Stern-Bach, Y., Greenberg-Ofrath, N., Flechner, I., & Schuldiner, S. (1990) *J. Biol. Chem. 265*, 3961-3966.
Van Tan, H., Fischer, S., & Fagard, R. (1988) *Eur. J. Biochem. 172*, 67-72.
Vincent, M. S., & Near, J. A. (1990) *Biochem. Pharmacol. 39*, 1823-1825.
Wouters, W., Van Dun, J., Leysen, J. E., & Laduron, P. (1985) *J. Biol. Chem. 260*, 8423-8429.

pH-Dependent Membrane Fusion and Vesiculation of Phospholipid Large Unilamellar Vesicles Induced by Amphiphilic Anionic and Cationic Peptides†

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ABSTRACT: We studied fusion induced by a 20-amino acid peptide derived from the amino-terminal segment of hemagglutinin of influenza virus A/PR/8/34 [Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987) *J. Biochem. (Tokyo) 102*, 957-962]. To extend the study, we have prepared several water-soluble amphiphilic peptides derived from the HA peptide; the anionic peptides D4, E5, and E5L contain four and five acidic residues and the cationic peptide K5 has five Lys residues in place of the five Glu residues in E5. Fusion of egg phosphatidylcholine large unilamellar vesicles induced by these peptides is assayed by two different fluorescence methods, lipid mixing and internal content mixing. Fusion is rapid in the initial stage (12-15% within 20 s) and remains nearly the same or slightly increasing afterward. The anionic peptides cause fusion at acidic pH lower than 6.0-6.5, and the cationic peptide causes fusion at alkaline pH higher than 9.0. Leakage and vesiculation of vesicles are also measured. These peptides are bound and associated with vesicles as shown by Ficoll discontinuous gradients and by the blue shift of tryptophan fluorescence. They take an α-helical structure in the presence of vesicles. They become more hydrophobic in the pH regions for fusion. When the suspension is made acidic or alkaline, the vesicles aggregate, as shown by the increase in light scattering. The fusion mechanism suggests that the amphiphilic peptides become more hydrophobic by neutralization due to protonation of the carboxyl groups or deprotonation of the lysyl amino groups, aggregate the vesicles together, and interact strongly with lipid bilayers to cause fusion. At higher peptide concentrations, E5 and E5L cause fusion transiently at acidic pH followed by vesiculation.

Membrane fusion activity of enveloped viruses has been studied extensively (White et al., 1983; Ohnishi, 1988; White, 1990). The envelope contains fusogenic glycoproteins whose hydrophobic segments are related to the fusion activity. In HA[1] of influenza virus, the hydrophobic segment exists at the N-terminus of the HA2 segment, a posttranslational product of HA. We have synthesized an eicosapeptide with the same amino acid sequence as that of the hydrophobic segment of the influenza virus A/PR/8/34 strain. We showed that the HA peptide caused fusion of egg yolk PC sonicated vesicles at acidic pH but not at neutral pH, in a manner similar to that of the parent virus (Murata et al., 1987a). We propose that the low-pH-induced fusion may arise from protonation of the carboxyl groups of the peptide (Maeda & Ohnishi, 1980; Ohnishi, 1988). In our study of succinylated melittin, the protonation of carboxyl groups was detected by ^{13}C-NMR and its pH dependence was nearly the same as that of fusion activity (Murata et al., 1987b).

Lear and DeGrado (1987) synthesized a 20-amino acid peptide from the HA2 N-terminal sequence of influenza virus B/Lee/40 strain. They showed fusion of dioleoyl-PC sonicated vesicles at neutral pH. They also prepared a 16-amino acid peptide which did not show fusion activity. Duzgunes and Gambale (1988) prepared a 17-amino acid peptide from influenza virus X-31. The peptide did not cause fusion of sonicated vesicles at pH values of 7 or 5 but did cause leakage of vesicles. Recently, Rafalsky et al. (1990) synthesized peptides representing the N-terminal 23 residues of gp41 of the human immunodeficiency virus. They showed evidence for fusion of sonicated phosphatidylglycerol, but not of PC, vesicles.

In the present study, we have synthesized several water-soluble amphiphilic anionic peptides, D4, E5, and E5L derived from the HA peptide (Figure 1). We have also prepared a cationic peptide K5 with the same sequence as E5 except for five Lys residues, to investigate the pH dependence of fusion. We have studied the fusion of egg PC LUVs induced by these peptides by two different fluorescent methods, lipid mixing and internal content mixing. The results clearly show that the anionic peptides cause fusion at acidic pH and the cationic

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[1] Abbreviations: HA, hemagglutinin; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); PC, phosphatidylcholine; LUV, large unilamellar vesicle; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *N*,*N*'-*p*-xylylenebis(pyridiniumbromide); R_{18}, octadecylrhodamine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; C9AC, cholesteryl anthracene-9-carboxylate.

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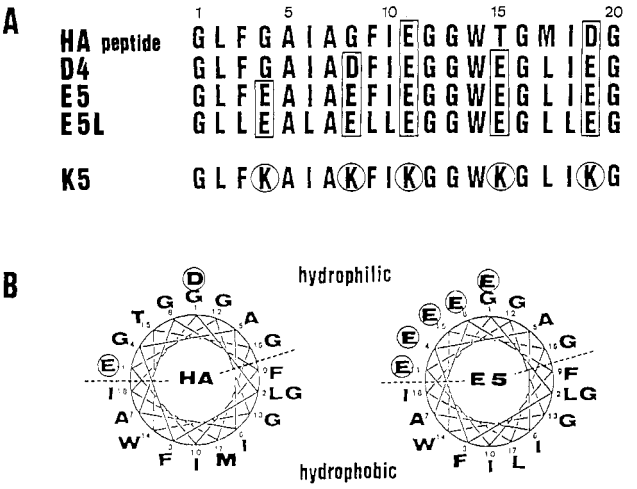

FIGURE 1: (A) Amphiphilic peptide analogues derived from HA peptide. D4, E5, and E5L are the anionic peptides with four, five, and five acidic residues, respectively, in comparison with the two residues in the HA peptide. K5 is the cationic peptide with five Lys residues versus five Glu residues in E5. (B) Helical diagram for HA peptide and E5.

one at alkaline pH. The peptides become more hydrophobic and take an α-helical structure in the pH regions for fusion. At higher peptide concentrations, E5 or E5L causes transient fusion at acidic pH and then vesiculation.

Materials and Methods

Materials. Peptides were synthesized as described (Takahashi, 1990). The peptide concentration was determined from the absorbance at 280 nm using molar extinction coefficients (M^{-1} cm^{-1}) of 5500 for D4, 5220 for E5, 5670 for E5L, and 5500 for K5 at pH 7.0–8.6. Egg yolk PC was extracted and purified according to Singleton et al. (1965) and assayed by the method of Bartlett (1959). Fluorescent probes, ANTS, DPX, NBD-PE, and R_{18} were purchased from Molecular Probes. Ficoll was obtained from Nacalai Tesque (Kyoto).

Assay of Fusion and Leakage. Internal content mixing was assayed by using ANTS/DPX as described in Ellens et al. (1985). Egg PC LUVs were prepared by reverse-phase evaporation and contained either 25 mM ANTS, 40 mM NaCl, and 10 mM Tris-HCl (pH 7.5) or 90 mM DPX and 10 mM Tris-HCl (pH 7.5). LUVs were extruded through 0.2-μm and 0.1-μm polycarbonate membranes (Nuclepore Corp.), successively, and separated from unencapsulated materials on Sephadex G-75 (Pharmacia) by using 100 mM NaCl and 10 mM Pipes (pH 7.4) as an elution buffer. A 0.5 mM (phospholipid concentration) 1:1 mixture of ANTS-containing LUVs and DPX-containing LUVs was suspended in 0.4 mL of Pipes buffer (140 mM NaCl and 5 mM Pipes, pH 7.5), and a small volume of peptide in Pipes buffer was added at time zero. The decrease in ANTS fluorescence at 530 nm, with the excitation wavelength 384 nm, was monitored at room temperature (22–23 °C) with a Hitachi 850S fluorescence spectrometer. The first measurement was done at about 5 s after the addition of peptide. The fluorescence intensity of the LUV suspension before addition of peptide was taken as 0% fusion, and 100% fusion was set equal to the zero level of the recorder. The fusion % was 100 minus the recorded fluorescence percentage of the maximum quenching.

Leakage of vesicle contents was assayed by using LUVs containing 12.5 mM ANTS, 45 mM DPX, and 10 mM Tris-HCl (pH 7.5). The peptide solution was added to the LUVs at time zero and the increase in ANTS fluorescence was measured. One hundred percent leakage was taken as the fluorescence intensity of LUVs after addition of Triton X-100 at a final concentration of 0.3%.

Lipid mixing of vesicles was assayed as described by Struck et al. (1981) with some modifications. Egg PC LUVs containing 1 mol % each of NBD-PE and R_{18} were prepared in Pipes buffer. A 0.5 mM 1:1 mixture of labeled and unlabeled LUVs was suspended in 0.4 mL of Pipes buffer, and a small volume of peptide in Pipes buffer was added at time zero. The increase in NBD fluorescence at 530 nm, with the excitation wavelength 450 nm, was monitored. One hundred percent or 0% fusion was taken as the fluorescence intensity of LUVs after addition of 0.3% Triton X-100 or before addition of peptide, respectively.

Fusion or vesiculation of vesicles was also investigated by electron microscopy. Egg PC LUVs were incubated in the presence of peptide, stained with 2% uranyl acetate, and viewed by a JEOL 100B electron microscope.

The 90° light scattering of an LUV suspension was measured at 400 nm to obtain information on aggregation and fusion.

Physicochemical Characterization of Peptides. The hydrophobicity of the peptides was estimated by their partitioning into Triton X-114 as described by Bordier (1981). Peptides were mixed at 0 °C with buffer at various pH values containing 0.5% (w/v) (final concentration) Triton X-114. The mixed solution was incubated at 0 °C for 30 min and then at 37 °C for 10 min and overlaid on a cushion of 6% (w/v) sucrose containing 0.06% (w/v) Triton X-114. After centrifugation at a low speed, the upper aqueous phase and the lower detergent phase were separated carefully. Triton X-114 at a final concentration of 5% was added to the upper phase. The peptide concentrations of both phases were estimated by the fluorescamine (Roche) assay.

Binding of peptides to egg PC sonicated vesicles was assayed by flotation centrifugation with Ficoll, as described by Shen et al. (1982). A suspension (50 μL) of vesicles containing 1% fluorescent marker CA9C was mixed with 20 μL of peptide (1–300 μM) and 30 μL of buffer at various pH values and incubated at room temperature for 5 min. The suspension was mixed with 100 μL of 25% Ficoll in Pipes buffer or in citrate buffer (100 mM NaCl and 50 mM sodium citrate, pH 5.0). One milliliter of 10% Ficoll in the same buffer, and the buffer, were overlaid to the suspension successively and centrifuged at 3000 rpm for 30 min using a Hitachi 05PR-22 swing rotor. After centrifugation, 100-μL fractions were taken from bottom to top and the vesicle concentration in each fraction was measured by the CA9C fluorescence. Most vesicles were concentrated at the interface between 10% Ficoll and buffer solution. The peptide concentration was determined by the fluorescamine assay.

CD spectra were measured at room temperature using a JASCO J-600 spectrophotometer. The peptide concentration was 220–240 μM in phosphate buffer (140 mM NaCl and 10 mM NaP_i, pH 7.4) or citrate buffer, in the absence or presence of 5 mM egg PC sonicated vesicles. The ellipticities were expressed as the mean residue weight basis.

The fluorescence spectrum of the tryptophan residue of peptides was measured by the excitation wavelength at 280 nm with peptide at 28 μM in the absence or presence of sonicated PC vesicles at 0.5 mM.

Results

Amphiphilic Peptides and Their CD Spectra. The CD spectrum of the HA peptide showed a distorted helical structure with a minimum at 222 nm, at acidic and neutral pHs, in the presence and absence of vesicles (Takahashi, 1990).

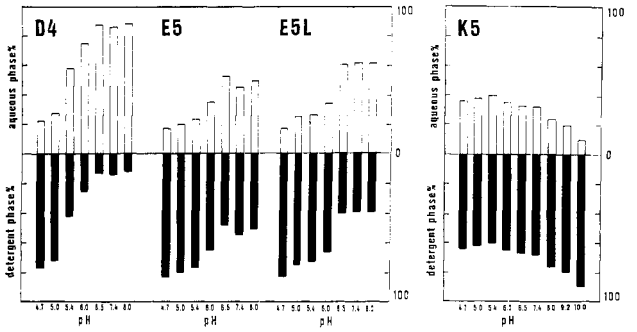

FIGURE 2: Hydrophobicity of peptides at various pH values as estimated by the Triton X-114 partitioning method. The concentrations of peptide in aqueous phase and in detergent phase were determined by the fluorescamine assay and plotted as a function of pH.

The ellipticity value $-[\theta] \times 10^{-3}$ at 222 nm was 9.0–9.5. In 50% trifluoroethanol, the HA peptide showed a typical α-helical structure with two minima at 206 and 222 nm. If we construct an α-helical wheel for the HA peptide, we have a segregated distribution of hydrophilic surface containing the acidic residues on one side and hydrophobic surface on the other side (Figure 1B).

To obtain more water-soluble peptide analogues, we reserved a row of residues Gly 1, Ala 5, Gly 12 and Gly 16 and changed another row of Gly 8 and Thr 15 into acidic residues. By this change D4 had four acidic residues. Both Gly residues 4 and 8 were converted into Glu residues in E5, which now contained five acidic residues. A cationic peptide K5 was synthesized with five Lys residues instead of the five Glu residues in E5. To make the peptide sequence more simple, with conservation of the amphiphilicity, we changed Ile residues 6, 10, and 18 and Phe residues 3 and 9 on the hydrophobic surface into five Leu residues in E5L. In all the peptide analogues, the oxidizable Met 17 was changed into Leu and the Asp 19 was converted into Glu to avoid the Asp-Gly sequence.

The CD spectra of the anionic peptides showed α-helical structure in the presence of vesicles (Takahashi, 1990). The helical content was larger at acidic pH than that at neutral pH. The ellipticity value ($-[\theta] \times 10^{-3}$) at 222 nm at neutral or acidic pH values was 7 or 14 for D4, 13 or 20 for E5, and 17 or 21 for E5L, respectively. In the absence of vesicles, D4 took a nearly random coil structure at both neutral and acidic pH values. E5 was random coil at neutral pH but showed distorted helix at acidic pH. E5L showed α-helical structure at both neutral and acidic pH values, with more ellipticity in the latter.

The CD spectrum for K5 in the presence of vesicles could not be measured because of a large increase in the light scattering. In the absence of vesicles, K5 took a nearly random coil structure, with signs of helical structure at neutral pH. However, K5 showed a distorted helical structure at alkaline pH, with $-[\theta] \times 10^{-3}$ at 222 nm of 9. These data indicate that the amphiphilic anionic and cationic peptides have α-helical or distorted helical structure as shown in Figure 1B in the presence of vesicles.

Hydrophobicity and Interaction with Lipid Vesicles. The hydrophobicity of peptides at various pH values was measured by partitioning into Triton X-114 (Figure 2). All these peptides were partitioned in both aqueous and detergent phases. However, the extent of partitioning into detergent phase became larger at pH values lower than 6.0 for the anionic peptides or higher than 8.0 for the cationic peptide.

The anionic and cationic peptides were bound to egg PC sonicated vesicles as shown by the Ficoll floating method. More than 95% of the peptide was bound to 1.25 mM vesicles for peptide concentrations lower than 300 μM in the pH range from 5.0 to 10.5.

The maximum wavelength of tryptophan fluorescence of peptides was 354–355 nm in the absence of vesicles. When egg PC vesicles (0.5 mM) were added to the peptide solution (28 μM), the wavelength blue-shifted by 10–20 nm within 2 s. The shift was from 349 to 340 nm for HA peptide and from 355 to 333 nm for D4, E5L, and K5 at various pH values. The shift for E5 was from 353 to 344 nm at neutral pH and from 353 to 333 nm at acidic pH. The blue shifts suggest an immersion of the tryptophan residue from aqueous medium into the low-polarity environment of the lipid bilayer (Dufourcq & Faucon, 1977).

Fusion Activity Induced by Anionic or Cationic Peptides. Fusion activity of egg PC LUVs induced by peptide was assayed by lipid mixing (Figure 3A) and by internal content mixing (Figure 3B). The two methods showed a rapid increase of 12–15% fusion within 20 s. Afterwards, the extent of fusion either remained at the same level or slightly increased.

The anionic peptides caused fusion at acidic pH values and the cationic peptide at alkaline pH values. Figure 4 shows the pH dependence of fusion measured at 3 min after addition of peptide. Fusion started to occur at pH values lower than 6.5–6.0 for the anionic peptides and higher than 7.5 for the cationic peptide. The degree of fusion was slightly dependent on peptides. The efficiency after 3 min was a little larger for E5L, with 22% for the internal content mixing (icm) and 29% for the lipid mixing (lm). The efficiency for other peptides was 13% (icm) and 19% (lm) for E5, 12% (icm) and 16% (lm) for D4, and 18% (icm and lm) for K5.

Fusion determined by internal content mixing was nearly the same or a little smaller than that determined by lipid mixing. One reason for the lower extent of content mixing may be the leakiness of the internal contents from LUVs before and/or during fusions (see Discussion). The leakage was measured independently by using egg PC LUVs containing both ANTS and DPX (Figure 3C). The peptide-induced leakage was also very rapid and extensive, 60–80% in 20 s in the pH values for fusion. The leakage induced by E5 or K5 was almost independent of pH, but that induced by D4 or E5L was dependent on the pH, being smaller at neutral pH.

In internal content mixing, the fluorescence level remained nearly constant after the initial rapid rise. The constant level suggests nonleakiness of fluorescent probes from the fused larger vesicles.

In the lipid mixing for K5 at neutral pH, the fluorescence intensity increased to a value of 7% after 3 min (Figure 3A). However, there was no increase in the internal content mixing (Figure 3B; see also Figure 4). Therefore, the small increase must arise from exchange of the fluorescent probes NBD-PE and R_{18} with phospholipid molecules between membranes in the absence of fusion or from mixing of outer monolayers.

The 90° light scattering of LUVs in the presence of anionic peptides was nearly the same at neutral pH as that of LUVs alone. However, when the pH was made acidic, the light scattering was greatly increased, indicating aggregation and fusion of vesicles. In the presence of cationic peptide, the light scattering of LUVs was already larger than that of LUVs alone at acidic and neutral pH values. However, when the pH was made alkaline, the light scattering increased further. The light scattering of all the peptides by themselves was negligibly small at various pH values.

Fusion was also investigated by negative-stain electron microscopy. On incubation of LUVs (0.5 mM) with an anionic peptide (14 μM) at pH 5.0 for 5 min at room temperature,

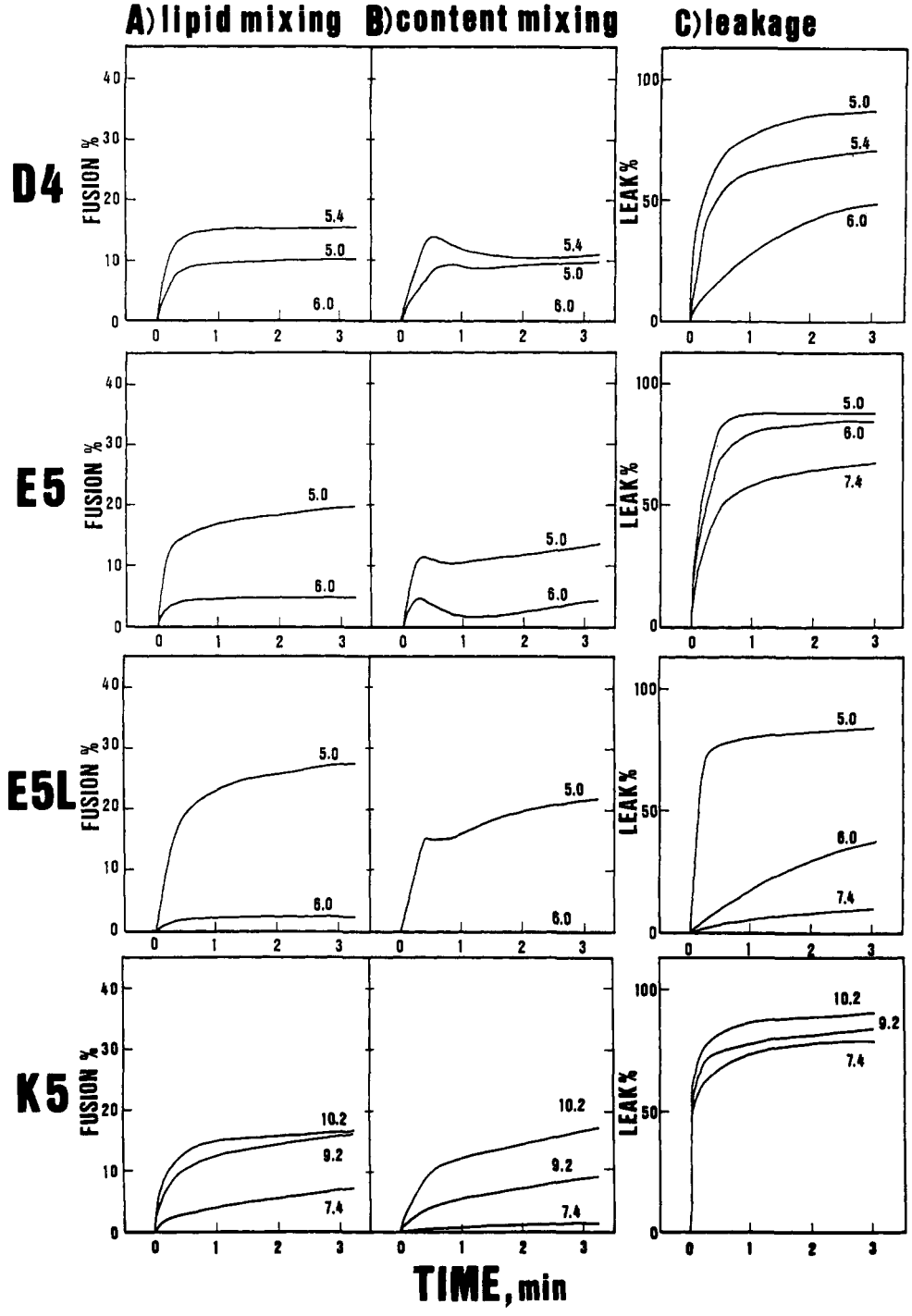

FIGURE 3: Time course of fusion and leakage of egg PC LUVs induced by anionic and cationic peptides at various pH values. Fusion was measured by the lipid mixing (A) and the internal content mixing (B). Leakage was measured by the release of ANTS (C). Peptide (12.5 μM) was added to a mixture of 0.25 mM LUVs containing 1 mol % of NBD-PE and R_{18} and 0.25 mM unlabeled LUVs (in panel A), to a mixture of 0.25 mM ANTS-containing LUVs and 0.25 mM DPX-containing LUVs (in panel B), or to 0.5 mM LUVs containing both ANTS and DPX (in panel C). The L/P ratio was 40.

the enlargement of some vesicles was observed with diameters of 1–1.2 μm (Figure 5B). When incubated at neutral pH, LUVs remained small and homogeneous with diameters of 0.1–0.3 μm (Figure 5A). Similar enlargement of vesicles was also observed when LUVs were incubated with K5 at alkaline pH.

Fusion and Vesiculation at Higher Concentrations of E5 or E5L. The peptide-induced fusion and leakage of egg PC LUVs were studied as a function of the phospholipid to peptide (L/P) ratio. The fusion efficiency and the extent of leakage were generally larger than those at larger L/P ratios (Figure 6). However, for higher concentrations of E5 and E5L, i.e., smaller L/P ratios of 20 for E5 or 30 for E5L, the extent of internal content mixing was smaller than at larger L/P ratios.

Figure 7 shows the time course of light scattering, lipid mixing, and internal content mixing for E5 at the small L/P ratio of 20 in comparison with larger L/P ratios of 75 or 90. The light scattering value was larger at larger L/P ratios of 75 and 90. However, the value at L/P = 20 did not follow this tendency. It increased rapidly within 10 s, then decreased, and became a little smaller than the initial scattering value I_0 after 1 min ($I_0/I = 0.95$). The internal content mixing at L/P = 20 was also increased rapidly in the initial stage up to 10 s and then decreased. The mixing ratios after 40 s were

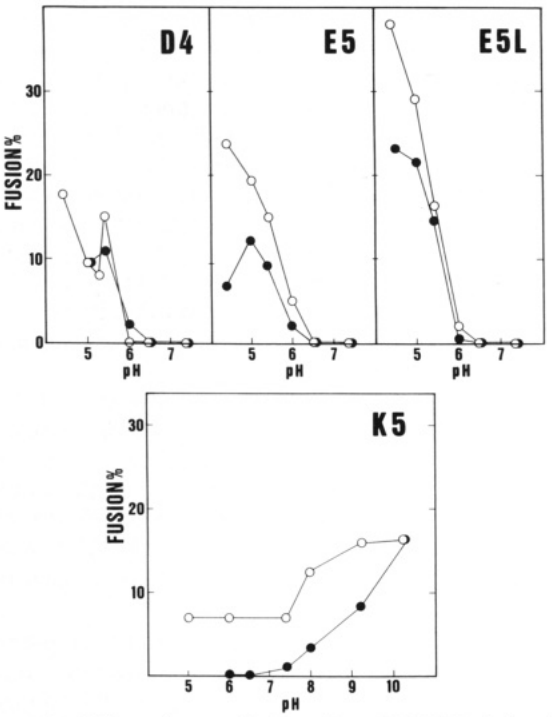

FIGURE 4: pH dependence of fusion of egg PC LUVs induced by anionic and cationic peptides. The fusion efficiency was measured at 3 min after addition of peptide using the lipid mixing (○) and the internal content mixing (●). The L/P ratio was 40.

now smaller than those obtained at L/P = 75. These results suggest that, after rapid fusion in the initial stage, the internal contents leaked out, probably by rupture of bilayers.

A negative-stain electron micrograph also showed rapid fusion and then vesiculation at an L/P ratio of 20 for E5. The electron micrograph observed at *R* (fusion for 20 s with the largest value of light scattering) showed larger vesicles of diameters around 1 μm and some smaller vesicles (0.1–0.3 μm) (Figure 5C). The smaller ones were similar to or a little smaller than egg PC LUVs. The micrograph obtained at *S* (fusion for 3 min with $I_0/I = 0.95$) only showed smaller vesicles of 0.1–0.3 μm (Figure 5D). These data suggest a rapid transient fusion and then vesiculation. The tubular structure was noticeable only in the vesiculation processes.

The other peptides, D4 and K5, and also the HA peptide did not cause such vesiculation at acidic pH when the L/P ratios were changed from 150 to 12. They only caused fusion.

Discussion

We have shown that the amphiphilic anionic peptides caused fusion at acidic pH values and the cationic peptide at alkaline pH values. Fusion was very rapid to about 12–15% in the initial stage (<20 s) and changed only slightly afterward. The pH dependence of fusion for the anionic peptides was similar to that for the HA peptide (Murata et al., 1987a).

Fusion of LUV was measured by the two fluorescent methods of lipid mixing and internal content mixing. The fusion extent determined by internal content mixing was nearly the same or a little smaller than that determined by lipid mixing. The smallness may arise from leakage of the internal contents. We measure leakage with LUVs containing ANTS/DPX probes. When leakage occurs, the diffusion of ANTS to the aqueous phase directly increases the fluorescence. In internal content mixing, the fluorescence changes only by the formation of the ANTS/DPX complex after fusion of ANTS-containing LUVs and DPX-containing LUVs. The fusion reaction should occur more rapidly than leakage.

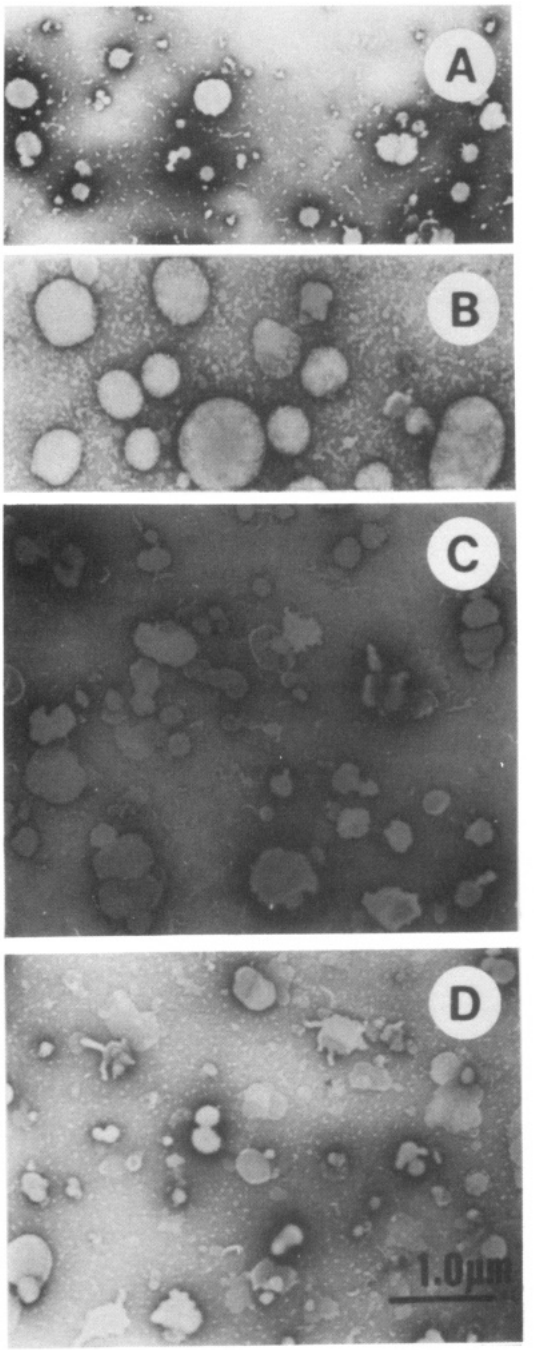

FIGURE 5: Electron microscopic observations of egg PC LUVs incubated with E5 for 5 min at room temperature at pH 7.4 (A) or pH 5.0 (B), at an L/P ratio of 75, and of egg PC LUVs incubated with E5 at pH 5.0 for 20 s (C) or 2 min (D) at an L/P ratio of 20.

The fluorescence probe should not leak from the fused larger vesicles in internal content mixing. Not all but some fractions of vesicles fused rapidly in the present case. The electron microscopic observation also suggests such a burst fusion followed by its arrest. The rapidness of fusion and nonleakiness of fused vesicles should enable the internal content mixing assay to measure fusion effectively.

Fusion of sonicated egg PC vesicles was also measured by lipid mixing with the same fluorescent probes (unpublished results). Internal content mixing was not able to be measured in this case because of the smaller internal volumes of sonicated vesicles. Fusion of egg PC sonicated vesicles induced by the anionic or cationic peptides was about 1.4 times larger than that with LUVs at the same peptide and lipid concentrations. This suggests that the fusion reaction is faster in sonicated

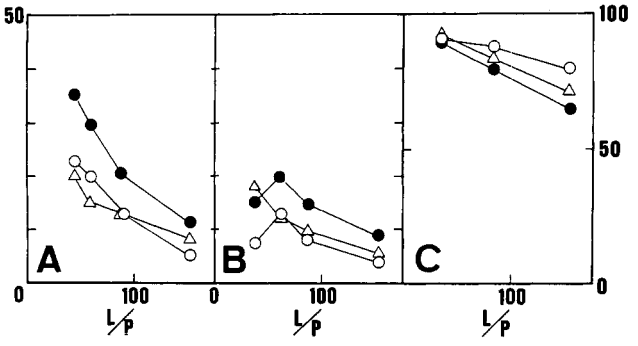

FIGURE 6: Dose dependence of fusion and leakage of egg PC LUVs induced by anionic peptides [(E5 (○) or E5L (●)] at pH 5.0 or by the cationic peptide K5 (△) at pH 9.6. The extent of fusion by lipid mixing (A) or by internal content mixing (B) and the extent of leakage (C) were measured at 3 min after addition of peptide and plotted against the L/P ratio. The vesicle concentration was held constant at 0.5 mM.

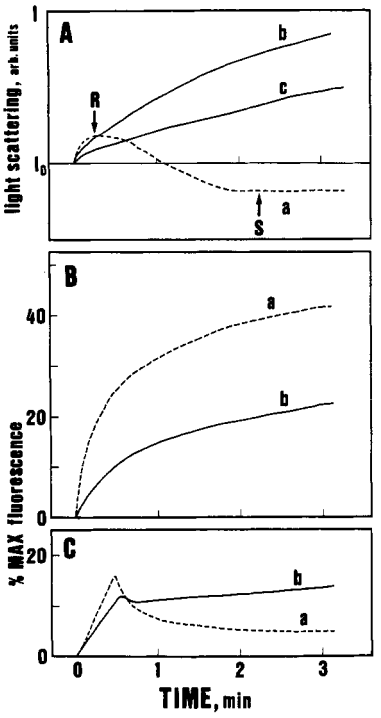

FIGURE 7: Time course in light scattering (A), lipid mixing (B), and internal content mixing (C) of egg PC LUVs incubated with E5 at L/P ratios of 20 (a), 75 (b), or 90 (c). The peptide [25 μM (a), 6.8 μM (b) or 5.5 μM (c)] was added to a mixture of 0.125 mM labeled LUVs and 0.375 mM nonlabeled LUVs for lipid mixing (panel B) or to a mixture of 0.125 mM ANTS-containing LUVs and 0.375 mM DPX-containing LUVs for internal content mixing (panel C). The electron micrographs C and D in Figure 5 were taken at the points R and S in (A). I_0 in (A) shows the initial light scattering value.

vesicles than in LUVs. Fusion of sonicated vesicles was also measured by using spin-labeled PC (Murata et al., 1990). When egg PC vesicles at 5 mM were incubated with the anionic or cationic peptides at 125 μM, 70–90% fusion occurred in 1 min. The higher extent of fusion in this case may be due to larger concentrations of peptide and vesicles than those for the fluorescent measurements.

The anionic and cationic peptides were bound to egg PC vesicles at various pH values as shown by the Ficoll flotation method. The blue shift in tryptophan fluorescence demonstrated that the residue was immersed in nonpolar regions of the lipid bilayer. However, such binding due to the amphiphilic nature of peptides at neutral pH does not cause fusion. The peptides caused leakage. E5 or K5 caused leakage independently of pH. D4 or E5L did not cause leakage at neutral pH but only at acidic pH. When the solution was made acidic or alkaline, the peptides became more hydrophobic, probably because of protonation of the carboxyl groups or deprotonation of the lysyl amino groups, respectively. The neutralized peptides had higher α-helical content. In acidic or alkaline solution, the peptides on the vesicle surface were more hydrophobic and complexed to cause aggregations, as shown by the increase in light scattering. The neutralized hydrophobic complexes may interact with the hydrocarbon region of closely associated lipid bilayer membranes to cause fusion. These processes may be involved in the fusion by the amphiphilic anionic or cationic peptides.

Recently we showed that an equimolar mixture of the anionic peptide E5 and the charge-reversed cationic peptide K5 caused fusion of egg PC LUVs at neutral pH (Murata et al., 1991). The peptides interact with each other by coulombic electrostatic interactions between the negative charges of E5 and the positive charges of K5 at neutral pH. They become more hydrophobic and take an α-helical structure when mixed together.

Lear and DeGrado (1987) showed fusion of sonicated dioleoyl-PC vesicles induced by the 20-amino acid peptide derived from influenza virus. The peptide had two acidic residues but caused fusion only at neutral pH. This is different from the present conclusion showing fusion at acidic pH but not at neutral pH. The 23-amino acid peptide derived from the human immunodeficiency virus had no acidic residues (Rafalsky et al., 1990). It caused fusion of phosphatidylglycerol-containing vesicles at neutral pH. Parente et al. (1988) showed a low-pH-induced fusion of sonicated PC vesicles induced by the amphiphilic peptide GALA. The hydrophobic complex in lipid bilayers should be important for fusions induced by these amphiphilic peptides.

In fusions of phosphatidylserine (PS) vesicles by Ca^{2+} (Portis et al., 1979) or by the positively-charged peptides like poly(L-lysine) (Walter et al., 1986), hydrophobic complex formations have been indicated, such as the trans Ca^{2+}–PS dehydrated complex in the former or the serine–cationic group–serine complexes in the latter.

Both leakage and fusion have been measured in various systems. In Ca^{2+}-induced fusion of PS LUVs, fusion was rapid in the initial phase and leakage came a little later (Wilschut et al., 1980; Ellens et al., 1985). The leakage of Tb^{3+}/dipicolinic acid or ANTS/DPX was attributed to collapse into cochleate cylinders and continued during the fusion reaction. In fusion of PS LUVs induced by the positively-charged peptides, the fluorescence increased rapidly and did not decrease afterward (Bondeson & Sundler, 1990). The encapsulated fluorescent probe Tb^{3+} did not leak from the fused vesicles, the same as in the present case and also in fusion of egg PC LUVs by an equimolar mixture of E5 and K5 at neutral pH (Murata et al., 1991). In the interaction of GALA with egg PC LUVs at acidic pH, the encapsulated fluorescence probe leaked out probably via pore formation of associated GALA molecules in bilayers (Parente et al., 1990). Leakage of fluorescent isothiocyanate–dextran from dioleoyl-PC LUVs induced by gramicidin A was caused by the formation of a precursor of the hexagonal II phase (Tournois et al., 1990).

The anionic and cationic peptides caused only fusion except at high concentrations. E5 and E5L at higher concentrations, i.e., at smaller L/P ratios of 20–30, caused fusion and then vesiculation to smaller vesicles at acidic pH. Thus, depending on the local concentration, the amphiphilic peptides can cause fusion or vesiculation. The HA peptide, D4, and K5 may cause

such vesiculation at L/P ratios smaller than 12. Lee and Kim (1988) showed the formation of micelles upon interaction of apomyoglobin with dimyristoyl-PC at a L/P ratio of 60, with an I/I_0 value of 0.25. The interaction of an amphiphilic α-helical segment of apomyoglobin with PC at acidic pH was proposed to cause micelle formation. We do not yet know if such micellar formation occurs in the present peptide system.

References

Bartlett, G. R. (1959) *J. Biol. Chem. 243*, 466-468.
Bordier, C. (1981) *J. Biol. Chem. 256*, 1604-1607.
Bondeson, J., & Sundler, R. (1990) *Biochim. Biophys. Acta 861*, 186-194.
Dufourcq, J., & Faucon, J.-F. (1977) *Biochim. Biophys. Acta 467*, 1-11.
Duzgunes, N., & Gambale, F. (1988) *FEBS Lett. 227*, 110-114.
Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532-1538.
Lear, J. D., & DeGrado, W. F. (1987) *J. Biol. Chem. 262*, 6500-6505.
Lee, J. W., & Kim, H. (1988) *FEBS Lett. 241*, 181-184.
Maeda, T., & Ohnishi, S. (1980) *FEBS Lett. 122*, 283-287.
Murata, M., Sugahara, Y., Takahashi, S., & Ohnishi, S. (1987a) *J. Biochem. (Tokyo) 102*, 957-962.
Murata, M., Nagayama, K., & Ohnishi, S. (1987b) *Biochemistry 26*, 4056-4062.
Murata, M., Takahashi, S., Kanaseki, T., & Ohnishi, S. (1990) in *Biophysics of Cell Surfaces* (Glaser, R., & Gingell, D., Eds.) pp 221-236, Springer, Berlin.
Murata, M., Kagiwada, S., Takahashi, S., & Ohnishi, S. (1991) *J. Biol. Chem. 266*, 14353-14358.
Ohnishi, S. (1988) *Curr. Top. Membr. Transp. 32*, 257-296.
Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1988) *J. Biol. Chem. 263*, 4724-4730.
Parente, R. A., Nir, S., & Szoka, F. C., Jr. (1990) *Biochemistry 29*, 8720-8728.
Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780-790.
Rafalski, M., Lear, J. D., & DeGrado, W. F. (1990) *Biochemistry 29*, 7917-7922.
Shen, D.-F., Huang, A., & Huang, L. (1982) *Biochim. Biophys. Acta 689*, 31-37.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53-56.
Struck, D., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093-4099.
Takahashi, S. (1990) *Biochemistry 29*, 6257-6264.
Tournois, H., Fabrie, C. H. J. P., Burger, K. N. J., Mandelsloot, J., Hilgers, P., van Dalen, H., de Gier, J., & de Kruijff, B. (1990) *Biochemistry 29*, 8297-8317.
Walter, A., Steer, C. J., & Blumental, R. (1986) *Biochim. Biophys. Acta 861*, 319-330.
White, J. (1990) *Annu. Rev. Physiol. 52*, 675-697.
White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 861*, 319-330.
Wilschut, J., Duzgunes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011-6021.

Lipophilic Cations: A Group of Model Substrates for the Multidrug-Resistance Transporter†

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ABSTRACT: The possibility that simple lipophilic cations such as tetraphenylphosphonium (TPP^+), tetraphenylarsonium (TPA^+), triphenylmethylphosphonium ($TPMP^+$), and diphenyldimethylphosphonium (DDP^+) are substrates for the multidrug-resistance transport protein, P-glycoprotein, was tested. Hamster cells transfected with and overexpressing mouse *mdr1* or mouse *mdr3* exhibit high levels of resistance to TPP^+ and TPA^+ (20-fold) and somewhat lower levels of resistance to $TPMP^+$ and DDP^+ (3-12-fold). Transfected cell clones expressing *mdr1* or *mdr3* mutants with decreased activity against drugs of the MDR spectrum (e.g., Vinca alkaloids and anthracyclines) also show reduced resistance to lipophilic cations. Studies with radiolabeled TPP^+ and TPA^+ demonstrate that increased resistance to cytotoxic concentrations of these lipophilic cations is correlated quantitatively with a decrease in intracellular accumulation in *mdr1*- and *mdr3*-transfected cells. This decreased intracellular accumulation is shown to be strictly dependent on intact intracellular nucleotide triphosphate pools and is reversed by verapamil, a known competitive inhibitor of P-glycoprotein. Taken together, these results demonstrate that lipophilic cations are a new class of substrates for P-glycoprotein and can be used to study its mechanism of action in homologous and heterologous systems.

Multidrug resistance is the phenomenon by which tumor cells in vivo and cultured cells in vitro become simultaneously resistant to a large group of structurally and functionally unrelated cytotoxic compounds (Gerlach et al., 1986; Moscow & Cowan, 1988). The phenomenon is caused by the overexpression of a group of membrane phosphoglycoproteins termed P-glycoproteins (P-gp) that are encoded by a small family of related *mdr* genes which become amplified and/or overexpressed in multidrug-resistant cells (Endicott & Ling, 1989). P-gp has been shown to bind ATP (Cornwell et al.,

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