the PE component of the membrane is already in a fusion-susceptible state. However, it is not clear whether OA functions merely by providing a titratable group or also by acting as a fusogen as demonstrated in studies of erythrocyte fusion (Ahkong et al., 1973). Creutz (1981) has shown that OA can induce the fusion of chromaffin granules preaggregated by Ca^{2+} and synexin. Experiments with other fatty acids will be necessary to elucidate the mechanism of action of OA and are in progress.

Ca^{2+} and Mg^{2+} also induce the fusion of OA/PE liposomes, but the leakage of aqueous contents is very low compared to the case with H^+. The divalent cations not only bind to the fatty acid (Seimiya & Ohki, 1973; Hauser et al., 1976), reducing the surface charge density, but also bind to the phospholipid (Seimiya & Ohki, 1973; Rojas & Tobias, 1965). Fusion of pure PE vesicles in the presence of divalent cations has been shown by freeze–fracture electron microscopy (Stollery & Vail, 1977) and the lipid dilution assay (T. M. Allen and D. Papahadjopoulos, unpublished results; Ellens et al., 1985b). The fusogenic effect of Mg^{2+} on large unilamellar vesicles composed of PS/PE is due to the presence of the PE component, since Mg^{2+} does not induce the fusion of large vesicles composed of pure PS (Düzgüneş et al., 1981a). When the electrostatic interbilayer repulsion caused by the ionized carboxyl group on OA is reduced upon divalent cation binding, the PE component of the aggregated vesicles would be able to interact and fuse if the ionic conditions are suitable for the fusion of pure PE membranes.

The reduction of pH results in the extensive release of aqueous contents of OA/PE liposomes. The rate of release appears to be faster than the rate of intermixing of contents as measured by the ANTS/DPX assay at 20 °C. In this respect, the fusion of OA/PE vesicles differs from the many other phospholipid vesicle systems fusing in the presence of divalent cations in which release is a considerably slower process or virtually nonexistent (Wilschut et al., 1980, 1981, 1983; Düzgüneş et al., 1981a,b). One exception is the fusion of vesicles composed of phosphatidate in the presence of Ca^{2+} at pH 8.5, where the rate of release exceeds the rate of fusion (Sundler & Papahadjopoulos, 1981). The rapid release of contents of OA/PE vesicles suggests that lowering the pH induces a destabilization of the membrane before inducing the fusion of the two membranes. The points of destabilization occurring in the region of contact between two membranes would be likely nucleation points for the intermixing of lipids during fusion (Düzgüneş & Papahadjopoulos, 1983). Ellens et al. (1984) have found that liposomes composed of cholesteryl hemisuccinate and PE leak their contents below pH 5.5 and that this leakage depends on liposome aggregation. In their

liposome system, however, lowering the pH results in lipid mixing but no intermixing of aqueous contents (Ellens et al., 1985a).

pH-induced destabilization and/or fusion of liposomes have been described recently by others. Liposomes composed of palmitoylhomocysteine, diheptadecanoyl-PC, and dipalmitoyl-PC, which release encapsulated carboxyfluorescein at a 5-fold higher rate at pH 6.0 than at pH 7.4, have been designed for possible therapeutic use for the treatment of primary tumors and metastases where the pH is thought to be less than physiological (Yatvin et al., 1980). Connor et al. (1984) have shown that sonicated vesicles composed of palmitoylhomocysteine and PE fuse when the pH is lowered below 7 and have suggested that the protonation of the *N*-acylamino acid leads to changes in electrostatic interaction between lipid head groups, or to phase separation of the lipid species. Sonicated PC vesicles have been shown to undergo fusion in the presence of albumin (Schenkman et al., 1981), or albumin fragments (Garcia et al., 1983) below pH 4, or clathrin (Blumenthal et al., 1983) at pH 6. Fusion of small asolectin vesicles with inner mitochondrial membranes (Schneider et al., 1980) and of vesicles composed of various negatively charged phospholipids in the presence of polyhistidine (Wang & Huang, 1984) has been demonstrated to take place at or below pH 6.5. Bondeson et al. (1984) have found that large unilamellar vesicles composed of phosphatidate, PE, and a glycolipid undergo fusion at pH 6.5 in the presence of lectin to initiate intermembrane contact and have proposed that partial protonation of the anionic lipid results in dehydration of the vesicle surface and induces fusion.

Our observations suggest the possibility that free fatty acids formed during the stimulation of secretion in various cell types (Michell, 1975; Irvine, 1982) may be components of fusion reactions involved in the budding off of new membranous structures from intracellular vacuoles as part of intracellular membrane traffic.

We have recently utilized OA/PE liposomes to deliver encapsulated fluorescent markers, which are themselves not pH sensitive, into the cytoplasm of CV-1 cells (Straubinger et al., 1983b, 1985a,b). Liposomes are thought to be internalized by coated vesicles and to encounter a low pH environment within endosomes (Straubinger et al., 1983a). The mildly acidic pH would cause the destabilization of the vesicles and their interaction with the endosome membrane. This interaction, which may involve the participation of protein components of the endosome membrane, may either destabilize the latter, allowing the entry of the marker from the lumen of the endosome into the cytoplasm, or lead to the fusion of the liposome with the endosome, with concomitant microinjection of the liposome contents directly into the cytoplasm.

ACKNOWLEDGMENTS

We thank Dr. Florea Lupu and Ivy Hsieh for assistance with the electron microscopy, Jean Swallow and Vera Vaughn for preparation of the manuscript, and TRW Semiconductors for their generous donation of electronic components.

Registry No. OA, 112-80-1; Ca, 7440-70-2; Mg, 7439-95-4.

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