Ca^{2+}-Induced Aggregation of Oligopeptides Having a Carboxyl Group in Phospholipid Bilayer Membrane

Kazuya OTODA, Shunsaku KIMURA, and Yukio IMANISHI*
Department of Polymer Chemistry, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606-01
(Received November 19, 1992)

Ca^{2+}-induced aggregation of peptides having an Asp residue in phospholipid bilayer membrane was studied on the basis of excimer formation of pyrenyl group in the peptide. A part of the peptides incorporated into the membrane formed aggregates. The degree of peptide aggregation in the membrane was maximum at the lipid/peptide molar ratio of 40—80, and decreased with increasing the amount of the lipid and dissociation of the carboxyl group. Cross-linking of the peptide by Ca^{2+} was observed only when the peptide concentration in the membrane was high and the carboxyl group was dissociated. The membrane fluidity at the surface and in the hydrophobic core was not influenced so much by the peptide aggregation, indicating that the peptide/Ca^{2+} complex is not very rigid.

It is well-known that Ca^{2+} is involved in various kinds of cellular regulatory systems.[1] For example, several Ca^{2+}-binding proteins such as calmodulin[2] and troponin C[3,4] exist in cytosol. They bind Ca^{2+} effectively by EF hand, in which several aspartic acid residues work as Ca^{2+}-binding sites. Ca^{2+}-binding proteins such as protein kinase C,[5] calpaine,[6] and lipocortin[7] have also been found in cell membranes. The Ca^{2+} binding generally induces conformation change of these proteins, leading to altered interactions with ligand or counterpart protein. Ca^{2+} has been shown to play an essential role in prothrombin binding to phospholipid.[8] In this case, Ca^{2+} acts as a bridge between γ-carboxyglutamic acid residue in prothrombin and the phospholipid. It is therefore interesting to study Ca^{2+} binding to peptides having a carboxyl group in phospholipid bilayer membrane.

Dimerization of receptor molecules in the membrane triggers signal transduction in the membrane.[9—11] We have reported that two glycopeptides in the membrane were cross-linked by lectin, which resulted in increase of membrane fluidity.[12] Since Ca^{2+} coordinates to two carboxyl groups, two protein molecules may be cross-linked by Ca^{2+} through a carboxyl group of each protein, and thereby the membrane structure might be disturbed. In the present study, oligopeptides having a carboxyl group were synthesized, and the aggregation of the peptides by Ca^{2+} was studied. The molecular structure of the oligopeptides used is shown in Fig. 1. These peptides carry a pyrenyl group, which works as a probe for excimer formation upon their aggregation. The peptides with a benzyl ester of the carboxyl group were used as reference.

Experimental

Materials. Dipalmitoylphosphatidylcholine (DPPC, Sigma), 5/6-carboxyfluorescein (CF, Sigma), 1,6-diphenyl-1, 3,5-hexatriene (DPH, Nacalai Tesq.), and *N*-(7-nitro-4-benzofurazanyl)phosphatidylethanolamine (NBD-PE, Molecular Probe) were commercially available and used without further purification.

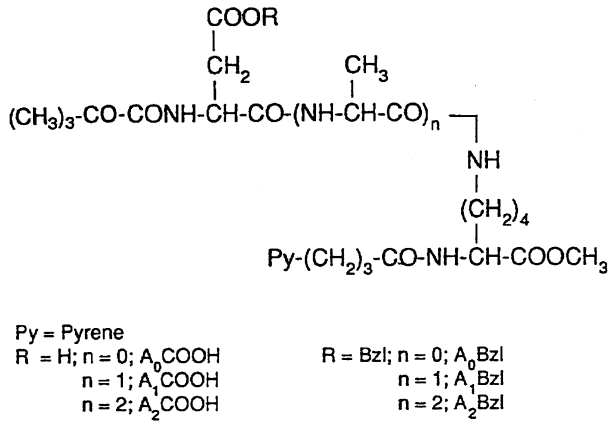

Fig. 1. Molecular structure of oligopeptides having a pyrenyl group. n=0, 1, 2.

Peptide Synthesis. The peptides were synthesized by a conventional liquid-phase method. The synthetic scheme is shown in Fig. 2. Peptide elongation was carried out by using dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) or *N*-hydroxybenzotriazole (HOBt) as coupling reagents. The yield of each coupling step was more than 50%. Each product was identified by IR and ^{1}H NMR measurements. Characteristic signals in these spectra are as follows.

IR data of A_2COOH: 1735 cm^{-1} (s), ester carbonyl; 1715 cm^{-1} (s), urethane carbonyl; 1625 cm^{-1}, amide I; 1520 cm^{-1}, amide II.

IR data of A_2Bzl: 1735 cm^{-1} (s), ester carbonyl; 1700 cm^{-1}, urethane carbonyl; 1640 cm^{-1}, amide I; 1520 cm^{-1}, amide II.

90 MHz ^{1}H NMR data of A_2COOH: δ=1.22 and 1.32, Ala–C$_\beta$H; 1.41, Boc; 3.70, OCH_3; 7.78—8.32 (m), pyrenyl group.

NMR data of A_2Bzl: δ=1.28, 1.33 and 1.36, Ala–C$_\beta$H; 1.40, Boc; 3.70, OCH_3; 7.30, OBzl; 7.78—8.37 (m), pyrenyl group.

The purity was checked by thin layer chromatography (TLC). Solvent systems for TLC were chloroform/methanol/acetic acid (95/5/3 v/v/v, CMA) and chloroform/methanol/pyridine (95/5/3 v/v/v, CMP). A_2COOH: R_f (CMA) =0.10 and R_f (CMP) =0.08. A_2Bzl: R_f (CMA)

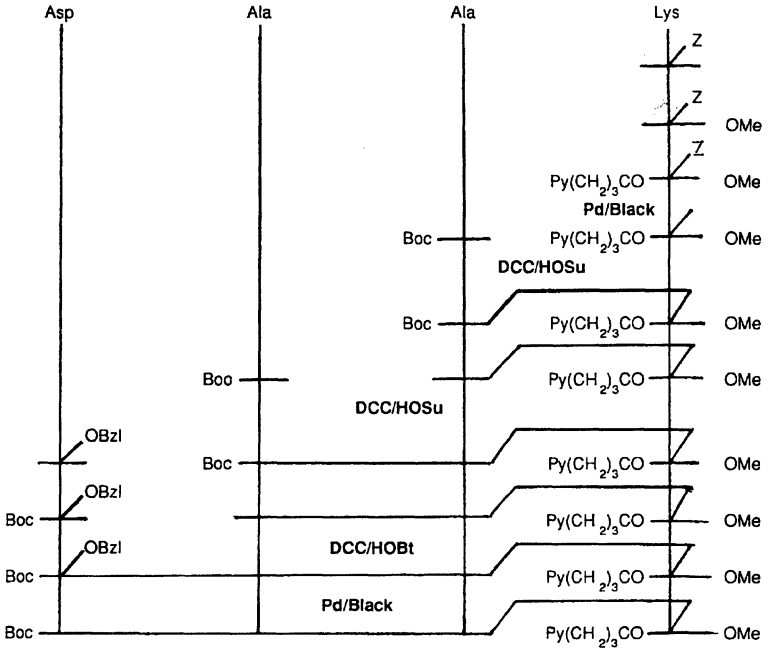

Fig. 2. Synthetic scheme of A_2COOH and A_2Bzl.

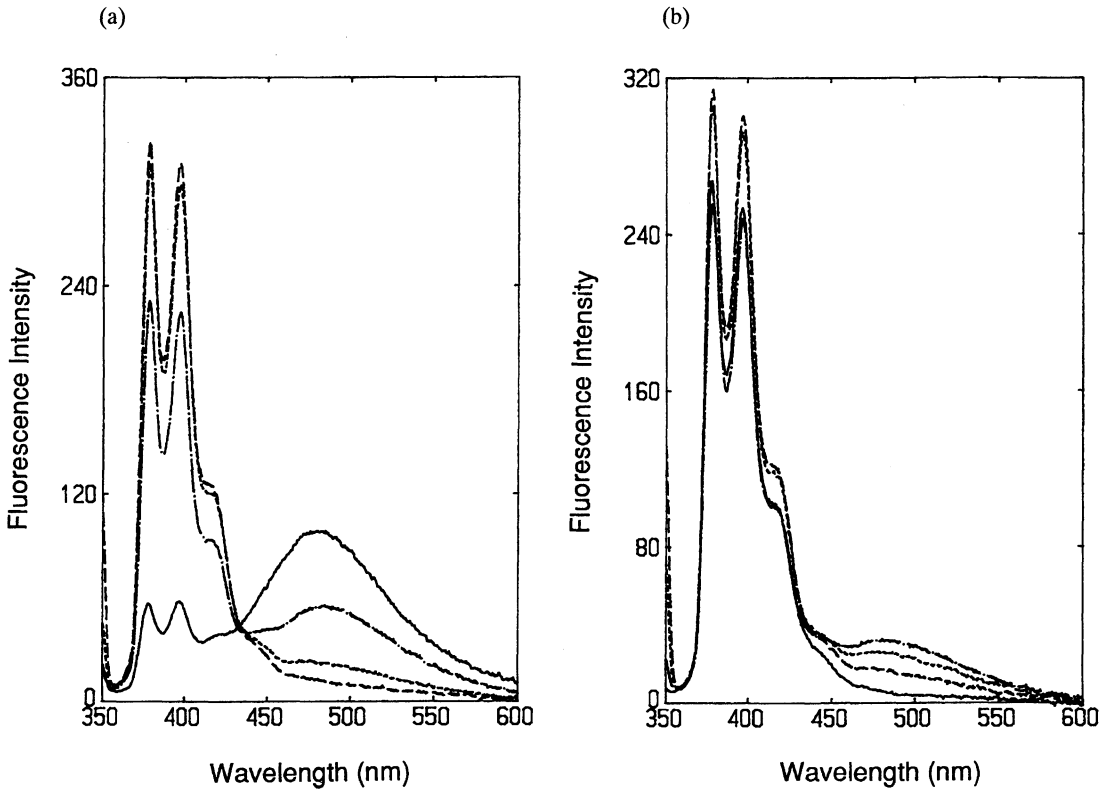

Fig. 3. Fluorescence spectra of (a) A_0Bzl and (b) A_0COOH in the presence of DPPC vesicles at the concentration of: —, 0; –·–, 78 μM; ····, 0.31 mM; ---, 0.90 mM. [A_0Bzl] and [A_0COOH] =2.3 μM. pH and temperature were 7.4 and 32 °C, respectively. Excitation wavelength was 345 nm.

=0.29 and R_f (CMP) =0.61. Elemental analyses of the final products are summarized in Table 1. Mp: A_2COOH 140—142 °C, A_2Bzl 156—158 °C.

Measurements. Fluorescence spectra were measured on a Hitachi MPF-4 fluorophotometer.

Small unilamellar vesicles were prepared by sonication of

DPPC dispersion in a buffer solution (10 mM Hepes and 0.1 mM EDTA, pH 7.4, 1 M=1 mol dm^{-3}) followed by ultracentrifugation at 100000 g_n. Fluorescenece depolarization of DPH and NBD-PE was measured by an equipment installed on the fluorophotometer as reported previously.[12] Excitation and monitor wavelengths were 360 and 425 nm for DPH and 478 and 540 nm for NBD-PE, respectively.

Lipid concentration was determined by a colorimetric method using phospholipase D (Diacolor, Toyobo).

CF Leakage. CF-encapsulated DPPC vesicles were prepared by the method reported by Barbet et al.[13] The excitation and monitor wavelengths of CF were 470 and 515 nm, respectively. Complete release of CF was attained by addition of Triton X-100 (0.3 wt%).

Results and Discussion

Distribution of Peptides to Lipid Membrane. Fluorescence spectra of A_0Bzl and A_0COOH at various concentrations of DPPC vesicles are shown in Figs. 3a and 3b, respectively. In a buffer solution of A_0Bzl, excimer emission was prevailing in the fluorescence spectrum, indicating aggregation of peptides due to hydrophobic interactions. With increasing lipid concentration, the excimer emission of A_0Bzl decreased, while the monomer emission increased. This fluorescence behavior indicates incorporation of A_0Bzl into the membrane with dissociation of aggregates. On the other hand, the fluorescence spectra of A_0COOH in the presence of DPPC vesicles were complex. The monomer emission was prevailing over the whole range of lipid concentrations examined. However, excimer emission appeared on addition of a small amount of lipid, and the excimer intensity decreased with increasing lipid concentration. The change of excimer intensity indicates that the peptide is concentrated in the lipid membrane of a small amount, and diluted with increasing amount of lipid. It is notable that excimer emission was detected in the presence of lipid molecules of a 470-fold molar excess, indicating attractive forces acting between the peptides facilitating the aggregation in the membrane.

The excimer to monomer intensity ratio (I_E/I_M) was investigated as a function of lipid/peptide ratio (Fig. 4). The solutions were adjusted at pH 7.4, where the carboxyl group of these peptides was dissociated. These experiments were carried out below the phase transition temperature of the membrane. Under these conditions, excimer is not formed by diffusion in the membrane, because the peptides are immobilized in the

Table 1. Elemental Analysis of Synthetic Peptides

Peptide			C	H	N
A_0COOH	$C_{36}H_{43}O_8N_3 \cdot 2/3H_2O$	Calcd	65.74	6.79	6.39
		Found	65.66	6.68	6.46
A_1COOH	$C_{39}H_{48}O_9N_4 \cdot 1/2H_2O$	Calcd	64.54	6.80	7.72
		Found	64.56	6.82	7.79
A_2COOH	$C_{42}H_{53}O_{10}N_5 \cdot H_2O$	Calcd	62.59	6.88	8.69
		Found	62.70	6.79	8.80
A_0Bzl	$C_{43}H_{49}O_8N_3 \cdot H_2O$	Calcd	68.51	6.82	5.57
		Found	68.64	6.86	5.76
A_1Bzl	$C_{46}H_{54}N_9O_4$	Calcd	68.47	6.74	6.94
		Found	68.24	6.80	6.95
A_2Bzl	$C_{49}H_{59}N_{10}O_5$	Calcd	67.03	6.77	7.98
		Found	66.63	6.74	7.87

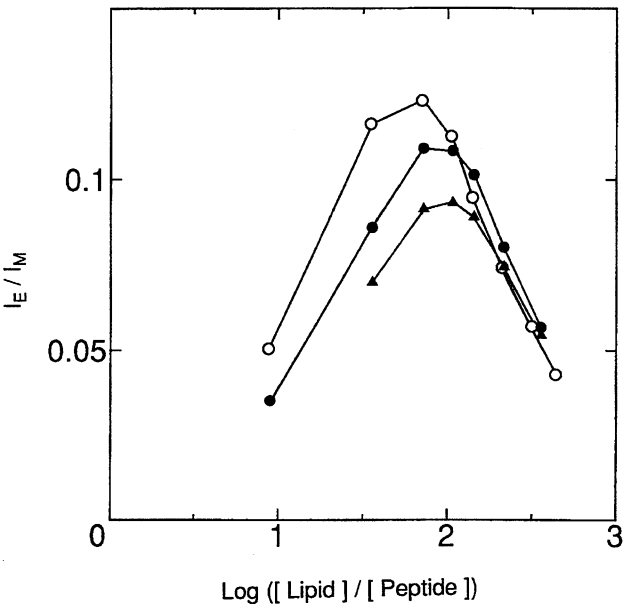

Fig. 4. I_E/I_M of A_0COOH (○), A_1COOH (●), and A_2COOH (▲) at different [Lipid]/[Peptide] molar ratios. I_E and I_M represent the fluorescence intensity at 480 and 377.5 nm, respectively. The peptide concentration was 2.2 μM. Temperature, 32 °C.

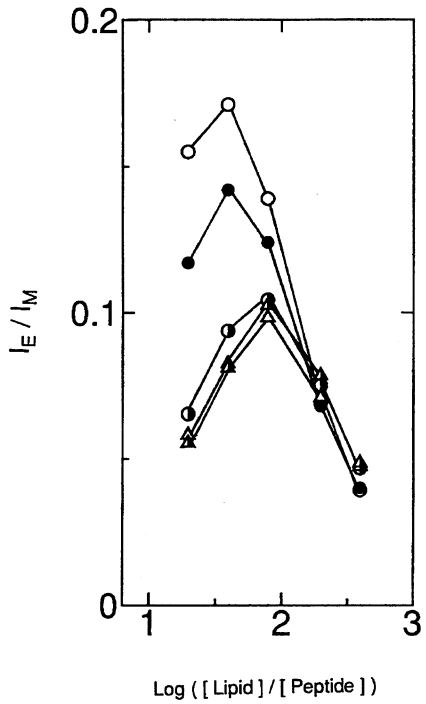

Fig. 5. pH dependence of I_E/I_M of A_2COOH at 32 °C. pH; ○, 3.0; ●, 4.0; ◑, 5.0; ▲, 6.0; △, 8.0. [A_2COOH] =2.2 μM.

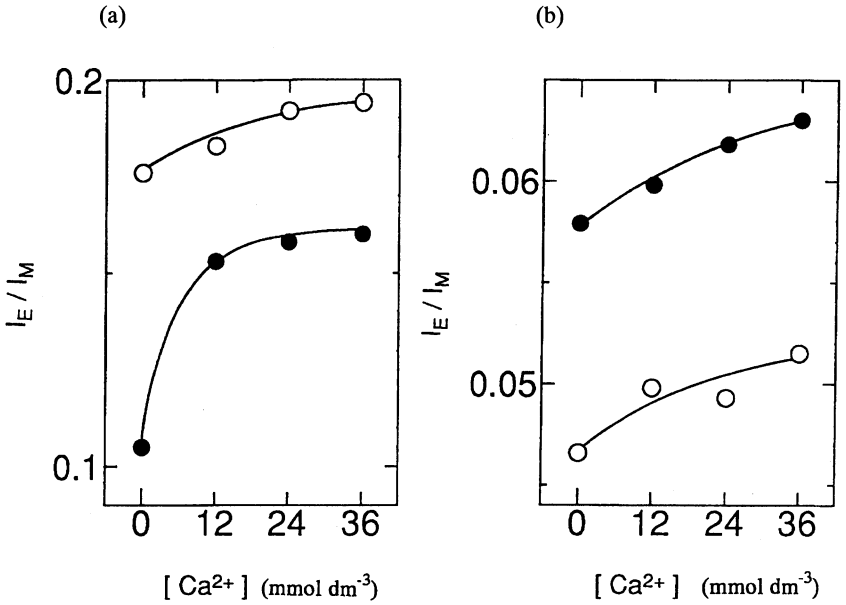

Fig. 6. Change of I_E/I_M of A_2COOH with addition of Ca^{2+} in (a) concentrated state ([DPPC]/[A_2COOH] =47) and (b) a diluted state ([DPPC]/[A_2COOH] =470) at 32 °C. pH; ○, 3.0; ●, 7.4. [A_2COOH] =2.2 μM.

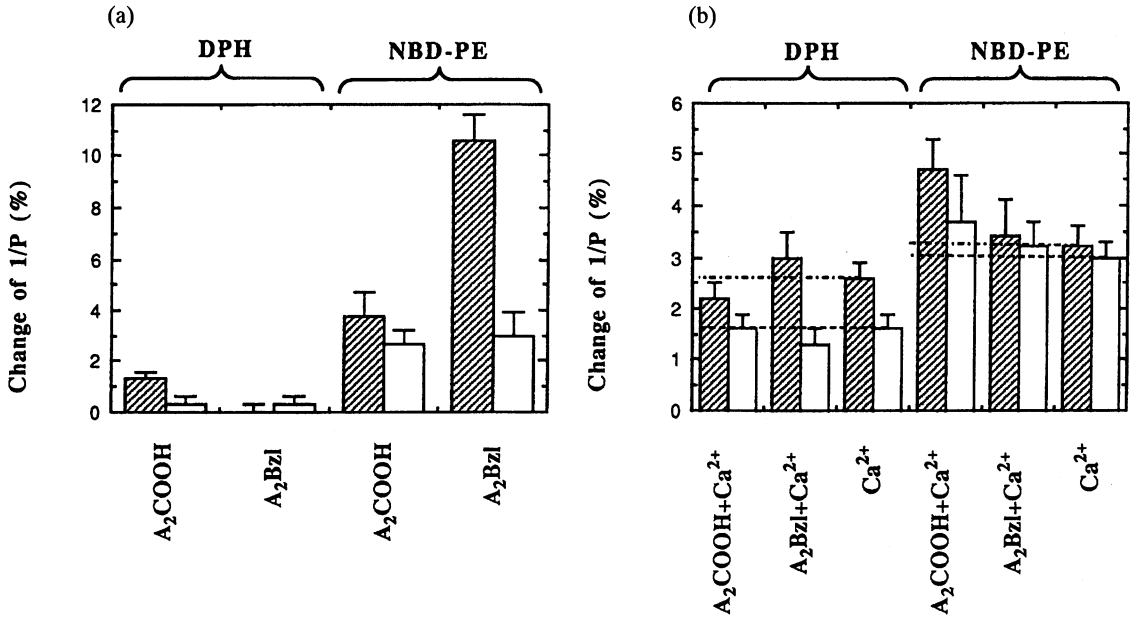

Fig. 7. Change of membrane fluidity by the addition of (a) peptide and (b) peptide plus Ca^{2+}. The ordinate of figures a and b represent $((1/P)_0-(1/P)_P)/(1/P)_0$ and $((1/P)_P-(1/P)_C)/(1/P)_P$, respectively, where $(1/P)_0$, $(1/P)_P$, and $(1/P)_C$ mean 1/P without additives, with peptide, and with peptide and Ca^{2+}, respectively. The concentrated state is shown by ▨, [DPPC] =0.12 mM, [DPH] =6.6 μM, and [NBD-PE] =6.2 μM, and the diluted state by □, [DPPC] =0.62 mM, [DPH] =1.4 μM, and [NBD-PE] =1.2 μM. For the measurements using DPH, [Peptide] was 3.1 μM, and for those using NBD-PE, [Peptide] was 2.5 μM. [Ca^{2+}] =14 mM. Temperature was 32 °C.

gel-state membrane. Therefore, I_E/I_M represents the static distribution of peptides in the membrane. As described above, I_E/I_M reached the maximum value at the lipid/peptide molar ratio of ca. 80. The maximum value of I_E/I_M decreased in the order of $A_0COOH > A_1COOH > A_2COOH$, indicating that the more hydrophobic the peptide is, the better dispersed in the lipid membrane.

pH Effect on Distribution of Peptide to Lipid Membrane. The change of I_E/I_M of A_2COOH against lipid/peptide molar ratio was measured at various pH (Fig. 5). A maximum value appeared at the lipid/peptide molar ratio of ca. 40 at pH 3 and 4, while ca. 80 at pH 5, 6, and 8, indicating that more lipid molecules are needed to concentrate A_2COOH of a dissociated state in the membrane. I_E/I_M values at pH 3 and 4 are higher than those at pH 5, 6, and 8 in the range of lipid/peptide molar ratio of 20—80. A_2COOH in a dissociated form may be difficult to aggregate due to electrostatic repulsion between carboxylate ions.

It should be noted that excimer emission might usually be negligible under the lipid/peptide molar ratio of more than 100 without any interaction between the peptides solubilized in the lipid membrane of a gel state. Therefore, the excimer formation of the charged peptides indicates a strong tendency toward aggregation of peptides in lipid membrane.

Ca^{2+}-Induced Peptide Aggregation. I_E/I_M of A_2COOH at the lipid/peptide molar ratio of 47 increased with addition of Ca^{2+} at pH 7.3, indicating cross-linking of the peptides of Ca^{2+} (Fig. 6a). Since Ca^{2+}-induced increase of I_E/I_M was not so remarkable at pH 3.0, dissociation of the carboxyl group is necessary for Ca^{2+} binding. On the other hand, I_E/I_M increased only slightly by the addition of Ca^{2+} at the lipid/peptide molar ratio of 470 (Fig. 6b). Besides, nearly the same degree of increase of I_E/I_M ratio was observed either at pH 7.3 or at 3.0. These results exclude the possibility that the interaction of Ca^{2+} and the carboxyl group of the peptide causes cross-linking of the peptides in the diluted conditions. Nonspecific binding of Ca^{2+} to the surface of phospholipid membrane[14] might induce phase separation of the membrane to concentrate slightly the incorporated peptides, leading to the small increase of I_E/I_M ratio.

Cross-linking of the peptides by Ca^{2+} occurs only when the peptides are concentrated in the lipid membrane. On the other hand, cross-linking of vesicles containing the peptides by Ca^{2+} was not detected in either conditions from turbidity measurement of the vesicle dispersion. These results suggest that the interaction of Ca^{2+} and the carboxyl group of the peptides should not be so strong.

Membrane Perturbation. The effects of peptide and Ca^{2+} additions on the membrane fluidity in the hydrophobic core and the membrane surface were measured by fluorescence depolarization of DPH and NBD-PE, respectively. The addition of A_2COOH and A_2Bzl decreased the membrane fluidity at the membrane surface, indicating the peptide incorporation in the membrane surface (Fig. 7a). The addition of A_2Bzl decreased the membrane fluidity more intensively than A_2COOH. It was more significant when the peptides are concentrated in the membrane than when diluted. The decreased fluidity might be explained in terms of dehydration of polar head groups of lipid molecules.[15] Water molecules should be dislocated from the polar head groups by intermolecular hydrogen bondings with the peptide incorporated in the membrane surface.

The addition of peptide plus Ca^{2+} also decreased the membrane fluidity (Fig. 7b). This observation is consistent with a previous report that Ca^{2+} dehydrates the membrane surface, and thereby decreases the membrane fluidity.[15] The extent of fluidity decrease at the membrane surface caused by A_2COOH plus Ca^{2+} was 1.5-fold of that without A_2COOH (lipid/peptide molar ratio of 47). On the other hand, A_2Bzl plus Ca^{2+} decreased the membrane fluidity by nearly same extent that without A_2Bzl. Therefore, the decrease of membrane fluidity caused by A_2COOH plus Ca^{2+} should be ascribed partly to Ca^{2+} binding to the peptides.

This result is in a sharp contrast with the case of lectin/glycopeptide interaction, in which lectin binding to a glycopeptide increased the membrane fluidity due to intravesicular cross-linking through the glycopeptides.[12] The lipid bilayer structure was considered to be distorted because of the formation of a ster-

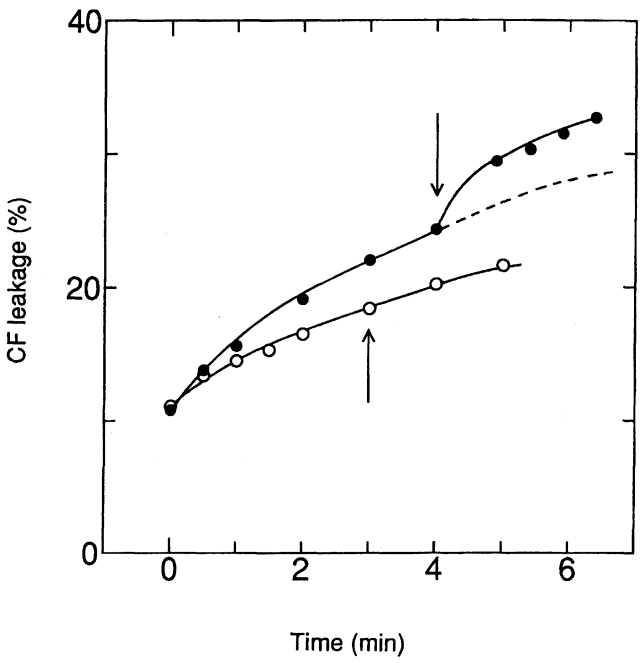

Fig. 8. CF leakage induced by the addition of Ca^{2+} in the presence of A_2COOH (●), and without the peptide (○). At 32 °C. [A_2COOH] =2.5 μM. [DPPC] =28 μM. Ca^{2+} addition (39 mM) was indicated by an arrow.

ically skewed "cis" complex.[16] However, in the present case, the molecular structure of the peptide/Ca^{2+} complex should not be so rigid that the cross-linking of the peptide is unlikely to disturb the membrane structure. It is plausible that the binding of Ca^{2+} to the peptide dehydrates the carboxyl group of the peptide, resulting in decreased fluidity of the membrane.

Membrane perturbation was also studied by leakage of CF encapsulated in DPPC vesicles. The time-course of CF leakage became discontinuous upon Ca^{2+} addition when the vesicles were incubated with A_2COOH in advance (Fig. 8). The membrane structure should have been disturbed by redistribution of the peptide upon cross-linking by Ca^{2+}. However, the rate of CF leakage after the Ca^{2+} addition was nearly the same as that without the peptide. The defects produced in the membrane upon formation of peptide/Ca^{2+} complex should be repaired by local rearrangement of the peptides and lipids in the membrane. This explanation holds itself on the assumption that the structure of the peptide/Ca^{2+} complex is not so rigid.

References

1) H. Rasmussen and P. Q. Barret, *Physiol. Rev.*, **64**, 938 (1984).
2) Y. S. Babu, J. S. Sack, T. J. Greenhough, C. E. Bugg, A. R. Meansf, and W. J. Cook, *Nature*, **315**, 37 (1985).
3) O. Herzberg and M. N. G. James, *Nature*, **313**, 653 (1985).
4) M. Sundaralingam, R. Bergstrom, G. Strasburg, S. T. Rao, P. Roychowshury, M. Greaser, and B. C. Wand, *Science*, **227**, 945 (1985).
5) Y. Nishizuka, *Nature*, **308**, 693 (1984).
6) K. Suzuki, *Trends Biochem. Sci.*, **12**, 103 (1987).
7) M. J. Geisow and J. H. Walker, *Trends Biochem. Sci.*, **11**, 420 (1986).
8) G. L. Nelsestuen, *J. Biol. Chem.*, **251**, 5648 (1976).
9) Y. Schechter, L. Hernaez, J. Schlessinger, and P. Cuatrecasas, *Nature*, **278**, 835 (1979).
10) A. Debant, G. Ponzio, E. Clauser, J. O. Contreres, and B. Rossi, *Biochemistry*, **28**, 14 (1989).
11) M. E. Cavis, T. Akera, T. M. Brody, and L. Watson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5764 (1977).
12) T. Ohtoyo, M. Shimagaki, K. Otoda, S. Kimura, and Y. Imanishi, *Biochemistry*, **27**, 6458 (1988).
13) J. Barbet, P. Machy, A. Truneh, and L. D. Leserman, *Biochim. Biophys. Acta*, **772**, 347 (1984).
14) R. P. Rand and S. Sengupta, *Biochim. Biophys. Acta*, **255**, 484 (1972).
15) H. Hauser, E. G. Finer, and A. Darke, *Biochim. Biophys. Res. Commun.*, **76**, 267 (1977).
16) A. Portis, C. Newton, W. Pangborn, and D. Papahadjopoulos, *Biochemistry*, **18**, 780 (1979).