J. Biochem. **130**, 393–397 (2001)

Membrane Fusion between Liposomes Composed of Acidic Phospholipids and Neutral Phospholipids Induced by Melittin: A Differential Scanning Calorimetric Study[1]

Yukako Higashino, Akira Matsui, and Kazuo Ohki[2]

Department of Physics, Graduate School of Science, Tohoku University, Aramaki, Aoba-ku, Sendai 980-8578

Received April 23, 2001; accepted June 18, 2001

Melittin-induced membrane fusion between neutral and acidic phospholipids was examined in liposome systems with a high-sensitivity differential scanning calorimeter. Membrane fusion could be detected by calorimetric measurement by observing thermograms of mixed liposomal lipids. The roles of hydrophobic and electrostatic interactions were investigated in membrane fusion induced by melittin. Melittin, a bee venom peptide, is composed of a hydrophobic region including hydrophobic amino acids and a positively charged region including basic amino acids. When phosphatidylcholine liposomes were prepared in the presence of melittin, reductions in the phase transition enthalpies were observed in the following order; dimyristoylphosphatidylcholine (DMPC) > dipalmitoylphosphatidylcholine (DPPC) > distearoylphosphatidylcholine (DSPC) > dielaidoylphosphatidylcholine (DEPC). The plase transition enthalpy of an acidic phospholipid, dipalmitoylphosphatidylserine (DPPS), was raised by melittin at low concentrations, then reduced at higher concentrations. DPPC liposomes prepared in melittin solution were fused with DPPS liposomes when the liposomal dispersions were mixed and incubated. Similar fusion was observed between dipalmitoylphosphatidylcholine and dimyristoylphosphatidic acid (DMPA) liposomes. These results indicate that a peptide including hydrophobic and basic regions can mediate membrane fusion between neutral and acidic liposomes by hydrophobic and electrostatic interactions.

Key words: differential scanning calorimetry, hydrophobic interaction electrostatic interaction, melittin, membrane fusion.

Membrane fusion is a key event in the biological function of living organisms. Fertilization involves membrane fusion of a sperm with an egg, and cell division requires membrane fusion to re-seal plasma membranes after cell division. Membrane fusion is also observed in endocytosis, exocytosis, and cellular membrane traffic (*1*). Recently, a scheme for the whole process of membrane fusion has been proposed based on the analysis of the structure of the SNARE complex containing syntaxin, SNAP-25, and synsptobrevin, which mediate membrane fusion (*2*), and the structure of the SNARE complex was revealed by X-ray crystallography (*3*). Participation of the SNARE complex is also manifested in membrane fusion of the endoplasmic reticulum (*4*). However, membrane fusion is a phenomenon that occurs in the lipid bilayer, and the molecular mechanism by which lipids participate in membrane fusion has not yet been elucidated. We believe that the essential part of membrane fusion proceeds on the lipid bilayer membranes since the two-dimensional structure of biomembranes is due to the molecular assembly of phospholipids in an aqueous environment. Indeed, liposomes have been adopted as the simplest model system in which to study membrane fusion (*5*). The fusion of model membranes containing acidic phospholipids initiated by Ca^{2+} has been well investigated, and S. Ohki has proposed that an increase in the hydrophobicity of the membrane surface caused by the binding of divalent cations mediates membrane fusion among acidic phospholipids (*6*). However, the Ca^{2+}-concentration needed to induce fusion in these systems is at least three orders of magnitude higher than the intracellular Ca^{2+} concentration required to trigger membrane fusion, which makes a direct role for Ca^{2+} unlikely. Therefore, we attempted to find a peptide factor that can induce fusion of membranes that include acidic phospholipids in place of Ca^{2+}. Since Eytan and Almary have shown that melittin induces the fusion of liposomes containing phosphatidylethanolamine, phosphatidylcholine, and cardiolipin (50, 20, 30%, respectively) (*7*), we choose melittin as a potential peptide to mediate membrane fusion. Here we have proved that melittin induces membrane fusion between phosphatidylcholine and acidic phospholipid, by observing DSC thermograms that reflect the mixing state of liposomal lipids. In this study, we investigated the effects of melittin on phospholipids from the viewpoints of hydrophobic and electrostatic interactions.

[1] This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture and the "Ground Research for Space Utilization" promoted by NASDA and the Japan Space Forum.

[2] To whom all correspondence should be addressed. E-mail: ohki@bio.phys.tohoku.ac.jp

Abbreviations: DSC, differential scanning calorimetry; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphate; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

We have also elucidated the hydrophobic and electrostatic interactions between melittin and phospholipids at the molecular level in detail.

MATERIALS AND METHODS

Synthetic phospholipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS), 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA), and bee venom, melittin (purity 91% by HPLC) were purchased from Sigma Chemical (St. Louis, MO). The desired pure phospholipid or mixture of phospholipids (2.0 μmol) in chloroform solution was placed into a small glass vial, and the solvent was evaporated under a nitrogen stream and then under reduced pressure. One milliliter of HEPES buffer (50 mM, pH 7.3) containing various amounts of melittin was poured into the vial, and the lipids were dispersed in a bath-type sonicator, Iuchi VS10U (Tokyo), at 80°C for 2 min. All preparations contained 5 mM of EDTA to inhibit the phospholipase activity included in melittin (*8*). Calorimetric scans were performed with a differential adiabatic microcalorimeter, Privalov calorimeter DASM-4 (Sinku Riko, Yokohama) (*9*), at a heating scan rate of 0.5 K/min in 0.5-ml cells under a pressure of 2.0 atm to prevent bubble formation (*10*). Before injection of the sample, the cells for the sample and reference were cooled to 10°C. Then the liposomal sample incubated for 2 h at the desired temperature was injected into the cell and the DSC measurement was started immediately. Data were processed on a personal computer, PC-9801RX (NEC, Tokyo).

RESULTS AND DISCUSSION

When phospholipid molecules are dispersed in water, they hydrate and then assemble into lipid bilayer membranes spontaneously. The membranes manifest a phase transition at a characteristic temperature that is determined primarily by the molecular species. Multi-lamellar vesicles of DPPC give a major main transition peak at 41°C, which is due to the melting of the acyl chains on the molecule, and a small pre-transition peak at 35°C. For DPPS dispersions at neutral pH, only the main transition is observed at 53°C. The cooperativity of phase transition is reflected in the transition profile on DSC thermograms. As the cooperativity of single-bilayer vesicles is smaller than that of multi-lamellar ones (*11*), the transition peaks in sonicated vesicles of DPPC and DPPS shown in Fig. 1 become broader compared to those of multi-lamellar vesicles. This result indicates that single- or oligo-lamellar vesicles are formed in the preparations using the sonicator, and also because negatively charged lipids form single-lamellar vesicles spontaneously (*12*). A high-sensitivity DSC, as used in the present experiments, can detect fine structures on lipid bilayer membranes, and the main transition peaks are clearly split into two peaks, which probably reflects different states of protonation of the polar moiety (Fig. 1, a and b). The single- and oligo-lamellar vesicles are suitable for membrane fusion experiments since the outer layers of liposomes are more important in the fusion process. Figure 1c shows a thermogram of liposomes prepared from a mixture of DPPC and DPPS at a molar ratio of 1:1. In a binary mixture of phospholipids, the profile of the thermogram depends on the miscibility of the components (*13*). A binary mixture of DPPC and DPPS gives one transition peak, which indicates that the miscibility of these phospholipids is fairly good. Therefore, as the mixing of these phospholipids proceeds, the transition peak of the binary mixture grows with a decrease in the separated peaks of each phospholipid. In the present study, this phenomenon was used to detect membrane fusion. This method has an advantage over popular techniques based on the mixing of aqueous vesicle contents (*14*) and resonance energy transfer between fluorescent lipids (*15*) since the mixing of membrane lipids is monitored directly without any probes.

Melittin is composed of 26 amino acids including 5 basic amino acids. In an aqueous environment, it forms tetramers and is easily soluble in water (*16*). The authors examined the interaction between melittin and various species of phosphatidylcholine by DSC. Phase transition enthalpies of phosphatidylcholines were reduced in the presence of melittin. The reduction in phase transition enthalpy depended markedly on the length of the fatty acyl chain, as summarized in Table I. The largest reduction was observed for DMPC, which has a 12-carbon acyl chain, with no reduction observed for DEPC and DSPC, which have 18-carbon acyl chains. In the present membrane fusion experiments, liposomes of 2 mM DPPC were prepared in buffer containing melittin. The enthalpy of the main transition was reduced in proportion to the melittin concentration; *e.g.* a 27% reduction was observed in buffer containing 0.1 mM melittin. This result indicates that melittin molecules penetrate the hydrophobic core of DPPC membranes (*17*). Liposomes of acidic phospholipids were prepared independently in buffer without melittin. In order to investigate membrane fusion, DPPC liposomes prepared with melittin were mixed with liposome preparations of acidic phospholipids

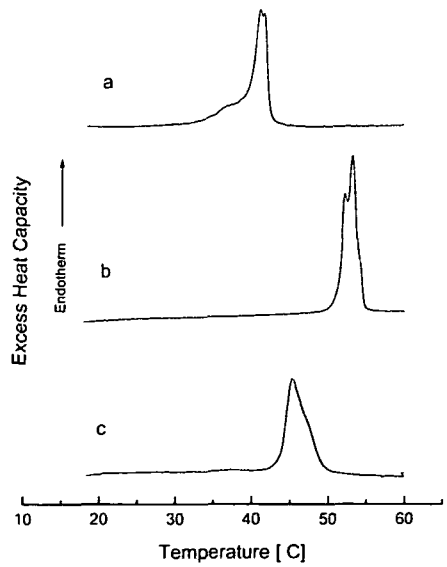

Fig. 1. **Thermograms of liposomes of (a) DPPC, (b) DPPS, and (c) a binary mixture of DPPC and DPPS (molar ratio, 1:1).** Liposomes were prepared by dispersing 2 μmol phospholipid(s) in 1 ml HEPES buffer (50 mM HEPES, 5 mM EDTA; pH 7.3) in a bath-type sonicator at 80°C for 2 min. Thermograms were obtained at a heating rate of 0.5 K/min.

formed without melittin.

At first, membrane fusion between DPPC and DPPS liposomes were examined by the method described above. In a control experiment, both DPPC and DPPS liposomes were prepared independently in HEPES buffer without melittin, and then these liposomes were mixed and incubated for 2 h at 55°C. DSC measurements of the mixture showed two separated peaks on the thermogram (Fig. 2a), One peak at 41°C and the other at 53°C, corresponding to the main transition peak of DPPC and DPPS liposomes, respectively (*cf.* Fig. 1, a and b).

The separated transition peaks indicate that membrane fusion does not occur between these liposomes in the absence of melittin. Then, DPPC liposomes were prepared in HEPES buffer containing the desired amounts of melittin and 5 mM EDTA. When these DPPC liposomes were mixed with DPPS liposomes, a new transition peak appeared around 44°C after 2-h incubation at 55°C (Fig. 2b), and increased in a melittin dose-dependent manner (Fig. 2, c and d). Finally, the transition peak coincided with that of the binary mixture of DPPC and DPPS (Fig. 2f). At an incubation temperature of 55°C, both DPPC and DPPS bilayer membranes are in the fluid phase. The same experiment at 25°C showed that melittin induces membrane fusion between DPPC and DPPS even when the membranes are in the gel phase (Fig. 2e). Hence, the phase structures of lipid-bilayer membranes hardly affect the fusion induced by melittin. Preliminary experiments using analogs of hemagglutinin (kindly provided by Dr. S. Takahashi of Kyoto University) have shown that the fusion activity of the peptide is markedly lower in gel phase liposomes than in liquid crystalline (fluid) phase liposomes. Compared to this result, the fusion activity of melittin is strong enough to induce fusion regardless of the phase structure of the membrane.

In order to examine electrostatic interactions between lipid bilayer membranes and the peptide, changes in the thermograms of DPPS liposomes induced by melittin were measured by DSC. DPPS liposomes without melittin show two transition peaks, probably because of the existence of different dissociation states of the phosphatidic acids (Fig. 3a). Figure 3b shows that 1 mol% of melittin results in one peak, and the transition enthalpy is raised to 12.0 kcal/mol from 8.29 kcal/mol (Fig. 3a). This may be because the basic amino acids of melittin interact with DPPS molecules electrostatically to make the two states merge into one, since the higher temperature peak merges into the lower temperature peak. At 2 mol% melittin, new transition peaks appear at 30 and 38°C (Fig. 3c). More peaks appear at 5 mol% melittin (Fig. 3d), and these peaks begin to merge at 15 mol% melittin (Fig. 3e), finally, becoming a single peak with a transition enthalpy of 3.62 kcal/mol at 27°C at 20 mol% melittin (Fig. 3, e and f). The melittin molecule includes 5 basic amino acids, so under these conditions all DPPS molecules should interact with melittin electrostatically. It is supposed that the complex of DPPS and melittin gives a transition peak at 27°C. Ohki *et al.*, using lipid monolayers, have shown that melittin tends to be adsorbed on the surface of the negatively charged phosphatidylserine membrane due to electrostatic interactions, and that the melittin molecule remains on the surface (*18*).

Control experiments were performed using DMPC and DPPC liposomes in order to examine the fusion activity of

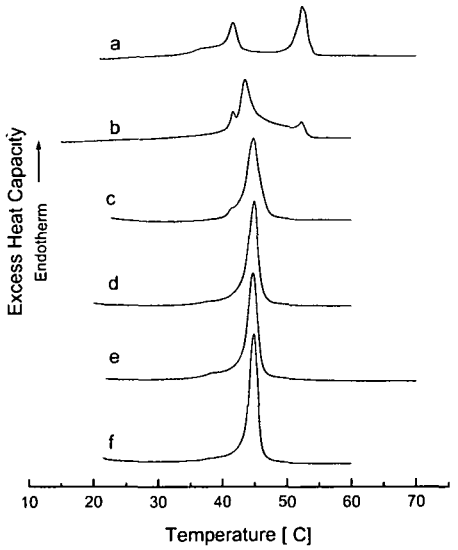

Fig. 2. **Thermograms of liposomal mixtures of DPPC and DPPS after 2-h incubation.** DPPC liposomes were prepared by dispersing 2 μmol phospholipid in HEPES buffer containing (a) 0 mM, (b) 0.02 mM, (c) 0.04 mM, (d) 0.06 mM, and (e) 0.06 mM melittin. In the absence of melittin, DPPS liposomes were prepared by the same method. The DPPC and DPPS liposomes were mixed and incubated for 2 h at (a)–(d) 55 or (e) 25°C. (f) Liposomes of binary mixtures of DPPC and DPPS (1:1) were prepared in 0.06 mM melittin at 80°C. After incubation, thermograms were obtained at a heating rate of 0.5 K/min.

TABLE I. **Melittin-induced reduction of phase transition enthalpy of liposomes composed of various phosphatidylcholines.**

Phospholipid species	Melittin (mol%)	Temperature (°C)	Phase transition	Enthalpy (kcal/mol)	Relative enthalpy (%)
DEPC	0	10.4		9.13	100
	1	10.4		9.14	100
	5	10.5	(peak 1)	3.72	98.7[a]
		12.6	(peak 2)	5.29	
DMPC	0	22.3		4.70	100
	1	22.4		4.52	96.2
	2	22.3		4.12	87.7
	5	22.3		2.82	60.0
DPPC	0	42.0		7.02	100
	2	40.4		6.45	91.9
	5	40.2		5.11	72.7
DSPC	0	53.7		10.1	100
	1	53.8		10.3	102
	5	53.1		9.39	93

[a]Total of separated peaks.

melittin between neutral liposomes. Liposomes of one phosphatidylcholine species were prepared in the presence of melittin and mixed with the other species of liposomes prepared without melittin. The thermograms after 2-h incubation showed two separated transition peaks of DMPC and DPPC up to 0.06 mM melittin (data not shown). These results indicate that melittin does not induce membrane fusion between neutral phospholipids and that acidic phospholipids are essential for melittin-induced fusion.

The participation of phosphatidic acid, another acidic phospholipid, in the melittin-induced membrane fusion was examined. Phosphatidic acids show a growth factor–like action (*19*) and are converted to diacylglycerol, which activates protein kinase C (*20*). Therefore, the fusion activity of melittin was further investigated between liposomes of DPPC and dimyristoylphosphatidic acid (DMPA). When DPPC liposomes were prepared in the presence of melittin, the melittin reduced the enthalpies but did not change the temperatures of the pre- and main transitions (Fig. 4a). DMPA liposomes gave a main transition peak at 52°C (Fig. 4b), and the addition of melittin shifted the peak to a lower temperature (Fig. 4c). While a broadening of the transition peak was observed, the transition enthalpy of DMPA was not reduced by the addition of melittin. These results indicate that melittin penetrates the hydrophobic core of DPPC membranes, and that it binds to the surface of DMPA membranes electrostatically (*17*). When DPPC liposomes were prepared in melittin solution and mixed with DMPA liposomes, the DSC thermogram of the mixture showed a new transition peak between a peak around 42°C and a peak around 50°C (Fig. 4d). The new peak corresponds to the transition peak of a binary mixture of DPPC and DMPA, and the peaks on either side correspond to DPPC and DMPA, respectively. The binary peak appears between the DPPC and DMPA peaks even at 0.002 mM melittin (Fig. 4d). At 0.02 mM melittin, only the peak of the binary mixture is observed (Fig. 4e). These results indicate that membrane fusion is similarly induced by melittin in liposomal systems in which phosphatidylserine is replaced by phosphatidic acid.

The essential process of membrane fusion involves reorganization of lipid molecules with the adjacent membranes, a process that is probably mediated by non-bilayer intermediates (*21*). Although the detailed mechanism of membrane fusion remains unknown, a group of homologous proteins, annexins, play an important role in membrane fusion of exocytosis in cells (*22*). However, using liposomes as a model, membrane fusion can be investigated in a defined system. Calcium ions induce the fusion of liposomes containing acidic phospholipids. Stamatatos *et al.* have reported that liposomes with opposite surface charges aggregate and proceed to fuse (*23*). Murata *et al.* have shown that synthetic amphiphilic peptides with opposite charges can induce membrane fusion between phosphatidylcholine liposomes (*24*). In these liposomal systems, presumably, electrostatic forces keep the membranes in contact, and the close contact makes the fusion process progress. When melittin assumes an α-helix configuration, three-fourths of the surface becomes a hydrophobic region, with the positive charges of the basic amino acids in the carboxyl terminus. Indeed, the transition enthalpy of DPPC is reduced by the addition of melittin (Table I and Fig. 4a). A reduction in the transition enthalpy is observed when a protein partially penetrates the bilayer membranes of DPPC (*17*). The addition of melittin shifts the phase transition temperatures of

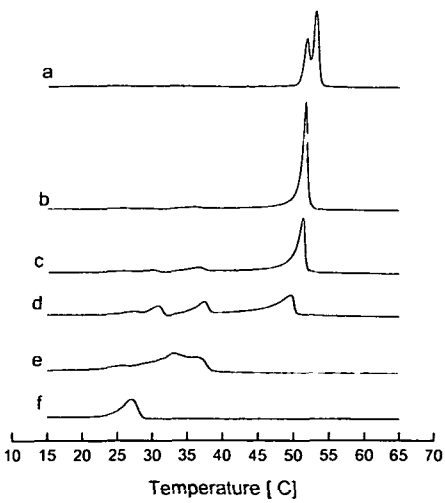

Fig. 3. **Thermograms of DPPS liposomes including desired amounts of melittin.** DPPS liposomes were prepared by dispersing 2 μmol phospholipid in 1 ml HEPES buffer in a bath-type sonicator at 80°C for 2 min. Then various concentrations of melittin were added to the liposomal dispersions: (a) 0 mM, (b) 0.02 mM, (c) 0.04 mM, (d) 0.1 mM, (e) 0.3 mM, and (f) 0.4 mM. Thermograms were obtained at a heating rate of 0.5 K/min.

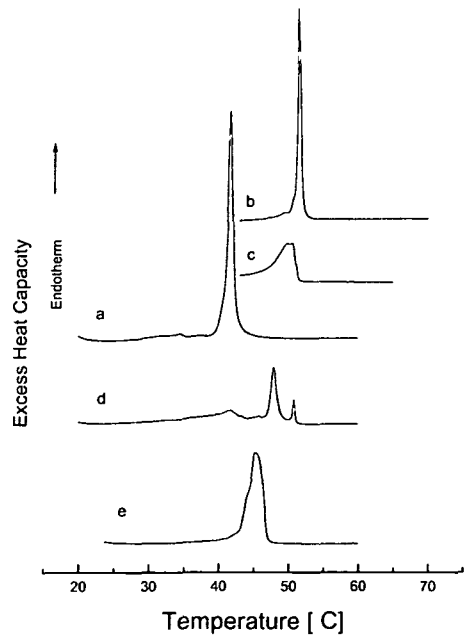

Fig. 4. **Thermograms of DPPC liposomes, DMPA liposomes, and liposomal mixtures of the two with and without melittin.** (a) DPPC and (c) DMPA liposomes were prepared by dispersing 2 μmol phospholipid in 1 ml HEPES buffer containing 0.06 mM melittin. In the absence of melittin (b) DMPA liposomes were prepared by the same method. (d) DPPC liposomes were prepared in 1 ml HEPES buffer containing 0.002 mM melittin and mixed with DMPA liposomes prepared without melittin, and then incubated for 2 h at 55°C. (e) DPPC liposomes were prepared in the presence of 0.02 mM melittin, and mixed with DMPA liposomes then incubated for 2 h at 55°C. After incubation, thermograms were obtained at a heating rate of 0.5 K/min.

DPPS and DMPA (Fig. 4c), which is consistent with an electrostatic interaction between melittin and acidic phospholipids. Two lysines and two arginines are located near the carboxyl end of the melittin molecule, with the rest of the molecule composed mainly of hydrophobic amino acids. Assuming that membrane fusion occurs when lipid bilayer membranes are in close contact, melittin may induce fusion by inserting its hydrophobic tail into one membrane and attracting the other membrane electrostatically. Monette and Lafleur have reported that melittin interacts with negatively charged lipids (*25*). The present results indicate that melittin can induce membrane fusion between acidic phospholipids and neutral phospholipids due to its amphiphilic nature. Melittin-induced fusion occurs in both the liquid crystalline and gel phases of lipid bilayers, which implies that melittin disturbs the membrane structure following the effect of membrane contact by electrostatic interaction. A recent study of membrane fusion revealed that SNARE (SNApREceptors) in vesicle membranes and SNARE in target membranes combine leading to membrane fusion (*4*). This result indicates that close contact between membranes is very important for progression to the next step of membrane fusion. When two membranes are in very close contact, the probability of going to the next step increases due to perturbation or fluctuation at the site of contact, which might trigger membrane fusion. The present results have shown that melittin induces a mixing of lipids between different liposomes, which indicates that melittin causes a close contact between membranes. Considering that the fusion activity of melittin does not depend on the phase structure of the membrane, lipid mixing probably leads to membrane fusion. Special mention should also be made of the method for measuring membrane fusion. In general, membrane fusion has been investigated by monitoring the mixing of vesicle lipids and the mixing of aqueous contents (*26*). Here, membrane fusion was examined by measuring the phase transition behavior of the liposomal system by DSC, which reflects the degree of lipid mixing. Recently, Pantazatos and MacDonald have observed membrane fusion between positively and negatively charged giant liposomes under an optical microscope (*27*).

REFERENCES

1. Gennis, R.B. (1989) *Biomembranes (Molecular Structure and Function)*, pp. 336–351, Springer-Verlag, New York
2. Hanson, P.I., Roth, R., Morisaki, H., Jahn, R., and Hauser, J.E. (1997) Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90**, 523–535
3. Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* **395**, 347–353
4. Patel, S.K., Indig, F.E., Oliveri, N., Levine, N.D., and Latterich, N. (1998) Organelle membrane fusion: A novel function in ER membrane fusion. *Cell* **92**, 611–620
5. Wilschut, J. and Hoekstra, D. (1986) Membrane fusion: Lipid vesicles as a model system. *Chem. Phys. Lipids* **40**, 145–166
6. Ohki, S. (1982) A mechanism of divalent ion-induced phosphatidylserine membrane. *Biochim. Biophys. Acta* **689**, 1–11
7. Eytan, G.D. and Almary, T. (1983) Melittin-induced fusion of acidic liposomes. *FEBS Lett.* **156**, 29–32
8. Ohki, K. (1989) A region-matched hydrophobic interaction between melittin and dimyristoylphosphatidylcholine in a ternary mixture of phosphatidylcholinesa. *Biophys. Res. Commun.* **164**, 850–856
9. Privalov, P.L., Plotnikov, V.V., and Filimonov, V.V. (1975) Precision scanning microcalorimeter for the study of liquids. *J. Chem. Thermodynamics* **7**, 41–47
10. Ohki, K., Takahasi, K., Kato, S., and Maesono, A. (1989) Short and long range calcium-induced lageral phase separations in ternary mixtures of phosphatidic acid, phosphatidylcholine and phosphatidylethanolamine. *Chem. Phys. Lipids* **50**, 109–117
11. Marsh, D., Watts, A., and Knowles, P.F. (1977) Cooperativity of the phase transition in single- and multibilayer lipid vesicles. *Biochim. Biophys. Acta* **465**, 500–514
12. Hauser, H. (1984) Some aspects of the phase behaviour of charged lipids. *Biochim. Biophys. Acta* **772**, 37–50
13. Cevec, G. and Marsh, D. (1987) *Phospholipid Bilayers*, pp. 369–406, John Wiley & Sons, New York
14. Wilschut, J., Duzgunes, N., Fraley, R., and Papahadjopoulos, D. (1980) Studies of the mechanism of membrane fusion: Kinetics of calcium ion induced fusion of phosphatidylserine vesicles followed by a new assay for mixing of aqueous vesicle contents. *Biochemistry* **19**, 6011–6021
15. Struck, D.K., Hoekstra, D., and Pagano, R.E. (1981) Use of resonance energy transfer to monitor membrane fusion. *Biochemistry* **20**, 4093–4099
16. Quay, S.C. and Condie, C.C. (1983) Conformational studies of aqueous melittin: Thermodynamic parameters of the monomer-tetramer. *Biochemistry* **22**, 695–700
17. McElhaney, R.N. (1986) Differential scanning calorimetric studies of lipid-protein interactions in model membrane systems. *Biochim. Biophys. Acta* **864**, 361–421
18. Ohki, S., Maucus, E., Sukumaran, D.K., and Arnold, K. (1994) Interaction of melittin with lipid membranes. *Biochim. Biophys. Acta* **1194**, 223–232
19. Moolenaar, W.H., Kruijer, W., Tilly, B.C., Verlaan, I., Bieman, A.J., and de Laat, S.W. (1986) Growth factor-like action of phosphatidic acid. *Nature* **323**, 171–178
20. Exton, J. (1990) Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265**, 1–4
21. Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leuissen-Bijvelt, L., and Cullis, P.R. (1979) Fusion of phospholipid vesicles in association with the appearance of lipidic particles as visualized by freeze fracturing. *Biochim. Biophys. Acta* **555**, 358–361
22. Creutz, C.E. (1992) The annexins and exocytosis. *Science* **258**, 924–931
23. Stamatatos, L., Leventis, R., Zuckermann, M.J., and Silvius, J. (1988) Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry* **27**, 3917–3925
24. Murata, M., Kagiwada, S., Takahashi, S., and Ohnishi, S. (1991) Membrane fusion induced by mutual interaction of the two charge-reversed amphiphilic peptides at neutral pH. *J. Biol. Chem.* **266**, 14353–14358
25. Monette, M. and Lafleur, M. (1995) Modulation of melittin-induced lysis by surface charge density on membranes. *Biophys. J.* **68**, 187–195
26. Hoekstra, D. (1982) Kinetics of intermixing of lipids and mixing of aqueous contents. *Biochim. Biophys. Acta* **692**, 171–175
27. Pantazatos, D.P. and MacDonald, R.C. (1999) Directly observed membrane fusion between oppositely charged phospholipid bilayers. *J. Membr. Biol.* **170**, 27–38