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Biochimica et Biophysica Acta 1194 (1994) 223–232

BBA Biochimica et Biophysica Acta

Interaction of melittin with lipid membranes

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Received 21 January 1994; revised 11 May 1994

Abstract

Interaction of melittin with lipid membranes was studied systematically with respect to its adsorption onto membranes, its effect on membrane leakage and fusion, and micellization at various melittin/lipid ratios. It was found that melittin has a strong affinity for adsorption onto lipid membranes. The analysis of the measured electrophoretic mobilities by use of a Gouy-Chapman double layer theory, shows that melittin is adsorbed onto the phosphatidylserine membrane several times more than the phosphatidylcholine membrane. However, it was observed that the phosphatidylcholine membrane is more susceptible to membrane leakage, vesicle fusion and micellization at a lower level of melittin adsorbed than the phosphatidylserine membrane. For small unilamellar phosphatidylcholine vesicles in 0.1 M NaCl, membrane leakage started at melittin to lipid ratio of 1:2000, a large increase in the rate of membrane leakage occurred at a ratio of about 1:500 or higher, membrane fusion occurred at a ratio of 1:200, and membrane micellization at a ratio of 1:10. On the other hand, for small unilamellar phosphatidylserine vesicles, the respective concentrations of melittin to result in membrane leakage, vesicle fusion, and membrane micellization were several times higher. Surface pressure measurements of lipid monolayers showed that the increase in surface pressure of the phosphatidylcholine monolayer due to the presence of melittin in the subphase solution was greater than that for the phosphatidylserine monolayer at any melittin concentration in the subphase solution. These experimental results indicate that melittin tends to be adsorbed on the surface of the negatively charged phosphatidylserine membrane due to the electrostatic binding so that the melittin molecule can stay out more on the surface of the membrane, while melittin appears to be adsorbed more into the hydrophobic membrane core for the electrically neutral phosphatidylcholine membrane.

Keywords: Melittin; Phospholipid membrane; Adsorption; Leakage; Fusion

1. Introduction

In order to gain insight into molecular mechanisms of biological membrane fusion, fusion in model membrane systems has been investigated by many authors. For lipid membrane fusion, a number of methods to induce fusion have been found [1,2]; divalent cation-induced fusion, temperature-induced fusion, osmotic pressure-induced fusion, electrical field-induced fusion, etc. Some macromolecules were also found to induce lipid membrane fusion [3]. Calcium binding proteins, such as synexin [4,5] and positively charged polypeptides such as polylysine [6] can enhance fusion of acidic lipid membranes in the presence of Ca^{2+} and other divalent cations. On the other hand, some non-charged macromolecules (e.g., polyethylene glycol) were found to induce fusion of both charged and neutral membranes in the absence of divalent cations [1,2,7,8]. Some proteins also induce membrane fusion without divalent cations. A typical example of these is the fusion proteins in virus-cell systems. These proteins (e.g., F protein in Sendai virus and HA proteins in various influenza viruses) possess a stretch of about 20 hydrophobic amino acid segments at their amino terminus which are considered to be relevant for the membrane fusion reactions in virus-biological cell systems [9–11]. It has been proposed that the degree of α-helical structure of the hydrophobic peptides is related to membrane fusion [12,13].

Melittin, the principal toxic peptide of bee venom, has hydrophobic segments somewhat similar to those found in the HA2 terminal chains [14] of influenza virus. Melittin is also capable of inducing membrane

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SSDI 0005-2736(94)00144-E

fusion [15–18], and lysing cells [19]. A number of structural studies of melittin interacting with lipid membranes have been done by NMR [20–23] and other spectroscopic techniques (fluorescence, EPR and EM, etc.) [24–29]. The secondary structure of melittin is well established. The recent studies with respect to the adsorption state of melittin on lipid membranes have concluded that, for fully hydrated lipid membranes, the location of melittin is at membrane surface regions [22,26–31]. New efforts have recently been made to consider electrostatic effects resulting from the melittin charge partitioned between membrane and aqueous phases [32–35] with respect to interfacial surface chemistry. Such studies would provide further information to elucidate the state of molecular adsorption. However, no work has been done on melittin adsorption on lipid membranes by either measuring the electrophoretic mobility of lipid vesicles or by measuring the penetration of melittin by monolayer studies. Also, the effect of melittin interaction with membranes on membrane leakage, aggregation and fusion has not been examined systematically. Lipid membrane fusion induced by melittin has been studied using internal content mixing fluorescence assay [15], membrane mixing assay using spin-labelled lipid [17], and electron microscopy [18]. In our fusion study, we used a fluorescence fusion assay using a membrane molecule-bound fluorophore, pyrene-PC [36]. This assay would not be affected by the external environmental changes and would have the least interference from the effects of membrane fusion and other artifacts. In order to understand the interacting of melittin with membranes and its functional role associated with membranes, we carried out a systematic study of melittin–lipid membrane interaction (adsorption onto membranes, membrane leakage, fusion, and micellization) with respect to melittin concentration.

2. Materials and methods

Phospholipids (egg-phosphatidylcholine (PC) and bovine brain phosphatidylserine (PS)) were purchased from Avanti Polar Lipids (Birmingham, AL). Melittin was obtained from ICN Biochemicals (Cleveland, OH) and used without further purification. Our HPLC analysis showed no noticeable impurities in melittin on a C_{18} μBondapak column (Reverse phase). The assay of the lipids for lysolipid formation, over the longest time of experiments and at the greatest ratio of melittin/lipid used for vesicle fusion in the present study, did not indicate detectable amounts of lysolipid within our experimental resolution (Table 1). In order to suppress the effect of the small quantities of phospholipase A_2 as impurity, all experiments were done in a solution containing 4 mM EDTA. It has been demonstrated that at this level of EDTA, the effect of phospholipase A_2 on lipids is virtually negligable [23]. 6-Carboxyfluorescein (CF) and 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (Pyr-PC) were purchased from Eastman Kodak (Rochester, NY) and Molecular Probes (Eugene, OR), respectively. 6-Carboxyfluorescein (CF) was repurified by chromatography on Sephadex LH-20 according to the published method [37]. All other chemicals used were of reagent grade from Baker. Water used was triply distilled in all glass apparatus, including an alkaline $KMnO_4$ process.

Table 1
Assay of lysophospholipids produced from egg-phosphatidylcholine in the presence of melittin in lipid suspension solution under various conditions at 24°C

Sample	Ratio (mol/mol)	EDTA	2 h	24 h
Melittin/PC	1:100	0 mM	ND (% yield)	ND
Melittin/PC	1:100	4 mM	ND	ND
Melittin/PC	1:10	0 mM	ND	0.8±0.4%
Melittin/PC	1:10	4 mM	ND	ND

An apropriate amount of melittin was applied to each lipid (egg phosphatidylcholine) vesicle suspension (1 mM/ml). After leaving the samples for the designated amounts of time at room temperature (24°C), each suspension solution was completely dried and the dried lipid was dissolved in chloroform/methanol (2:1) of 0.5 ml. Then, 50 μl of each lipid solution was applied on the TLC plate and it was developed on the plate with chloroform/methanol/acetic acid/water (25:15:4:2). Corresponding spots to lyso-PC and PC were collected and dissolved in chloroform/methanol (2:1) solution of 0.5 ml and centrifuged to separate solid materials from the lipid solution. The supernatants were collected and a same aliquot of each supernatant was used for phosphate analysis [40]. The mol ratio of lyso-PC to the total PC (lyso-PC and PC) was obtained which was converted to the quantity in percent of lyso-PC in the total PC. ND, not detectable.

2.1. Phospholipid vesicle preparation

Small unilamellar phospholipid vesicles composed of either PC or PS, were prepared in 0.1 M NaCl/5 mM Hepes/4.0 mM EDTA (pH 7.4) solution by sonication [38]. The lipid concentration of the stock vesicle solution was approx. 1.5 mM phospholipid in the above mentioned buffer solution. Fluorescence probe (Pyr-PC) labelled lipid vesicles were also made by the same sonication method after mixing PC or PS and 10% (w/w) of Pyr-PC. The average sizes of such unilamellar vesicles were about 400 Å in diameter as determined with a photon-correlation spectrometer (Coulter N4). Small unilamellar PS or PC vesicles encapsulated with CF were also prepared by the same sonication method in a solution of 80 mM CF/10 mM Hepes/4 mM EDTA (pH 7.4). Unencapsulated CF in the vesicle suspension solution was removed by gel-filtration on a Sephadex G-75 column.

Large unilamellar lipid vesicles were prepared by the modified reverse phase-evaporation method [39]. After large vesicles of various size distribution were formed, the vesicle suspensions were passed through a nuclear pore (pore size 0.1 μm) membrane filter twice and only the filtered vesicles were collected. The average size of the filtered vesicles was 0.9 ± 0.1 μm in diameter as determined with the photon-correlation spectrometer.

2.2. Electrophoretic mobility measurements

Electrophoretic mobility of lipid vesicles was measured with a microelectrophoresis apparatus (Malvern Zeta-sizer II X4 C, Worchester, UK). The stock lipid vesicles of large unilamellar PC and PS vesicles with an average diameter of 900 Å, were prepared in 0.1 M NaCl/4.0 mM EDTA/5 mM Hepes (pH 7.4) (lipid concentration 2 mM). A small amount of the stock vesicle solution was suspended in the same NaCl buffer solution at a lipid concentration of 0.1 mM. The concentration of phospholipids was determined by phosphate analysis [40]. Then, a certain amount of melittin was added to this lipid suspension solution to give a known concentration ratio of melittin to lipid (1:1000 to 1:1).

2.3. Release of CF from lipid vesicles

Release of CF from lipid vesicles into the vesicle suspension medium in the presence of melittin at different concentrations, was monitored with respect to time by measuring the relief of self-quenching of CF encapsulated in the vesicles. CF fluorescence excited at a 490 nm wave length was detected at a wave length of 520 nm by a fluorometer (Perkin-Elmer LS-5). The lipid concentration of vesicle suspensions used in the experiment was approx. 0.02 mM.

2.4. Lipid vesicle fusion

One part of the fluorophore (Pyr-PC) incorporated lipid vesicles (PC or PS vesicles) and four parts of PC or PS vesicles without the fluorophores were suspended in NaCl buffer solutions (various concentrations of NaCl containing 5 mM Hepes and 4.0 mM EDTA (pH 7.4)). The total lipid concentrations were approx. 0.02 mM for all fusion experiments. For the PC-pyrene fluorescence fusion assay the fluorophore, Pyr-PC, was excited at 340 nm and the fluorescence emission spectra were recorded in the range of wave length 350 nm–500 nm, and fluorescence intensities at 376 nm (monomer's emission maximum M) and 460 nm (excimer's emission maximum E) were recorded. According to the method of Amselem et al. [36], the dilution factor, D_p, of the fluorophore or the extent of vesicle fusion is expressed by

$$D_p - 1 = ((E/M)_o - (E/M)_m)/(E/M)_m \quad (1)$$

where $(E/M)_o$ and $(E/M)_m$ are the ratios of excimer to monomer intensities without melittin and with melittin, respectively. From Eq. (1), we have

$$D_p = (E/M)_o/(E/M)_m = ((E/M)_m/(E/M)_o)^{-1} \quad (2)$$

The probe dilution, D_p, is also expressed as $(L^* + L)/L^*$, where L^* and L are the total lipids of the fluorescence labelled vesicles and the lipids of unlabelled vesicles fused to labelled vesicles, respectively. Since large vesicles (diameter greater than 700 Å) are less fusable, binary fusion of vesicles would dominate for the vesicle fusion events. Therefore, the meaningful value of the probe dilution for vesicle fusion would be in the range of 1 to 2 or at most 3.

2.5. Determination of vesicle size

Lipid vesicles of either PC or PS were suspended at the lipid concentration of 1.0 mM in 100 mM NaCl/5 mM Hepes/4.0 mM EDTA (pH 7.4) and then various amounts of melittin were added to the vesicle suspension to obtain various ratios of melittin to lipid (1:1000–1.0). The average diameter of the lipid vesicles was measured with a photon correlation spectrometer (Coulter N-4). Changes in melittin concentration in the vesicle suspension were made at a time interval of about 10 min. Within this time, the change in vesicle size reached a steady value. The data of vesicle size measurements were taken after such a steady state was achieved for each melittin concentration.

2.6. Surface tension measurements

Phospholipid monolayers (PC or PS) were prepared by placing an aliquot of the lipid spreading solution (approx. 1 mM lipid in hexane) by means of a microsyringe (Hamilton) on an aqueous surface of constant area (about 64 cm^2 in a glass dish). The surface tension was measured after complete evaporation of solvent. The lipid was placed so that the surface tension was the same (20 dynes/cm) for each monolayer. Subphase solution was the 0.1 M NaCl/5.0 mM hepes/4.0 mM EDTA (pH 7.4). The surface tension of the monolayer was measured with an electronic balance (Beckman, LM500), using a microscopic cover glass ($18 \times 18 \times 0.2$ mm) as a Wilhelmy plate (accuracy of 0.1 dynes/cm). Then, a certain amount of melittin was injected into the subphase solution and the solution was stirred well with a magnetic stirrer so that a homogeneous concentration of melittin was attained. The detailed description of the method is given elsewhere [41]. The surface

pressure was obtained from the following relationship of the surface tension and pressure [42]:

$\pi = \gamma_0 - \gamma$

where π is the surface pressure, γ_0 the surface tension of water and γ the surface tension of the monolayer. All experiments were done at 24 ± 1°C.

3. Results

In order to examine the nature of adsorption of melittin onto lipid membranes, in the first series of experiments, lipid vesicle electrophoretic mobilities were measured for both PC and PS large unilamellar vesicles in the presence of various amounts of melittin. The results of vesicle electrophoretic mobility measurements indicated that melittin was readily adsorbed onto the lipid membrane surface which made the membrane surface charge more positive. The change in electrophoretic mobility was greater for the PS vesicle than for the PC vesicle. This indicates that more melittin was adsorbed onto the PS membrane than the PC membrane. This is confirmed by calculation (see Discussion). At zero concentration of melittin in the PC vesicle suspension solution, the PC vesicle mobility was virtually zero. At a ratio of melittin to lipid of 1:1000, there was a noticeable change (0.05 $\mu m\ V^{-1}\ cm\ s^{-1}$) in vesicle mobility (see Fig. 1(a)): the vesicle surface has a positive ζ-potential of about 0.7 mV. The vesicle electrophoretic mobility increased as the melittin/lipid ratio increased. At the ratio of 1:100, the vesicle mobility was found to be 0.3 $\mu m\ V^{-1}\ cm\ s^{-1}$. At a ratio of 1:10, the mobility increased to 1.3 $\mu m\ V^{-1}\ cm\ s^{-1}$, and the ζ-potential corresponding to this mobility was + 18.2 mV. For the PS vesicles, the vesicle electrophoretic mobility was $-4.4\ \mu m\ V^{-1}\ cm\ s^{-1}$ in 0.1 M NaCl at pH 7.4 without melittin, which corresponded to the ζ-potential −61.6 mV. The increase in melittin concentration reduced the vesicle mobility (see Fig. 1(b)). At the ratio of 1:100, the vesicle mobility was $3.6\ \mu m\ V^{-1}\ cm\ s^{-1}$, and at 1:10, the mobility was $-1.8\ \mu m\ V^{-1}\ cm\ s^{-1}$. The latter corresponds to ζ-potential of −25 mV. At a ratio of melittin to lipid of 1:2, the ζ-potential changed its sign from negative to positive: i.e., at this concentration of melittin, the positive charges of the adsorbed melittin exceeded the intrinsic negative charges of the phosphatidylserine membrane surface.

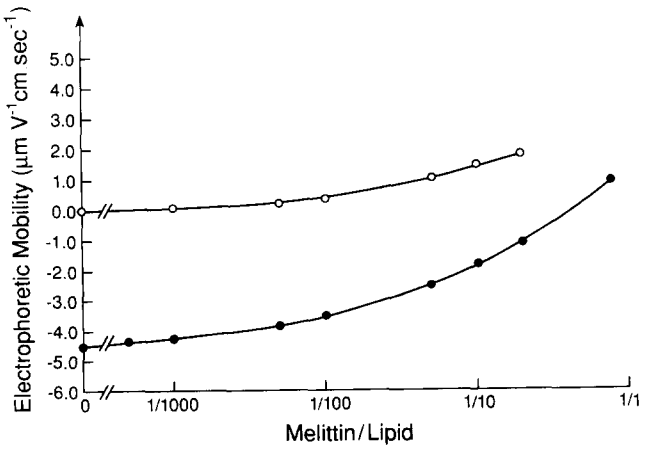

Fig. 1. Electrophoretic mobility of large unilamellar lipid (PC,PS) vesicles suspended in 0.1 M NaCl buffer, 5 mM Hepes/4 mM EDTA (pH 7.4) as a function of melittin concentration. The lipid concentration of the suspension solution was 0.1 mM. (a) ○, PC vesicles; (b) ●, PS vesicles.

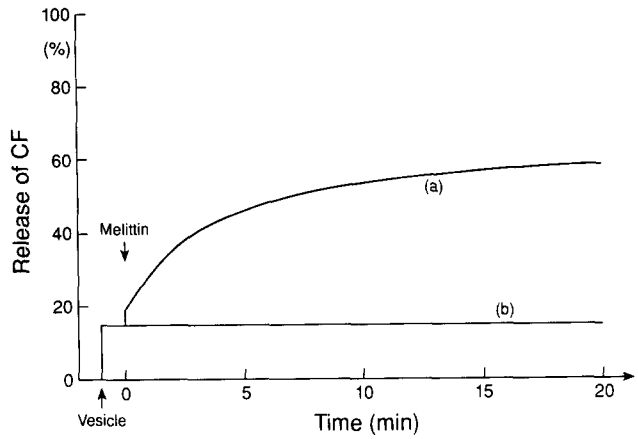

Fig. 2. Typical time-courses of 6-carboxyfluorescein (CF) released from small unilamellar phosphatidylcholine (PC) vesicles in the presence of melittin at a ratio of melittin to lipid of 1:400. (a) 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.2), (b) no melittin present but 0.1 M NaCl/5 mM Hepes/4 mM EDTA containing phospholipase A_2 the amount of which corresponds to the upper limit (5 units/mg melittin) allegedly contained in the melittin sample at a ratio of melittin/lipid of 1:250. The total lipid concentration was 0.02 mM.

In the second series of experiments, the membrane leakage caused by the melittin interaction with the membrane was examined for a fluorophore (6-carboxyfluorescein) encapsulated lipid vesicles. Typical time courses for the release of 6-carboxyfluorescein (CF) from small unilamellar lipid vesicles induced by an addition of melittin of a given concentration (melittin/lipid = 1:400 for PC, 1:50 for PS) are shown in Fig. 2 for PC vesicles and in Fig. 3 for PS vesicles. After the addition of melittin to the vesicle suspension, the release of CF reached a steady value in 10–15 min. For the PS vesicle, a greater amount (7–8-fold) of melittin was needed to induce the same amount of CF release as obtained for PC vesicles (see Fig. 4).

In order to examine the possible effect of phospholipase A_2 on the observed release of CF from the lipid vesicles, the following control experiments were performed: the maximum amount of phospholipase A_2 which may be contained as a contaminant in the melittin sample (5 units/mg melittin) was introduced into a

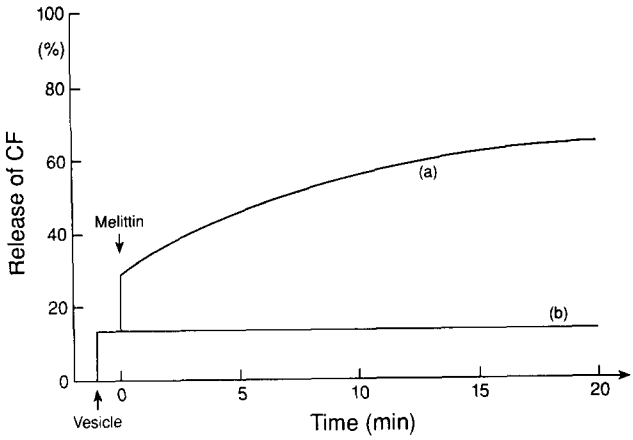

Fig. 3. Similar experiments as those shown in Fig. 2, except for use of the PS vesicles instead of PC, and melittin/lipid ratio of 1:50. (a) 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.2), (b) no melittin present but phospholipase A_2 in 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.2), the amount of which was allegedly contained in the melittin sample at the ratio of melittin/lipid of 1:10.

lipid vesicle suspension without melittin and its effect on membrane leakage was examined. In the presence of phospholipase A_2 the amount of which corresponds to the melittin/lipid ratio of 1:250, there was no appreciable effect of phospholipase A_2 on the CF release in either case of no EDTA or 4.0 mM EDTA presence in the suspension solution. However, for the presence of phospholipase A_2 corresponding to the ratio of melittin/lipid of 1:10, some (approximately a few % of the effect for the case of melittin application at melittin/lipid 1:10) extent of CF release was observed in the solution without EDTA. However, this was not observed when the suspension solution contained 4.0 mM EDTA. The presence of EDTA in the solution may have inactivated the enzyme activity of phospholipase A_2. Therefore, all solutions containing 4.0 mM EDTA used in the experiments clearly did not give any effect from phospholipase A_2.

The release of CF depended upon the concentration of melittin (expressed as a ratio of melittin to phospholipid molecules) in the vesicle suspension, which is shown in Fig. 4 for both PC and PS vesicles. The values in Fig. 4 are those measured at 10 min after melittin addition. A small extent of leakage from lipid vesicles started at the melittin/lipid ratio of as low as 1:2000 for PC vesicles and 1:500 for PS vesicles suspended in 0.1 M NaCl buffer solution at pH 7.4. An appreciable release of CF was observed at the melittin/lipid concentration ratio of 1:400 for PC and 1:60 for PS. At all ranges of melittin concentration, the PC vesicle is more susceptible to membrane leakage than the PS vesicle.

In the third series of experiments, the effect of melittin on lipid vesicle fusion was studied. One part of small unilamellar lipid vesicles of PC or PS containing 10% (M/M) of Pyr-PC, and four parts of small lipid vesicles made of PC or PS were suspended in various NaCl concentration solutions. The dilution of the fluoresence probes incorporated in lipid vesicles into unlabelled lipid vesicles due to fusion was observed by measuring the ratio of excimer (E: measured at 460

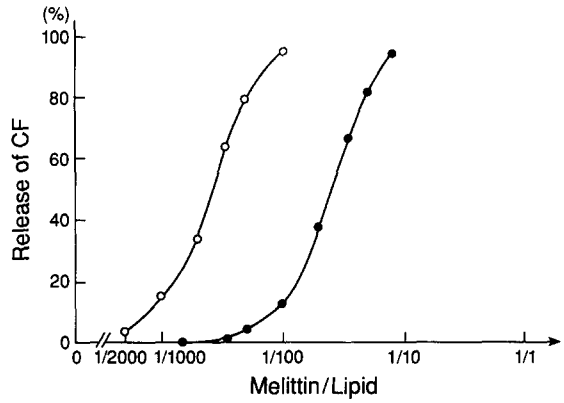

Fig. 4. Melittin-induced CF released from small unilamellar lipid vesicles in 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.2) at various melittin concentrations. The melittin concentration is expressed as the ratio of melittin to lipid. 80 mM CF encapsulated in lipid vesicles excited at 490 nm was self-quenched. When CF was released to the environmental solution, the fluorescence of CF was observed at 520 nm. A certain concentration of melittin was added to the 0.1 M NaCl buffer solution and the release of CF was monitored as time. The data above was taken at 10 min. after melittin was added. 100% release corresponded to the situation where 0.05% of Triton X-100 was added to vesicle suspension solution and the vesicles were supposed to be all lysed. ○, PC vesicles in 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.4); ●, PS vesicles in 0.1 M NaCl/5 mM Hepes/4.0 mM EDTA (pH 7.4).

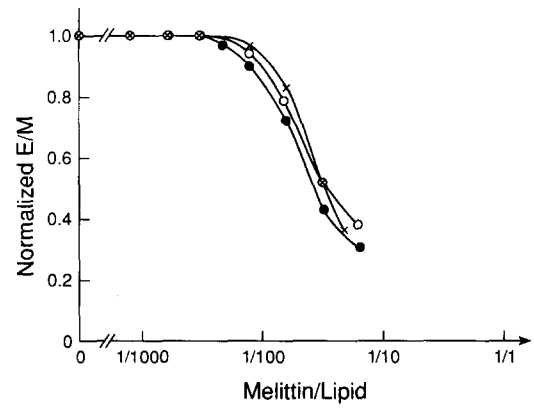

Fig. 5. Fusion of small unilamellar PC vesicles with small unilamellar PC vesicles incorporated with Pyr-PC is plotted with melittin concentration in the vesicle suspension. Melittin concentration is given by the ratio of melittin/lipid. Total lipid concentration = 0.02 mM. The ratio (E/M) of the Pyrene excimer (460 nm) to monomer (376 nm) fluorescence is plotted on the oridinate. A decrease in this quantity indicates a dilution of the fluorescence probe and therefore is related to an extent of vesicle fusion The fluorescence was measured 10 min after the addition of melittin at different amounts. All vesicle suspensions contained 5 mM Hepes and 4.0 mM EDTA (pH 7.4). ○, 0.1 M NaCl, ×, 0.5 M NaCl; ●, 2.0 M NaCl. Normalized $E/M = (E/M)_m/(E/M)_o$.

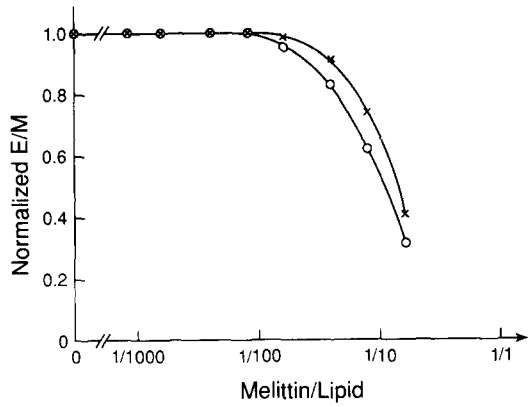

Fig. 6. Similar experimental results as in Fig. 5, except for using PS vesicles. ○, 0.1 M NaCl; ×, 0.5 M NaCl.

nm) to monomer (M: at 376 nm) fluorescence, when the fluorophore was excited at 340 nm. Further details are described in a published paper [36]. This ratio (E/M) which corresponded to dilution of the probes or the extent of vesicle fusion were obtained as a function of the ratio of melittin/lipid molecules. Figs. 5 and 6 show such experimental results for PC and PS vesicles, respectively. The fusion is expressed as the diluiton factor, D_p, of the fluorophore (Fig. 7). As mentioned earlier, the probe dilution factor up to 2 is relevant for vesicle fusion events in this system. As seen in these figures, the fusion of PC vesicles in 0.1 M NaCl buffer started and developed gradually at a melittin/lipid ratio of 1:200. The rate of fusion increased rapidly in the range of the melittin/lipid ratios of 1:100–1:50 (Fig. 5). Fusion of PS vesicles started at much higher melittin concentration as a large reduction of E/M was observed in the range of the ratios of 1:20–1:10 (see Fig. 6). Ionic strength did not appreciably affect the melittin-induced vesicle fusion in the range of 0–2.0 M NaCl.

In order to have further insight into the above observed vesicle fusion induced by melittin, the size of

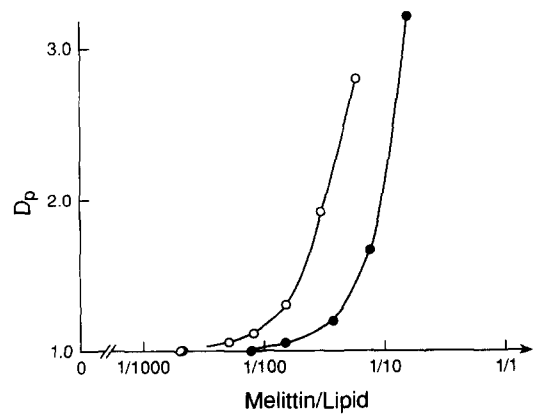

Fig. 7. The plot of the probe dilution factor, D_p, with respect to melittin concentration. The data of E/M were taken from those in Figs. 5 and 6. ○, PC; ●, PS.

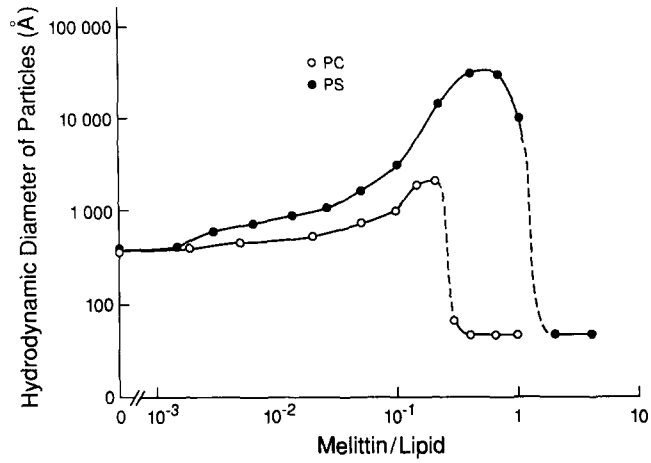

Fig. 8. Hydrodynamic radius of small unilamellar PC vesicles in 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.4) as a function of melittin concentration. The lipid concentration was 1.0 mM and the melittin concentration is expressed as the ratio of melittin/lipid. ○, PC; ●, PS.

vesicle was measured as a function of melittin concentration using a photon-correlation spectrometer in the fourth series of experiments. The results of the size of PC vesicles with various amounts of melittin in 0.1 M NaCl are shown in Fig. 8. As the melittin/lipid ratios increased from 1:800 to 1:500 in 0.1 M NaCl, a small increase in vesicle size or vesicle aggregates was observed. Further increase in melittin concentration induced a further increase in an average size of lipid particles probably due to lipid vesicle fusion as well as vesicle aggregation. However, the addition of melittin of more than 1:10 ratio induced a sudden and dramatic decrease in the size of the particles. These observations are similar to those reported earlier [18]. Beyond a certain concentration of melittin (e.g., a ratio of melittin/lipid greater than 1:5 for the PC vesicles in 0.1 M NaCl), lipid vesicles appeared to form micellar configurations because the average diameter of the particles measured was about 50–60 Å. From these measurements, it was learned that the significant reduction of the excimer to the monomer ratio observed in the range of melittin/lipid ratio less than 1:10 in the vesicle fusion experiments (see Figs. 6 and 7) was not due to the dilution of the fluorescence molecules among lipid vesicles resulting from vesicle fusion, but it was probably due to the micelle formation of melittin/lipid molecules. Similar experimental results for the PS vesicles are shown in Fig. 8.

Two obvious different observations for the size changes of PC and PS vesicles due to melittin interaction were: (1) the melittin concentration at which the average size of vesicles was muximum, was lower for the case of PC vesicles than for the PS vesicles. These results are similar to those obtained from both vesicle membrane leakage as well as vesicle fusion experi-

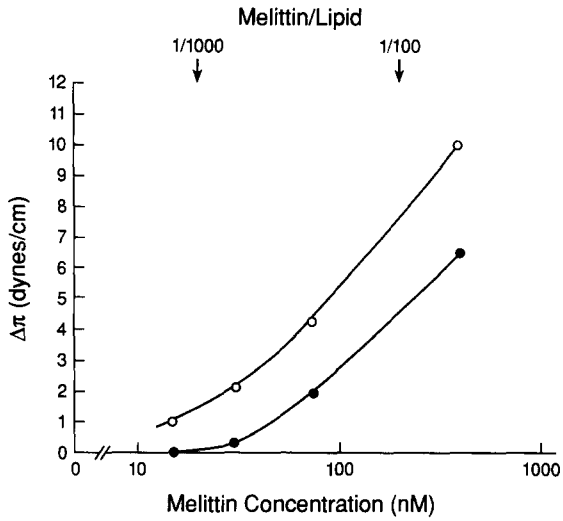

Fig. 9. Surface pressure increase in lipid monolayers formed on 0.1 M NaCl/5 mM Hepes/4 mM EDTA (pH 7.4) with respect to melittin concentration in the subphase solution. The initial film pressure of the monolayer was 20 dynes/cm for all monolayers. ○, PC; ●, PS.

ments. (2) The maximum average size of the fused and/or aggregated PS vesicles induced by melittin was about ten times greater than that of the PC vesicles. Similar results were obtained for vesicle aggregation by measuring turbidity of vesicle suspension with respect to melittin concentration (data not shown).

In order to elucidate other aspects of melittin adsorption onto lipid membranes, a study of the surface pressure of lipid monolayer was done as a function of concentration of melittin in the subphase solution. As the concentration of melittin increased, the surface pressure of the monolayer increased, which can be interpreted as the result of melittin penetration into the monolayer phase. An electrostatic adsorption does not contribute appreciably to the change in surface tension [43]. It was found that the monolayer pressure increase for the PC monolayer was greater than that for the PS monolayer at all melittin concentrations, where the initial pressures of monolayers (without melittin in the subphase) were maintained at 20 dynes/cm for all monolayers. Experimental results are shown in Fig. 9 with respect to the increase in surface pressure $\Delta\pi$ at a certain melittin concentration:

$$\Delta\pi = \pi(\text{melittin}) - \pi(\text{no melittin})$$

Although the melittin concentrations in this system and vesicle fusion system are expressed in different ways, a rough correspondence in concentration is given in the figure. At the melittin concentration corresponding to the melittin/lipid ratio of 1:200, melittin increased the PC monolayer pressure considerably but not for the PS monolayer.

4. Discussion

All experimental results described above enable us to draw some conclusions on the interaction mode of melittin with lipid membranes.

(1) For the PC membrane in 100 mM NaCl solution, it indicates that at very low concentration of melittin (molar ratio of melittin/lipid less than 1:2000), melittin molecules are adsorbed onto the surface of the membrane. The measurements of vesicle electrophoretic mobility and membrane leakage experiments indicate this possibility. From the electrophoretic mobility data and the Eqs. (A-1), (A-2) and (A-3) in the Appendix, the surface charge density of the PC membrane at a melittin/lipid ratio of 1:1000, is calculated to be about 3 positive electronic charges/100000 Å^2. Since the concentration of melittin ions (less than μM) is so small compared to other ions (100 mM Na^+ and Cl^-) in the vesicle suspension solution, the contribution of melittin to the total ionic strength is neglected in the above calculation. By assuming a net five positive charges per melittin molecule and the area per melittin molecule adsorbed onto the membrane to be 150 Å^2 [35], and by using the surface charge density calculated, the adsorption of melittin (e.g., how many lipids per melittin) on the PC membrane at this melittin concentration is calculated to be one melittin per about 2500 lipids by use of the following equation:

$$\sigma = e\frac{Z_m X_m}{A_L X_L + A_m X_m}$$

where σ is the surface charge density, A_L and A_m the average areas per PC molecule (68 Å^2) and melittin (150 Å^2) adsorbed on the PC membrane, respectively, and Z_m is the effective charge number, 5, per melittin molecule adsorbed. X is the mol fraction of lipid or melittin in the membrane.

(2) The leakage of internal contents of even a relatively large molecule like CF occurred at melittin/lipid ratios of less than 1:2000 for PC vesicles. Vesicle fusion started to occur at about the ratio of 1:200. The greatest increase in the extent of fusion occurred at the ration of 1:100–1:50. At the melittin/lipid ratio of 1:100, the surface charge density was about 1.0 positive electronic charge/4200 Å^2 which corresponds to one melittin molecule per 300 lipid molecules. At a ratio of 1:20, the surface charge density increased to 1.0 electronic charge/1300 Å^2 which corresponds to one melittin molecule per about 100 lipids. By assuming that melittin molecules are adsorbed on the outer side of lipid vesicle membrane only, these values correspond to about 1:10 of melittin applied to the bulk solution to be adsorbed onto the lipid membrane. An increase in melittin concentration more than the ratio of 1:10, seemed to produce micelles of melittin and lipid complexes.

(3) For the PS membrane, the analysis is similar to the PC, except for taking into account the effect of Na^+ binding to the lipid molecule. For the PS membrane, the surface charge density is expressed by

$$\sigma = e\frac{Z_{ex}X_L + Z_mX_m}{A_LX_L + A_mX_m}$$

where Z_{ex} is the effective charge of a PS molecule of the membrane, which is $-0.37e$ per lipid (due to the binding of Na^+) in 0.1 M NaCl [38]. X_L is the fraction of the PS molecules in the melittin adsorbed PS membrane, $X_L + X_m = 1$. Other parameters, A_L, X_m, Z_m, A_m are the same as for the PC case.

For the PS case, it is deduced that almost all melittin molecules are adsorbed onto the lipid membranes, leaving little melittin in the bulk solution. This agrees with the observation made for another acidic lipid-melittin system [35]. This means that the phosphatidylserine membrane adsorbs melittin about 10-times more than the PC membrane. One of the main reasons for this may be due to the strong electrostatic attraction between melittin and the phosphatidylserine membrane.

Other authors [33,34] have used an effective charge number of 2 for a melittin molecule instead of 5 or 6 in order to analyze their experimental results wtih use of the Gouy-Chapman theory. When bulky molecules having multiple charge distribution are in an electrolyte solution, it is necessary to assign an effective charge number for such a molecule to make a point charge approximation for use of the Gouy-Chapman theory [44] However, once these ions are adsorbed on the membrane surface facing an aqueous solution, it may be reasonable to assume that the full charges of the adsorbed molecules contribute to the charge density of the adsorbed surface. This is supported by the direct evidences of the pK_a values, as measurements of amino acid chains of membrane-bound melittin [22] and the surface charge effects induced by PS in mixed bilayer with PC with respect to melittin bound [45].

The increase in vesicle size occurred with the increase in the amounts of melittin adsorbed onto the membrane surfaces (Figs. 1 and 7). As the amount of adsorbed melittin is increased (Fig. 1), the extent of vesicle fusion (Figs. 5 and 6) increased. There appears to be some vesicle aggregation due to melittin interaction with membranes in these stages but there must also be vesicle fusion judging from the results obtained by the fluorescence fusion assay (Figs. 5, 6, and 7). It should be noted that the probe dilution between 1–2 may be significant for vesicle fusion because the first binary fusion events may be predominant in this vesicle fusion system as described earlier. For all experimental results of membrane leakage, vesicle fusion and micellization, the common and clear feature was that the PC vesicle was more susceptible to the melittin effect than the PS vesicle; the concentration of melittin necessary to produce the same effect on the above membrane properties was several times higher for the PS membrane than the PC, in spite of the amount of melittin adsorbed being about ten times more for the PS membrane than the PC membrane.

(4) It has been shown that melittin molecule is adsorbed at the surface region for fully hydrated lipid membranes [22,28–31] rather than spans through the membrane. The latter occurs for dry oriented lipid membranes [26,31] or lipid membranes under application of transmembrane potential [46–49]. Our experimental results suggest that the interaction modes of melittin with the neutrally charged and negatively charged membranes may be different. The melittin molecule has three positively charged regions; amino-terminal, the peptide positions 7, and 21–24. Therefore, when melittin interacts with a negatively charged membrane surface, it may be adsorbed parallel to the membrane surface where these three positions of the melittin molecule bind to the negatively charged sites on the membrane surface. This will result in the hydrophobic portion of melittin having more difficulty associating with the hydrophobic portion of the membrane. While, when interacting with a neutrally charged membrane surface, it may not bind with the membrane electrostatically but be adsorbed in hydrophobic interaction. This will leave the bulky hydrophilic portions of the molecule at the membrane surface (peptide positions above 20) and the other portion may penetrate into the membrane hydrocarbon phase in competition with the charged portion of the molecule at position 7. This scheme is similar to those proposed by others [22,28,31]. This may be the reason why the PC membrane is more susceptible to the melittin effect (membrane leakage, vesicle fusion and micellization) than the PS membrane, in spite of lower adsorption of melittin on the PC membrane surface than the PS membrane. This interpretation is also supported by the monolayer pressure measurements upon melittin interaction with lipid monolayers (Fig. 9).

From these results, it can be predicted that at a certain concentration of melittin (e.g., the ratio of melittin to lipid of 1:100), the increase in the mole fraction of PS molecules in the PC membrane should reduce the effect of melittin on membrane leakage and fusion. Besides less penetration of melittin molecules into the PS membrane, another factor contributing to low suceptibility for membrane leakage and fusion of the PS membrane, may be the electrostatic repulsive forces exerted onto two interacting PS membranes due to their intrinsic negative charges. However, when sufficient melittin molecules are adsorbed onto the PS vesicle membrane reducing the negative charges on surface, hydrophobic forces due to adsorbed melittin

would bring two interacting PS vesicles closer than those for the PC vesicles. These may be the reasons why, once the amount of adsobed melittin exceed a certain threshold value, the extent of the size increase of the PS vesicles is much greater and drastic than that of the PC vesicles. Once two vesicles interact closely with each other through adsorbed melittin molecules, two membranes might be bridged by means of a hydrophobic portion of the melittin molecule and result in fusion. In such a situation, the degree of penetration of hydrophobic peptide into the membrane phase seems to correlate with the degree of fusion.

Membrane disruption at high melittin concentrations may be caused by the membrane instability due to asymmetric adsorption of melittin molecules on one side of the membrane. At the ratio of melittin to lipid greater than 1:10, micellization occurs for the PC membrane. At this concentration of melittin, melittin is adsorbed to the membrane at the ratio of 1 melittin to 50–60 lipids. Both steric and electrical free energy differences on both sides of the membrane could induce the disruption of membranes. The membrane instability may also be caused by a large amount of melittin penetrated into the lipid phase which may cause membrane leakage, thus weakening membrane stability.

Appendix

The surface potential $\psi(0)$ and the surface charge density σ of a membrane surface facing an electrolyte solution are expressed by the following equation:

$$\sigma = \tfrac{1}{273}\left(\sum_i C_i(\infty)\left[\exp\left(\frac{-eZ_i\Psi(0)}{kT} \right) - 1\right]\right)^{1/2} \qquad \text{(A-1)}$$

where the surface charge density σ is expressed in an electronic charge per Å^2; $C_i(\infty)$ the molecular concentration of the i-th ionic species in the bulk phase; Z_i the valency of the i-th ion, k the Boltzmann's constant and $T = 297$ K. The surface potential of the Gouy Chapman diffused layer in an uni-uni valent electrolyte neglecting the contribution of the melittin ions due to its low concentration compared with other ions is expressed as:

$$\psi(x) = \frac{2kT}{e} \ln\left(\frac{1 + \alpha \exp(-\kappa x)}{1 - \alpha \exp(-\kappa x)} \right) \qquad \text{(A-2)}$$

where

$$\alpha = \frac{\exp\left(e\psi(0)/2kT\right) - 1}{\exp\left(e\psi(0)/2kT\right) + 1}$$

and κ is the Debye constant.

The ζ potential is calculated from the measured value of vesicle electrophoretic mobility, u, by the Helmholtz-Smoluchowski equation:

$$u = \epsilon\epsilon_0\zeta/\eta \qquad \text{(A-3)}$$

where η is the viscosity of the aqueous solution and ϵ and ϵ_0 are the permittivities of the aqueous medium and free space (vacuum), respectively. With use of Eqs. (A-1) and (A-2) and the ζ-potential obtained from the measured vesicle electrophoretic mobility using Eq. (A-3), the surface potential $\psi(0)$ and the surface charge density σ can be calculated by choosing the plane of sheer relation to the ζ to be 2.0 Å from the membrane surface.

References

[1] Sowers, E.A. (1987) Cell Fusion, Plenum Press, New York.
[2] Ohki, S., Doyle, D., Flanagn, T.D., Hui, S.W. and Mayhew, E. (1988) Molecular Mechanisms of Membrane Fusion, Plenum Press, New York.
[3] Blumenthal, R. (1987) in Current Topics in Membrane Transport, Vol. 29 (Klausner, R.D. et al., eds.), pp. 203–254, Academic Press, New York.
[4] Hong, K., Duzgunes, N. and Papahadjopoulos, D. (1982) Biophys. J. 37, 297–305.
[5] Creutz, C.E. (1981) J. Cell Biol. 91, 247–256.
[6] Gad, A.E., Silver, B.L. and Eytan, G.D. (1982) Biochim. Biophys. Acta 690, 124–132.
[7] Tilcock, C.P.S. and Fisher, D. (1979) Biochim. Biophys. Acta 577, 53–61.
[8] Knutton, S. (1979) J. Cell. Sci. 36, 61–72.
[9] Gething, M.J., White, J.M. and Waterfield, M.D. (1978) Proc. Natl. Acad. Sci. USA 75, 2737–2740.
[10] Hsu, M.C., Scheid, A. and Choppin, P.W. (1979) Virology 95, 467–491.
[11] Novick, S.L. and Hoekstra, D. (1988) Proc. Natl. Acad. Sci. USA 85, 7433- 7437.
[12] Wharton, S.A., Martin, S.R., Ruigrok, R.W.H., Skehel, J.J. and Wiley, D.C. (1988) J. Gen. Virol. 69, 1847–1857.
[13] Takahashi, S. (1990) Biochemistry, 29, 6257–6264.
[14] Murata, M., Nagayama, K. and Ohnishi, S.-I. (1987) Biochemistry 26, 4056–4602.
[15] Morgan, C.G., Williamson, H., Fuller, S. and Hudson, B. (1983) Biochim. Biophys. Acta 732, 668–674.
[16] Bradrick, T.D. and Georghiou, S. (1987) Biochim. Biophys. Acta 905, 494–498.
[17] Eytan, G.D. and Almary, T. (1983) FEBS Lett. 156, 29–32.
[18] Dufourcq, J., Faucon, J.-F., Fourche, G., Dasseux, J-L., LeMaire, M. and Gulik-Krzywicki, T. (1986) Biochim. Biophys. Acta 859, 33–48.
[19] Sassa, G., Freer, J.H., Colacicco, G. and Weismann, G. (1969) J. Biol. Chem. 244, 3575–3582.
[20] Brown, L., Braun, W., Kumar, A. and Wuthrich, K. (1982) Biophys. J. 37, 319–328.
[21] Dufourc, E.J., Smith, I.C.P. and Dufourcq, J. (1986) Biochemistry 25, 6448–6455.
[22] Stanislawski, B. and Ruterjans, H. (1987) Eur. Biophys. J. 15, 1–12.
[23] Dempsey, C.E. and Watts, A. (1987) Biochemistry 26, 5803–5811.
[24] Batenburg, A.M., Hibbeln, J.C.L. and De Kruijff, B. (1987) Biochim. Biophys. Acta 903, 155–165.

[25] Schulze, J., Mischeck, U., Wigand, S. and Galla, H.-J. (1987) Biochim. Biophys. Acta 901, 101–111.
[26] John, E. and Jahnig, F. (1991) Biophys. J. 60, 319–328.
[27] Faucon, J.F., Dufourcq, J. and Lussan, C. (1979) FEBS Lett. 102, 197–193.
[28] Altenbach, C., Froncisz, W., Hyde, J.S. and Hubbell, W.L. (1989) Biophys. J. 56, 1183–1191.
[29] Brauner, J.W., Mendelsohn, R. and Prendergast, F.G. (1987) Biochemistry 26, 8151–8158
[30] Dempsey, C.E. and Butler, G.S. (1992) Biochemistry 31, 11973–11977.
[31] Frey, S. and Tamm, L.K. (1991) Biophys. J. 60, 922–930.
[32] Schwarz, G. and Beschiaschvili, G. (1989) Biochim. Biophys. Acta 279, 82–90.
[33] Stankowski, S. and Schwarz, G. (1990) Biochim. Biophys. Acta 1025, 164–172.
[34] Kuchinka, E. and Seelig, J. (1989) Biochemistry 28, 4216–4221.
[35] Beschiaschvili, G. and Seelig, J. (1989) Biochemistry 28, 52–58.
[36] Amselem, S., Barenholz, Y., Loyter, A., Nir, S. and Lichtenberg, D. (1986) Biochim. Biophys. Acta 860, 301–313.
[37] Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137.
[38] Ohki, S. (1982) Biochim. Biophys. Acta 689, 1–11.
[39] Ohki, S. (1984) J. Membr. Biol. 77, 265–275.
[40] Allen, R.J.L. (1940) Biochem. J. 34, 858–865.
[41] Ohki, S., Ohki, C.B. and Duzgunes, N. (1976) In Colloid and Interface Science, Vol. 5 (Kerker, M., ed.), pp. 271–284, Academic Press, New York.
[42] Davies, J.T. and Rideal, E.K. (1961) Interfacial Phenomena, Academic Press, New York.
[43] Arnold, K., Hoekstra, D. and Ohki, S. (1992) Biochim. Biophys. Acta 1124, 88–94.
[44] Ohshima, H. and Ohki, S. (1991) J. Colloid Interface Sci. 142, 596–598.
[45] Dempsey, C.E., Bitbol, M. and Watts, A. (1989) Biochemistry 28, 6590–6596.
[46] Tosteson, M.T. and Tosteson, D.C. (1981) Biophys. J. 36, 109–116.
[47] Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswonde, J., Pincus, M.R. and Blumenthal, R. (1982) J. Biol. Chem. 257, 2469–2476.
[48] Tosteson, M.T., Alvarez, O., Hubbell, W., Bieganski, R.M., Altenbach, C., Caporales, L.H., Levy, J.J., Nutt, R.F., Rosenblatt, M. and Tosteson, D.C. (1990) Biophys. J. 58, 1367–1375.
[49] Schwarz, G., Zong, R. and Popescu, T. (1992) Biochim. Biophys. Acta. 1110, 97–104.