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Basic amphipathic helical peptides induce destabilization and fusion of acidic and neutral liposomes

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We have studied the fusion of small unilamellar vesicles composed of egg PC and of a mixture of egg PC plus egg PA using various basic amphipathic peptides. Fusion was monitored by carboxyfluorescein leakage assay, light scattering, membrane intermixing assay, contents mixing assay and electron microscopy. Ac-(L-Leu-L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (peptide 4_3) and Ac-(L-Leu-L-Ala-L-Lys-L-Leu)$_3$-NHCH$_3$ (peptide $4'_3$), which have high hydrophobic moments, caused transformation of small unilamellar vesicles into larger and relatively homogeneous ones. Ac-(L-Leu-L-Leu-L-Ala-L-Arg-L-Leu)$_2$-NHCH$_3$ (5_2), which has medium hydrophobic moment, induced weak but appreciable fusion, while Ac-(L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (3_3) which has no helical structure did not show any fusion. However, peptides 4_3, $4'_3$ and 5_2 caused massive leakage of the contents from small unilamellar vesicles. These results indicated that interaction of the peptides with artificial membranes caused extensive perturbation of the lipid bilayer, followed by fusion. The fusogenic capacity of model basic peptides was correlated with the hydrophobic moment of each peptide when the peptides adopted an α-helical structure in the presence of acidic liposomes. Peptides 4_3 and $4'_3$ also showed weak fusogenic ability for neutral liposomes, while 5_2 and 3_3 showed no ability, suggesting that highly amphipathic peptides, such as 4_3, interact weakly but distinctly with neutral liposomes to fuse them.

Introduction

Many biological processes call for membrane fusion. In spite of the accumulation of a large amount of data during the past decade, the molecular mechanisms of membrane fusion are still scarcely known [1,2]. A variety of peptides and proteins has been shown to participate in the fusion process. The fusogenic effects of synexin [3,4], clathrin [5] and a viral protein [6] have been considered to depend on their acidic amino acid moiety. Their mechanism of action was explained as follows: protonation of charged acidic residues of proteins by lowering the pH leads to conformational change in proteins, followed by the exposure of their hydrophobic surface, which in turn facilitates the membrane fusion [7].

On the other hand, a variety of cationic peptides and proteins have been found to be fusogenes of artificial membrane, e.g., polylysine [8,9], polymyxin B [9,10], gramicidin S [11], melittin [12–14], and extension peptide of mitochondrial protein [15] as peptide, and lysin [16] and myelin basic protein [17] as protein. Peptides such as polymyxin B and melittin are amphiphiles with an N-terminal hydrophobic moiety and a hydrophilic one composed of several positive charges. However, the amphipathic features of gramicidin S are considerably different from those of other peptides. Gramicidin S is a cyclic decapeptide containing an antiparallel β-structure. A hydrophobic moiety with leucines and valines lies on one side of the decapeptide ring and a hydrophilic one with ornithines lies on the other side, forming an amphipathic tertiary structure [11]. A study of the

Abbreviations: DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DPPG, dipalmitoyl-DL-α-phosphatidylglycerol; DSPC, distearoyl-DL-α-phosphatidylcholine; egg PC, egg yolk phosphatidylcholine; egg PA, egg yolk phosphatidic acid produced from egg yolk PC; N-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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secondary structure of lysin, which has a function in the fusion of mollusk spermatozoa and ova, indicated that there are some hydrophobic domains and positively charged regions [18]. These findings suggest that an appropriate orientation of the hydrophobic moiety and cationic charge in the peptide structure satisfies the requirements for fusogenic ability. However, a detailed study on the relationship between the ability of amphiphiles to cause fusion and their structure has not yet been undertaken.

We have previously reported that some of the basic model peptides designed from a common feature of the extension peptides, e.g., Ac-(L-Leu-L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (4_3), showed some perturbing activity against various biomembranes [19,20] and that this activity was closely related to the amphipathy due to the stable α-helical structure of the peptides in the presence of liposomes [21]. The mode of perturbation of the biomembrane was proposed to be that the peptides lie parallel to the surface of the bilayers by shallowly penetrating their hydrophobic regions in the amphipathic structure and entering the bilayers [22]. These observations prompted us to study any possible fusogenic ability of the model peptides. This paper discusses the interaction of model peptides having various extents of α-helical moment (Fig. 1) with unilamellar liposomes composed of egg PC containing egg PA, and it attempts to demonstrate that the peptides caused extensive perturbation of the lipid bilayers which resulted in the fusion of the neutral or acidic small unilamellar vesicles. Their amphipathy induced by α-helical structure in the presence of liposomes was important for the fusogenic ability.

Materials and Methods

Materials

Ac-(L-Leu-L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (4_3), Ac-(L-Leu-L-Ala-L-Lys-L-Leu)$_3$-NHCH$_3$ ($4_3'$), Ac-(L-Leu-L-Leu-L-Ala-L-Arg-L-Leu)$_2$-NHCH$_3$ (5_2) and Ac-(L-Ala-L-Arg-L-Leu)$_3$-NHCH$_3$ (3_3) were prepared by the solution method and their purity was confirmed by paper electrophoresis, amino-acid and elemental analyses as previously reported [23]. DPPC, DPPG, egg PC, egg PA and polymyxin B were purchased from Sigma Chemical. N-NBD-PE and N-Rh-PE were purchased from Avanti Polar Lipids. Carboxyfluorescein was purchased from Eastman and further purified as previously reported [21]. All other reagents were of an analytical grade.

Phospholipid concentration was determined by an assay using the phospholipids-test Wako reagent purchased from Wako Pure Chemical Industries and was expressed in terms of phosphorus concentration. The buffer used in the present work comprised 5 mM Hepes (pH 7.4)/100 mM NaCl, except for the leakage experiment using synthetic phospholipids (DPPC and DPPG), in which the buffer comprised 20 mM Hepes (pH 7.6)/32 mM NaCl/128 mM KCl.

Release of liposome contents

The ability of peptides to induce leakage of liposome contents entrapped in liposomes was checked by monitoring the change of the fluroescence intensity of carboxyfluorescein which had been encapsulated in liposomes at high self-quenching concentrations [24].

Lipid film obtained by the evaporation of a chloroform solution of phospholipids (20 mg) was hydrated in Hepes buffer (pH 7.4) containing 100 mM carboxyfluorescein by repeated vortexed-mixing at 50°C for synthetic phospholipids (DPPC and DPPG) and at room temperature for natural ones (egg PC and egg PA) for 30 min. The suspension was sonicated at 50°C for synthetic phospholipids and at room temperature for natural ones for 60 min using a Tomy Seiko ultrasonic disrupter Model UR-200P. Liposomes trapping carboxyfluorescein were subjected to gel filtration through a Sepharose 4B column (1 × 20 cm) in Hepes buffer (pH 7.4). 2-ml fractions were collected and the small unilamellar vesicles collected in fraction 7, which was the fraction just before the non-encapsulated dye-elution, were used for the release experiment. The lipid concentration of the fraction 7 was about 2.8 mM.

A liposome solution (50 μl) of fraction 7 was added to a solution of Hepes buffer (pH 7.4, 2 ml) to give a final concentration of 70 μM phospholipids. 50 μl of an appropriately diluted solution of the peptide in Hepes buffer was then added to the mixture. Carboxyfluorescein was excited at 470 nm and emission at 540 nm was monitored with a Toshiba VY-50 cut-off filter which cut off below the wavelength of 505 nm. The fluorescence intensity was measured 3 min after the addition of peptides in the liposomes. Complete release of carboxyfluorescein was obtained by the addition of Triton X-100 (0.1%, v/v).

90° light scattering

Scattering experiments were performed on a JASCO FP-550A spectrofluorometer with both excitation and emission monochrometers set at 545 nm at a temperature of 25°C. Small unilamellar vesicles used for this experiment were prepared as described above without carboxyfluorescein. The concentration of phospholipids was adjusted to 70 μM. The light-scattering intensity was determined with 10 min incubation with peptides in the presence of liposomes.

Fusion of liposomes

The NBD/Rh resonance energy-transfer assay was used to monitor membrane intermixing [25]. The Tb-dipicolic acid fusion assay was used to further confirm the results of the NBD/Rh resonance energy-transfer assay [26].

Two kinds of small unilamellar vesicles containing N-NBD-PE and N-Rh-PE (each 2 mol%) were prepared by sonication as described above. Equimolar aliquots were mixed at 25°C and the final concentration was adjusted to 70 μM. The peptides were added to the mixed liposomes and the decrease in NBD fluorescence was recorded continuously at an excitation wavelength of 450 nm and an emission wavelength of 530 nm. The fluorescence intensity of the mixed solution of both liposomes before the addition of peptides was taken as 100%. It has been reported that some amines weakened the fluorescence intensity of N-NBD-PE during the fusion assay [11]. Under our experimental conditions, however, no decrease in intensity of N-NBD-PE was observed in the range of peptide concentrations when the fusion assay was monitored.

The Tb-dipicolic acid fusion assay was performed as follows. Two populations of small unilamellar vesicles were prepared in (a) 15 mM $TbCl_3$/150 mM sodium nitrotriacetate and (b) 150 mM dipicolic acid sodium salt in Hepes buffer adjusted to a final pH of 7.4. The elution buffer on gel filtration consisted of 100 mM NaCl/1 mM EDTA/5 mM Hepes (pH 7.4). Liposomes were separated from non-encapsulated materials by gel filtration on Sepharose 4B. Equimolar aliquots of liposomes containing trapped Tb^{3+} and dipicolic acid were mixed, and the final phospholipid concentration was adjusted to 70 μM. Then, the peptides were added to the mixed liposomes. Fluorescence was monitored 10 min after the addition of peptides at an excitation wavelength of 276 nm and an emission wavelength of 545 nm.

Electron microscopy

Liposomes were prepared by sonication and the concentration was adjusted to 10-times higher (about 700 μM) than that in other experiments in order to easily obtain electroscopic images. Electron microscopy was examined by negative-stain and freeze-fracture methods both with and without the addition of peptides. The peptide concentration in this experiment was 150 μM (lipid/peptide molar ratio, $R_i = 5$) for which a maximum light scattering for acidic liposomes was observed. All the peptides were incubated for about 1 min with liposomes before staining or fixing.

For negative-stain electron microscopy, the sample solutions were placed on formvar, carbon-coated grids and stained with 2% phosphotungstic acid adjusted to pH 7.4 with NaOH at room temperature. A JEM-100C electron microscope was used.

For freeze-fracture electron microscopy, the sample solutions after about 1 min of incubation were fixed for 30 min by 1% glutaralaldehyde and suspended in 30% glycerin. A few drops of the suspended solution were rapidly frozen in Freon 22 precooled in liquid nitrogen, and fractured in a Balzers freeze-fracture unit (Balzers type BAF 301; Balzers, Lichtenstein) at −110°C. The replica was made by shadowing with platinum-carbon (2 nm thick) at an angle of 45°, followed by evaporating carbon (25 nm thick) in a vertical position. The replicas, after washing with sodium hypochlorite, were collected and used.

Results

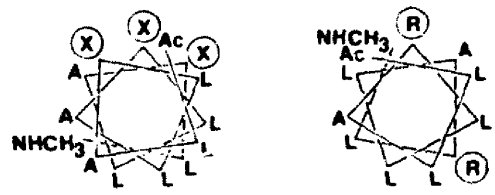
Hydrophobic moment of peptides

Four peptides among ten models designed from mitochondrial extension peptides [21] were used in this experiment (Fig. 1). Polymyxin B, which has a high fusion ability, was used as a reference in the fusion experiment. The contents of α-helix were 70% for peptide 4_3, 50% for $4_3'$ and 5_2 and 0% for 3_3 in the presence of liposomes [21]. Their hydrophobic moments were defined as the sum of vectors of the hydrophobicities of the side-chains in the α-helical structure according to the Eisenburg method [27]. The values of hydrophobicity for individual amino acids used for the computations were the consensus values. The values of the hydrophobic moments of 4_3, $4_3'$ and 5_2 were 0.51, 0.39 and 0.16, respectively. The value of 3_3 was not calculated because of the absence of an α-helical structure in the presence of liposomes.

Peptide-induced dye leakage from liposomes

It is well-known that the interaction of peptides or proteins with lipid bilayers can alter the structure of lipid bilayers and change the permeability of lipid bilayers. The leakage of encapsulated carboxyfluorescein from liposomes can be easily used for examining lipid–peptide interactions. Our previous dye-leakage experiment at the phase transition temperature showed that peptides such as 4_3 and $4_3'$ with a high ability to form α-helical structures in the presence of liposomes induced complete dye release at extremely low concentrations from neutral liposomes [21]. Peptide 5_2 also

Ac-(Leu-Ala-Arg-Leu)$_3$-$NHCH_3$ 4_3
Ac-(Leu-Ala-Lys-Leu)$_3$-$NHCH_3$ $4_3'$
Ac-(Leu-Leu-Ala-Arg-Leu)$_2$-$NHCH_3$ 5_2
Ac-(Ala-Arg-Leu)$_3$-$NHCH_3$ 3_3



4_3 or $4_3'$ 5_2

Fig. 1. Primary structure of the basic model peptides and the α-helical wheel of 4_3 (X = R), $4_3'$ (X = K) and 5_2.

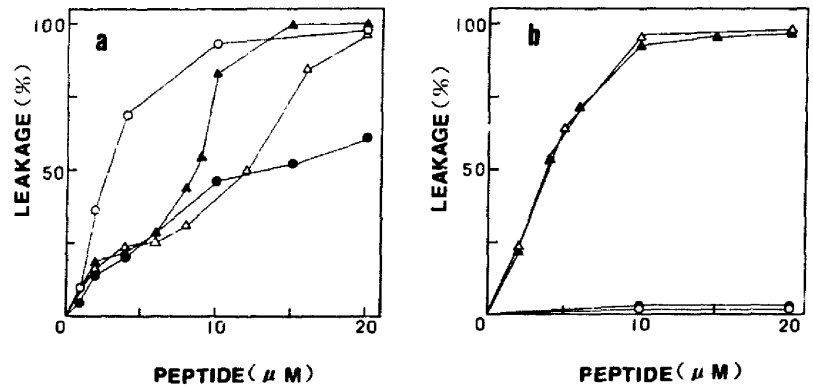


Fig. 2. Release of encapsulated carboxyfluorescein from liposomes. The effect of DPPC/DPPG composition on peptide $4'_3$ (a) and polymyxin B (b). The composition ratios of DPPC/DPPG were 1 : 0 (○), 3 : 1 (●), 1 : 3 (▲) and 0 : 1 (△).

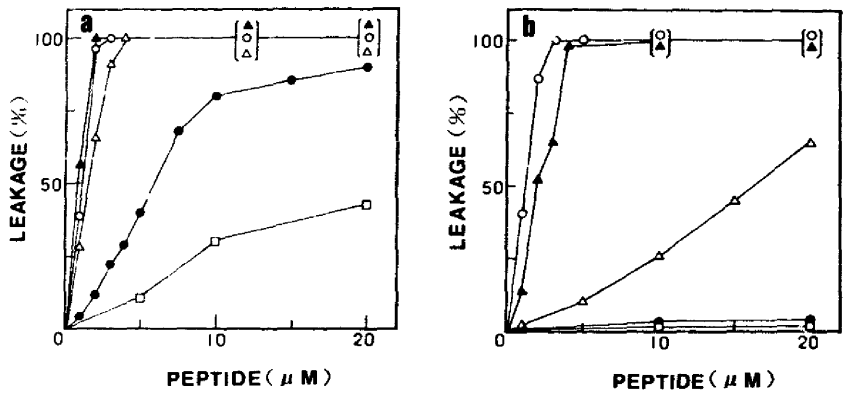


Fig. 3. Release of encapsulated carboxyfluorescein from egg PC/egg PA (3 : 1) (a) and egg PC (b) liposomes in the presence of polymyxin B (●), $4'_3$ (○), 4_3 (▲), 5_2 (△) and 3_3 (□) as a function of peptide concentration.

released the dye completely at a relatively low concentration, while 3_3 showed an extremely low ability to release dye. In this paper, the ability of leakage from liposomes was re-examined by an alteration of the composition of DPPC and DPPG as a function of a concentration of $4'_3$ at 25 °C. The ability of $4'_3$ to induce leakage did not depend on changes in the composition of DPPC and DPPG (Fig. 2a). However, a lower rate of release was observed for DPPC/DPPC liposomes compared to neutral DPPC liposomes at a low range of concentration. The results show that $4'_3$ interacted with neutral liposomes in a gel state to perturb the bilayers. Interestingly, liposomes composed of DPPC/DPPG (3 : 1) were unable to reach 100% leakage even at a high

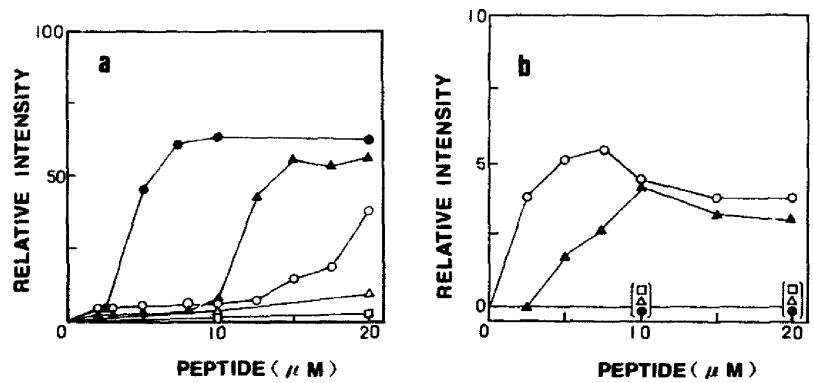


Fig. 4. Light-scattering profiles of egg PC/egg PA (3 : 1) (a) and egg PC (b) liposomes in the presence of polymyxin B (●), $4'_3$ (○), 4_3 (▲), 5_2 (△) and 3_3 (□) as a function of peptide concentration.

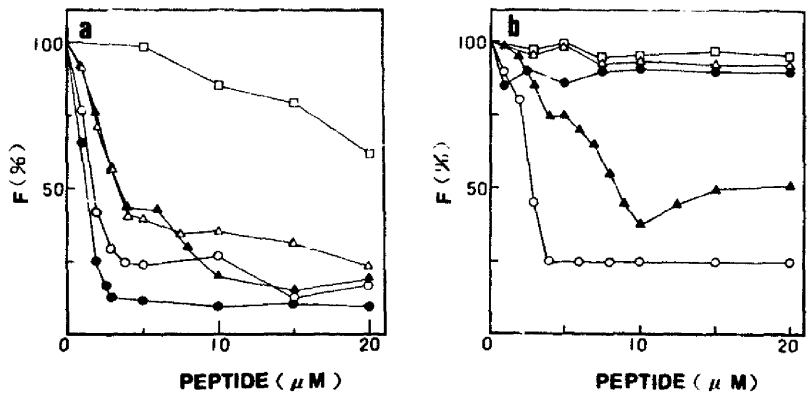


Fig. 5. Fusion of egg PC/egg PA (3:1) (a) and egg PC (b) liposomes induced by the peptides by a membrane-mixing assay as a function of peptide concentration. Polymyxin B (●), $4_3'$ (○), 4_3 (▲), 5_2 (△) and 3_3 (□).

peptide concentration. On the other hand, polymyxin B showed no leakage for neutral liposomes or liposomes with a low DPPG content, while it showed a strong leakage ability for liposomes with a higher DPPG content as shown in Fig. 2b.

The leakage experiment was also performed using the natural phospholipids, egg PC and egg PA (Fig. 3). peptides 4_3 and $4_3'$ showed an extremely high leakage ability for egg PC and egg PC containing 25% egg PA. The leakage ability of 4_3 for acidic liposomes was much higher than for neutral liposomes. The leakage ability of 5_2 was moderate for egg PC and similar to that of 4_3 and $4_3'$ for egg PC containing 25% egg PA. Polymyxin B showed no perturbing ability for neutral liposomes but a high ability for acidic liposomes similar to that for the liposomes prepared from synthetic phospholipids DPPG and DPPC. The extent of 100% dye-leakage ability of the peptides from synthetic lipids (DPPC and DPPC), which were in a gel state under experimental conditions at 25°C, was much higher than that from natural lipids (egg PC and egg PA), which are in a fluid state, indicating that the peptides may interact differently with the gel or fluid phase of the bilayers.

Peptide-induced aggregation of liposomes

Peptide-induced aggregation of liposomes was monitored by light scattering. For PA-containing liposomes, 4_3 effectively induced aggregation, though slightly less compared to polymyxin B (Fig. 4a). Compound 5_2, which is less amphipathic than 4_3, was fairly effective but 3_3, which is not amphipathic, did not show any ability of aggregation. Aggregation efficiency of peptides is correlated to the calculated hydrophobic moments of peptides, indicating that amphipathy of the peptides is closely related to the ability of turbidity. Maximum aggregation-inducing concentrations of polymyxin B and 4_3 were about 10 and 15 μM, respectively, and the relative intensity did not change upon the further addition of either peptide. These results suggest that the peptides did not lead to the fragmentation of the aggregated or fused large liposomes into the small micelles as in the case of the normal detergent. This was further confirmed by an electron microscopic experiment which is described later. Interestingly, a considerable difference was observed between the aggregation abilities of 4_3 and of $4_3'$, which have different basicities and amphipathies, indicating that fusogenic activity of guanidino cation is stronger than that of amino cation.

On the other hand, 4_3 and $4_3'$ induced aggregation of neutral liposomes at about 2.5 and 1 μM, respectively. Aggregation reached a maximum at about 10 μM (Fig. 4b) and then gradually decreased. However, the intensities for egg PC liposomes are 10-times less than those for egg PA-containing liposomes. Polymyxin B failed to induce aggregation of neutral liposomes. These results imply that the charge interaction between peptides and liposomes is important, especially essential to polymyxin B, in order to show the ability of peptides on fusion of liposomes.

Effects of peptides on fusion of liposomes

In order to determine the ability of fusion, two assays using intermixing of liposome membranes and liposome contents mixing were performed. Fig. 5 shows the intermixing of liposome membranes as a function of peptide concentration for egg PC and egg PC/egg PA liposomes containing N-NBD-PE and N-Rh-PE. For neutral liposomes (Fig. 5b), intermixing by 4_3 was observed at a concentration of 1 μM and reached the maximum at 4 μM. Peptide $4_3'$ also showed an ability for fusion, though this was less than that of 4_3. However, polymyxin B, 5_2 and 3_3 had no intermixing abilities.

For acidic liposomes (egg PA/egg PC) (Fig. 5a), polymyxin B had an extremely strong intermixing effect and 5_2 had a slightly less intermixing effect compared to that of 4_3 and $4_3'$. Peptide 3_3 showed an extremely low intermixing effect. These results suggest that α-heli-

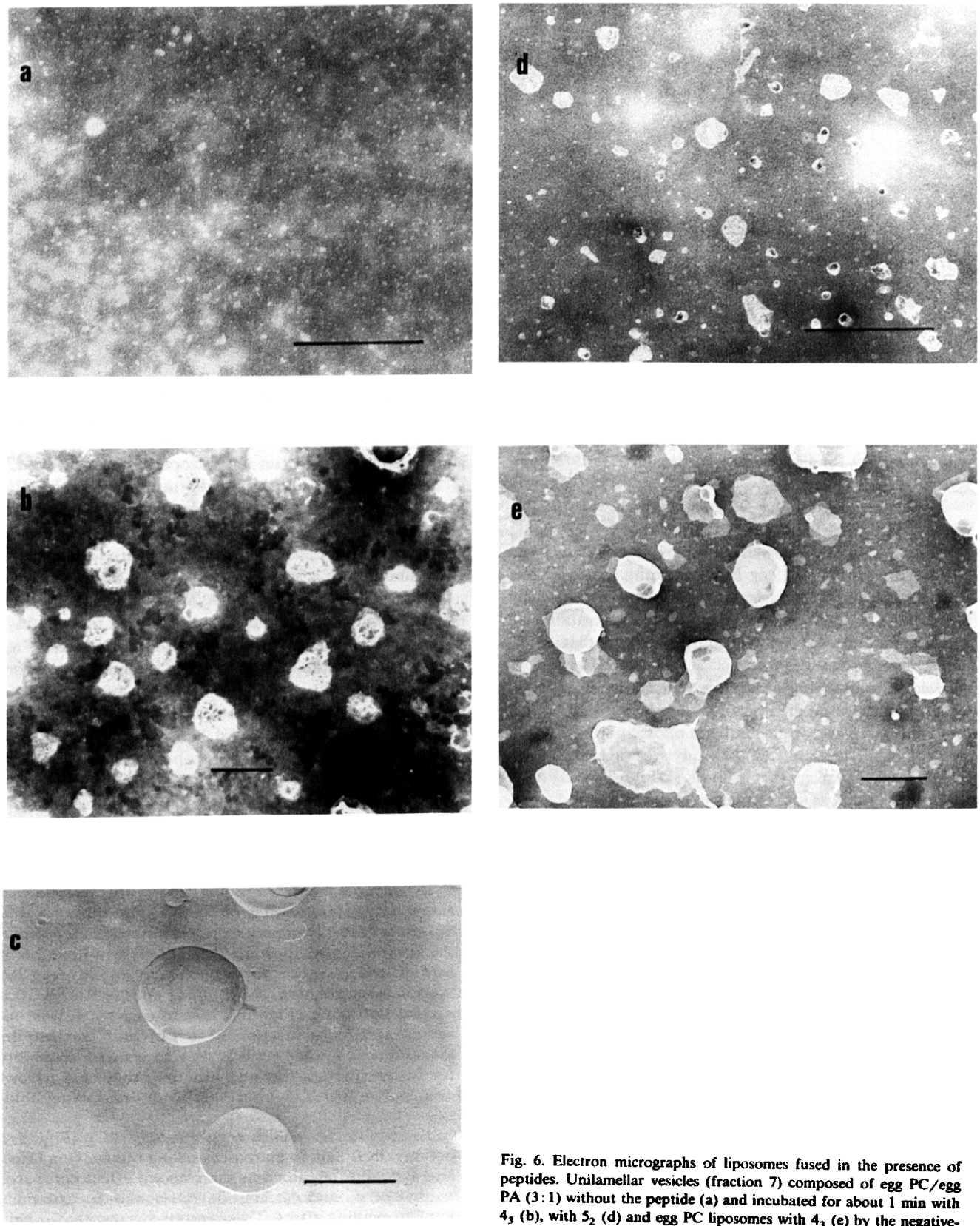


Fig. 6. Electron micrographs of liposomes fused in the presence of peptides. Unilamellar vesicles (fraction 7) composed of egg PC/egg PA (3:1) without the peptide (a) and incubated for about 1 min with 4_3 (b), with 5_2 (d) and egg PC liposomes with 4_3 (e) by the negative-stain method. Egg PC/egg PA (3:1) liposomes with 4_3 by freeze-fracture method (c). The bar represents 500 nm.

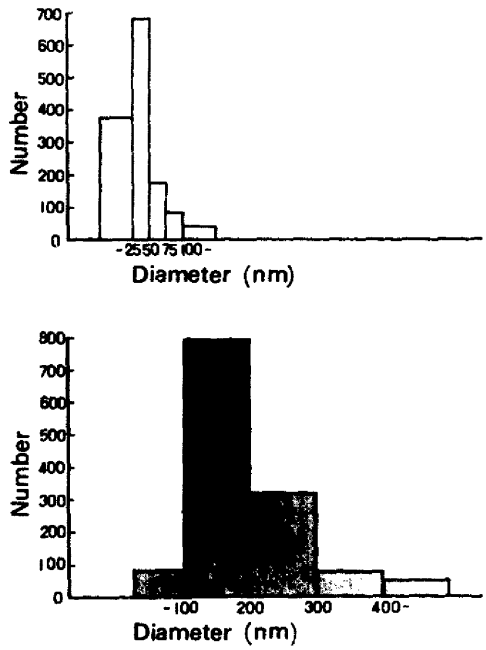


Fig. 7. Weight-averaged liposome size distribution in the absence (a) and in the presence (b) of 4_3. Liposomes were composed of egg PC/egg PA (3:1).

cal amphipathy is an important factor for artificial membrane fusion.

When 4_3 and $4_3'$ were added to egg PA-containing egg PC liposomes with entrapped Tb^{3+} or dipicolic acid, only a small rise in fluorescence was detected, while 5_2 and 3_3 showed no rise in fluorescence (data not shown). However, these observations do not rule out fusion of liposomes by 4_3 and $4_3'$. If the association of 4_3 with the liposomes results in a rapid release of trapped liposome content, the Tb^{3+}-dipicolinate assay would not be able to monitor the fusion. A similar result has been observed concerning the interaction of polymyxin B with small unilamellar acidic liposomes [28].

Electron microscopy by both negative staining and freeze-fracture has confirmed that 4_3 induced a fusion of liposomes. The structure of liposomes composed of egg PC/egg PA (3:1) prepared by sonication was observed as a relatively homogeneous mass and their diameters were in the range of about 25–50 nm (Fig. 6a). After about 1 min of incubation with 4_3, a large mass of liposomes was found in the range of almost 100–300 nm in diameter, although liposomes exceeding 500 nm in diameter were sometimes seen (Figs. 6b and 7). In spontaneous fixation of the liposomes after incubation of peptide, large liposomes which were smooth spheres were found in the freeze-fracture electron micrograph (Fig. 6c), indicating that large liposomes are unilamellar and the fusion velocity is extremely fast. Fusion by $4_3'$ was also observed for acidic liposomes and the liposomes were slightly smaller than those induced by 4_3 (data not shown). Peptide 5_2 also fused acidic liposomes, though the liposomes were much smaller and much more heterogeneous than those induced by 4_3 (Fig. 6d). Neutral liposomes were also fused by 4_3 in much smaller sizes (Fig. 6e). Morphological changes, such as the formation of micelles, were not observed in this experiment, though melittin caused such a change [14].

Discussion

We have investigated the interaction of peptides having various α-helical hydrophobic moments with artificial membranes composed of egg PC and egg PC plus egg PA (3:1). Our results indicate that the interaction of peptides having large hydrophobic moments, such as 4_3 or $4_3'$, with small unilamellar vesicles leads to extensive perturbation of the phospholipid membranes to cause fusion of acidic liposomes and release of the trapped liposome contents. The morphological experiment by both negative-staining and freeze-fracture methods showed that the peptides caused the transformation of small unilamellar vesicles to form large unilamellar ones. In this connection, it has been reported that polymyxin B formed multilamellar vesicles of DPPC/DSPC [28]. Peptide 4_3 had no lytic activity such as fragmentation of phospholipid bilayers into disc and micelles when the lipid-peptide ratio (R_i) was 5. This was also confirmed by the fact that the addition of a high amount of 4_3 of up to 100 μM did not lead to a decrease in turbidity. On the other hand, melittin, which induced the fusion of small unilamellar vesicles, led to the fragmentation of egg PC liposomes into discs and micelles upon the addition of a further amount ($R_i = 5$) of it [14]. Thus, melittin has lytic activity for various membranes, while 4_3 does not solubilize membrane-bound enzymes from mitochondria and microsomes, even at a concentration of 100 μM of peptide, as reported previously [19,20]. The lack of any lytic property of 4_3, such as fragmentation of the bilayers into disc and micelles, may be one answer why the membrane-bound proteins were not solubilized. Peptide $4_3'$ showed a weak ability of contents mixing, suggesting that the basic intensity is also an important factor.

Peptide 5_2 showed relatively high intermixing and a perturbing effect on acid liposomes in spite of having no effect on neutral liposomes. Although 5_2 showed a low aggregation ability, electron microscopic observations showed that this peptide clearly fused acidic liposomes, but had no ability to form large liposomes as did 4_3. These findings indicate that 5_2 was less effective in the enlargement of liposome size by fusion than 4_3. Peptide 3_3 did not show any association or membrane-mixing effect. The values of the hydrophobic moment ($4_3 > 4_3' > 5_2 > 3_3$) generally paralleled their abilities in fusion. These results clearly show that the amphipathic intensity of the peptides is important for revealing an

effective fusogenic ability. The size of liposomes fused also depends on the amphipathic intensity of the peptides.

For neutral liposomes, 4_3 and $4_3'$ showed a strong ability for dye leakage but a weak ability for membrane mixing and light scattering. Contents mixing using Tb^{3+}-DPA gave unsatisfactory results because of extremely strong dye leakage. Peptides 5_2, 3_3 and polymyxin B either did not interact or hardly interacted with phospholipid bilayers. Peptides 4_3 and $4_3'$ fused neutral liposomes, as seen by electron microscopy. This means that only the peptide having a large amphipathy can interact with neutral liposomes.

The generally accepted concept of the fusion process is as follows. Liposomes have to aggregate initially and be apposed closely, and then the destabilization of the bilayer structure occurs [29]. The present study has indicated that 4_3 was able to meet some or all of these requirements, irrespective of acid or neutral liposomes. Our previous fluorescence study on pyrenylalanine-containing peptide analogs of 4_3 suggested that these peptides adopt an amphipathic α-helix in the presence of lipid membranes in which the axis of the helix lies parallel with the surface of the phospholipid bilayer. The hydrophilic side of the helix interacts with the acidic moiety of phospholipids in the membrane and the hydrophobic side penetrates and faces the hydrocarbon moiety in the membrane [22]. The insertion of the hydrophobic part of 4_3 into the liposomes may cause destabilization of the bilayer structure and the release of the encapsulated dye from the liposomes. The positively charged hydrophilic side in the helix existing at the membrane surface in one liposome can attract negative charges located on another liposome, which may lead to apposition, followed by fusion. Peptide $4_3'$ attracts the negative charges on the liposomes more weakly, and this may be the reason why the fusogenic ability of $4_3'$ is less than that of 4_3.

Lysin, which is responsible for the dissolution of the egg vatelliane layer, has been proposed to promote fusion between sperm and egg plasma membranes [16]. A prediction of the secondary structure of this cationic protein has shown that it has four α-helical amphipathic regions [18], one of which has a sequence comprising amino-acid residues from 31 to 40 (-Leu-Val-Lys-Trp-Leu-Arg-Val-His-Gly-Arg-) similar to 4_3. The hydrophobic moment of the sequence (0.46) is almost the same as that of 4_3 (0.52). Interestingly, there are five positively charged dipeptides (3 Lys-Lys and 2 Arg-Arg) in the lysin sequence. It is possible that the protein is processed prior to biological action. If so, the fragment 21–47 that is produced by porcessing will act as a fusogen.

Fusion of liposomes can occur by one of the following mechanisms: (a) fusion of liposomes, in which case the contents are not leaked as fusion events in cells; (2) cracking and reannealing, in which case the contents are spilled; and (3) transfer of lipid molecules [29]. Though 4_3 showed a high ability to increase liposome size, massive leakage was also observed, suggesting that it seems to cause fusion by cracking and reannealing.

Acknowledgments

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