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Interaction of melittin derivatives with lipid bilayer membrane. Role of basic residues at the C-terminal and their replacement with lactose

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Melittin possesses an amphiphilic property in the primary sequence in which hydrophilic residues are located at the C-terminal region from Lys-21 to Gln-26. A part of the hydrophilic sequence was cleaved off by endopeptidase Arg-C to obtain melittin 1–22. The affinity of melittin 1–22 for neutral phospholipid membrane was reduced to 1/3 that of melittin, indicating that the basic residues, Lys-23 and Arg-24, are important in binding of melittin to the membrane. The melittin 1–22 was extended toward the C-terminal end by connection of lactose (melittin-lac),the membrane affinity of which was slightly higher than the melittin 1–22, but lower than melittin. The leakage experiment of 5,6-carboxyfluorescein encapsulated in DPPC liposomes showed that the activities of melittin 1–22 and melittin-lac in membrane lysis were much lower than melittin. However, the melittin 1–22 formed a voltage-dependent ion-channel in an azolectin bilayer membrane. It is thus considered that Lys-23 and Arg-24 residues of melittin play an important role in binding to the polar region of membrane for lysis, but not for ion-channel formation.

Introduction

Melittin is a major component of bee venom with 26 amino-acid residues. Melittin displays an amphiphilic property since the primary sequence is divided into the hydrophobic 1–20 segment and the hydrophilic 21–26 segment [1]. Pleiotropic activities of melittin have been found in enhancement of ion permeability through lipid bilayer membrane [2,3], formation of voltage-dependent ion-channels [4,5], lysis [6], fusion of membranes [7] and activation of phospholipase A_2 [8,9].

Conformation and assembly formation of melittin have been studied by using various methods: X-ray diffraction [10], NMR [11], CD [12,13], IR [14–16], fluorescence [17–20], dielectric relaxation [21], etc. It has been shown that Gly-1–Thr-10 and Leu-13–Gln-26 segments take an α-helical structure and axes of two helical segments intersect at Thr-11–Gly-12 with a 120° angle in a solid state. In an aqueous solution, monomeric melittin without taking any specific conformation [23] is in equilibrium with a tetramer taking an α-helical conformation [12]. The latter fraction increases with increasing melittin concentration or ionic strength [22]. Melittin has been reported to be distributed to phospholipid bilayer membrane in the form of tetramer [18,24,26] as well as monomer [19,25]. Different types of molecular orientation of melittin in lipid bilayer membrane have been proposed, wedge-like model [11,20,27] and trans-bilayer model [15,16,28]. In either of them, the hydrophilic 21–26 segment containing two Lys and two Arg residues stays at the membrane surface and acts for anchoring the peptide to the membrane.

Basic residues located at the edge of hydrophobic segment, examples of which are found in signal peptides of secretary proteins, are also reported important in incorporation the hydrophobic sequence into phospholipid bilayer [29]. In addition, it has been pointed out that Arg residues of dynorphin 1–13 increase the membrane affinity of the peptide and promote the perpendicular orientation of the peptide in the membrane [30]. Either effect should be important in relation with the biological activity of the peptides. However, it is controversial whether hydrophilicity, positive charge, or the hydrophobic methylene side chain of the basic residues is essential for the interaction. In the present investigation, two melittin derivatives were prepared; one is melittin 1–22, and the other is melittin-lac (Fig. 1) in which 23–26 segment of melittin is replaced with lactose. The replacement of a hydrophilic charged

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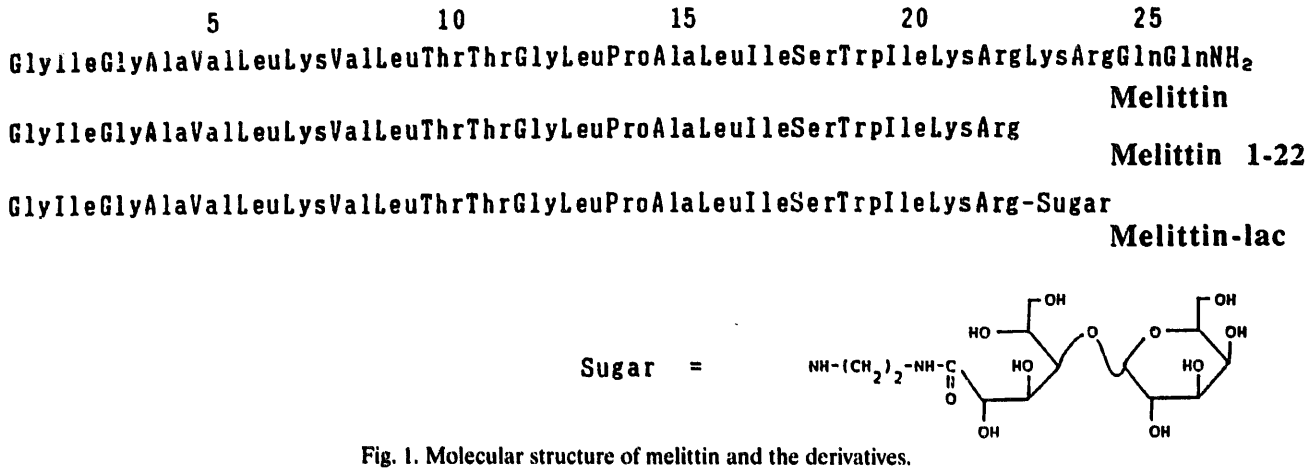

Fig. 1. Molecular structure of melittin and the derivatives.

moiety of the peptide with a hydrophilic lactose retains the hydrophilicity but excludes the charge interaction. These melittin derivatives were synthesized to investigate the role of positive charges at the C-terminal region of melittin in the interaction with phospholipid membrane and to find a guide line for controlling molecular orientation of synthetic peptides in phospholipid membrane.

Materials and Methods

Materials. Melittin (Sigma) and azolectin (Associated Concentrates) were commercially available and purified according to the methods reported by Quay and Condie [31] and Kagawa and Racker [32], respectively. Endopeptidase Arg-C (Sigma), dipalmitoylphosphatidylcholine (DPPC, Sigma), 5,6-carboxyfluorescein (CF, Sigma), and RCA_{60} (Seikagakukougyou) were commercially available and used without further purification.

Synthesis. Melittin 1–22 and melittin-lac were synthesized as follows. For melittin 1–22, Melittin (50 mg) was dissolved in a phosphate buffer (20 mM, pH 8.4, 50 ml) and was treated with endopeptidase Arg-C (20 unit) for 10 h. After removing precipitate by centrifugation, the supernatant was desalted by using a Sephadex G-50 column and lyophilized. The solid product was purified by a Sephadex G-25 column using 20 mM phosphate buffer (pH 7.4) as eluant to exclude melittin uncleaved by the endopeptidase. The major fraction eluted after the native melittin fraction was desalted and lyophilized. The synthesis of melittin 1–22 was confirmed by amino acid analysis. For melittin 1–22 · Msc_3, amino groups of melittin 1–22 were blocked by a 2-(methanesulfonyl)-ethoxycarbonyl (Msc) group by the method similar to that reported by Tesser and Balvert-Geers [33]. Melittin 1–22 (13 mg) was dissolved in water/acetonitrile (1:3, v/v) at 40°C. Methylsulfonylethyl *p*-nitrophenyl carbonate (Msc-ONp, 15 mg) and triethylamine (10 μl) were added and reacted overnight. The product was purified by a Sephadex G-25 column using 20 mM phosphate buffer eluant (pH 7.4). The major fraction was desalted and lyophilized. Yield 5 mg. The synthesis of melittin-lac was started by dispersion of melittin 1–22 · Msc_3 (5 mg) in dimethylformamide, dicyclohexylcarbodiimide (DCC, 3.6 mg) and *N*-hydroxybenzotriazole (2.6 mg) were added at 0°C. Lac-EDA (10 mg), which was prepared by the reaction of lactonolactone and *N*-benzyloxycarbonyl-ethylenediamine-HCl followed by catalytic hydrogenation, was added to the mixture at room temperature and reacted overnight. The solution was poured into a mixed solution of dioxane/methanol/4 M NaOH (14:5:1, v/v/v) to remove the protection groups. After 5 min, the solution was neutralized with 10% citric acid, and condensed to a small volume. The product was purified by a Sephadex G-25 column using 20 mM phosphate buffer eluant (pH 7.4). The major fraction was desalted and lyophilized. Yield 3.3 mg.

Preparation of liposomes. DPPC was dispersed in 10 mM Hepes buffer (pH 7.4), containing 0.1 M NaCl and 0.1 mM EDTA. The dispersion was sonicated at a temperature above the phase-transition temperature of the DPPC bilayerand centrifuged at $100000 \times g$ to obtain small unilamellar vesicles. Lipid concentration was determined by a colorimetric method (Diacolor, Toyobo) using phospholipase D.

Affinity for phospholipid membrane. Melittin, melittin 1–22 and melittin-lac possess Trp residue at the position 19. Upon binding to phospholipid membrane, the fluorescence intensity of the peptides was enhanced and the maximum wavelength of emission shifted to a shorter wavelength. The fluorescence intensity of the peptide in a Hepes buffer was measured with the addition of DPPC liposome at 48°C and analyzed according to the following equation presented by Nicolson and Blalustein [34] and Surewicz and Epand [35].

$$\epsilon - 1 = (\epsilon_b - 1) - (K_d/n) \cdot (\epsilon - 1)/m$$

(ϵ, fluorescence intensity ratio; ϵ_b, fluorescence intensity ratio in the presence of excess lipid; K_d, dissociation constant; n, the number of binding sites in a lipid molecule; m, lipid concentration) Fluorescence measurements were carried on a Hitachi MPF-4 spectrophotometer.

CF leakage. CF-entrapped DPPC liposome was prepared by the method reported by Barbet et al. [36]. A small aliquot of a peptide stock solution was added to the dispersion of CF-entrapped DPPC liposome under stirring at 48°C. The fluorescence intensity at 515 nm was measured. The excitation wavelength was 470 nm.

BLM measurement. A thin Teflon film (0.25 mm) with a hole of 0.2–0.3 mm diameter was clamped between two halves of a Teflon trough. The hole was precoated with hexadecane/hexane (6 : 4 v/v) mixture. The azolectin membrane was formed by the method reported by Montal and Muller [37]. Both water phases contained 1 M KCl. Before the measurement, AC 200 mV (peak-to-peak, 1 kHz) was applied to the membrane for 30 min, which brought virtually solvent-free membranes [38]. The membrane resistance of more than 10 Gohm was confirmed. The peptide was added to both phases.

Results and Discussion

Membrane affinity

Dissociation constants of melittin and the melittin derivatives from DPPC liposome in a liquid-crystalline state are shown in Table I together with the shift values of maximum emission wavelength (δ_{lamda}). Dissociation constants of the melittin 1–22 and the melittin-lac are much higher than that of melittin. The attachment of hydrophilic lactose to the C-terminal of melittin 1–22 cannot restore the high affinity for phospholipid membrane of melittin. This result indicates that positive charges at the C-terminal region of melittin plays an important role in binding to phospholipid membrane. The same conclusion was drawn by Brauner et al. [16] from the ATR-IR measurement that a melittin derivative lacking the hydrophilic C-terminal segment showed lower membrane affinity than melittin.

TABLE I

Binding parameters of peptide to phospholipid membrane

[melittin] = $2.2 \cdot 10^{-6}$ M; [melittin 1–22] = $2.0 \cdot 10^{-6}$ M; [melittin-lac] = $2.2 \cdot 10^{-6}$ M.

Peptide	λ_{max} (nm)		δ_{lamda}	$K_d/n \cdot 10^{-4}$ (M)
	Buffer	DPPC liposome		
Melittin	350	337	13	0.8
Melittin 1–22	351	345	6	2.9
Melittin-lac	351	344	7	2.4

The difference between melittin and the melittin 1–22 in free energies of transfer from an aqueous phase to a phospholipid membrane was calculated. Since CD measurement revealed that melittin takes a helical conformation in TFE, the same conformation may occur when incorporated into phospholipid membrane. Most of amino acid residues of the melittin 1–20, in particular, the residues 13–20 are hydrophobic. When the 13–20 residues of melittin 1–22 are incorporated into phospholipid membrane, hydrophilic Lys-21 and Arg-22 residues at the C-terminal region are necessarily brought into the membrane, because a transfer of these residues from an aqueous phase into the membrane gains −35 kJ/mol due to hydrophobic interactions [39]. In the case of melittin, Lys-23 and Arg-24 residues can be also brought in the membrane under the situation that the positive charges in the side-chains remain in aqueous phase. This state is rationalized under the condition that Lys and Arg residues are located within the third and fourth position, respectively, from the end of peptide segment incorporated in the membrane [39]. Consequently, the membrane affinity of melittin should be higher than the melittin 1–22 by −35 kJ/mol, but in experiment the difference was evaluated to be −9 kJ/mol (Table I). The marginal prevalence of melittin over the melittin 1–22 in the membrane affinity might be explained by considering a total incorporation of Lys-23 and Arg-24 of melittin into the membrane. The difference between the calculation and the experiment may be ascribed to ambiguous properties of interphase between bulk-water and the hydrocarbon layer of lipid membrane, where electric charges interacting with phosphate groups hinder the evaluation of hydrophobic interactions.

Although the hydrophobicity of the melittin 1–22 should be strongly reduced by introduction of lactose, melittin-lac has a slightly higher membrane affinity than the melittin 1–22. The higher affinity might be ascribed to a strengthened amphiphilicity in the primary sequence of melittin-lac. It has been proposed that the primary amphiphilicity as well as the secondary amphiphilicity which appears upon taking a secondary structure, influences interaction of peptide with phospholipid membrane [40].

Location of glycopeptide

Melittin-lac is expected to be distributed to the phospholipid membrane in a state that the hydrophobic peptide segment is buried into the hydrophobic region of the membrane and the hydrophilic lactose segment is exposed to the aqueous phase. In order to get to know the orientation of the glycopeptide in phospholipid membrane, the locations of the lactose segment and the Try residue of the glycopeptide in the membrane were examined in the following way.

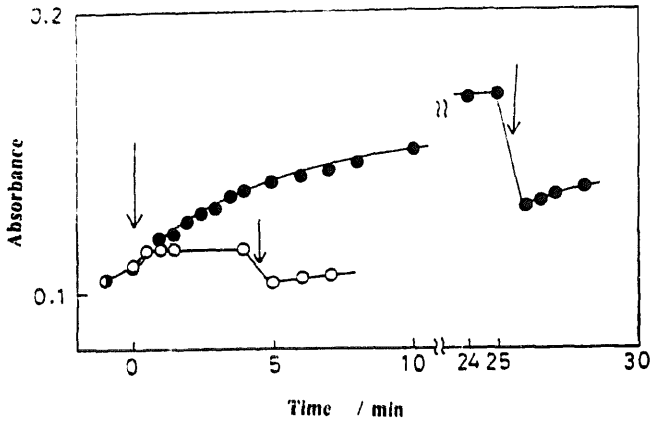

Fig. 2. Turbidity change of DPPC liposome with the addition of RCA_{60}. ○, without melittin-lac; ●, in the presence of melittin-lac (1.8 μM). The lectin was added at the first arrow and lactose (1.5 mM) at the second arrow. The turbidity change was monitored by absorbance at 400 nm. [DPPC] = 1.4 mM; [RCA_{60}] = 1.6 μM.

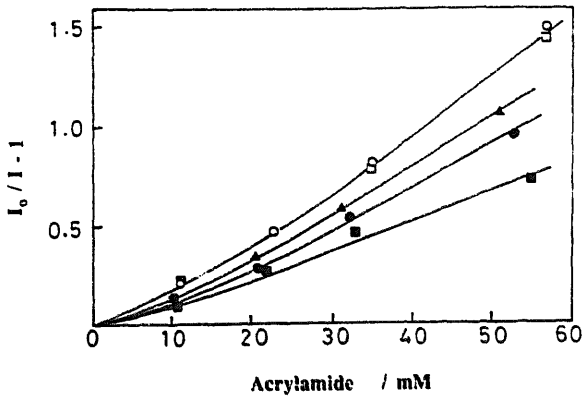

Fig. 3. Quenching of Try-19 of the melittin derivatives by acrylamide (Stern-Volmer plot). ■, [melittin] = 2.2 μM and [DPPC] = 1.2 mM; ▲, [melittin 1–22] = 1.6 μM and [DPPC] = 2.3 mM; ●, [melittin-lac] = 2.3 μM and [DPPC] = 1.8 mM; □, [melittin] = 2.2 μM, ○, [melittin-lac] = 2.3 μM.

RCA_{60} is a lectin which specifically binds to α-D-galactose. The addition of RCA_{60} to melittin-lac in a DPPC liposome increased the absorbance at 400 nm (Fig. 2). Since such a large increase in the absorbance was not observed in the absence of DPPC liposome, it means that RCA_{60} induced the aggregation of liposomes by binding to the lactose units of melittin-lac molecules which were distributed to the lipid membrane having the lactose unit held at the membrane surface.

Aggregation of liposome was partially dissociated by the addition of lactose, which competitively inhibits binding of RCA_{60} to melittin-lac. The interaction of RCA_{60} with melittin-lac should be reversible, but non-specific interactions of RCA_{60} with liposome also take place to induce aggregation.

Depth of the Try-19 residue of the melittin derivatives in the membrane was evaluated from a quenching experiment with a water-soluble quencher, acrylamide. Stern-Volmer plot of the quenching experiment is shown in Fig. 3. Melittin and melittin-lac in a buffer solution were similarly quenched by acrylamide. In the presence of DPPC liposomes, the quenching rate decreased in the order of melittin 1–22 > melittin-lac > melittin. The result indicates that the location of Try-19 in the membrane changes according to the nature of the C-terminal region of the peptide. Interestingly, the location of the Try residue of melittin in the membrane is deepest among the melittin derivatives. This result agrees with the result of the shift of the fluorescence maximum wavelength as shown in Table I, which shows that the largest shift upon binding to the lipid membrane is observed with melittin.

The higher membrane affinity and the deep invasion into the membrane of the Try-19 residue of melittin are explained as follows. The basic residues of melittin are incorporated into the membrane as described in the previous section. The C-terminal part takes an α-helical structure with a perpendicular orientation to the membrane surface due to a large amphiphilic dipole moment [41]. All of the charged groups, Lys-21, Arg-22, Lys-23 and Arg-24, interact with phosphate groups of phospholipid, thereby the positive charges are neutralized. Hence, the Try-19 residue of melittin is located inside the membrane with a distance of 9 Å from the polar region of the membrane. On the other hand, the Try-19 residue of melittin 1–22 resides close to the C-terminal Arg-22 residue and, hence, to the membrane surface than that of melittin. The lactose residue of melittin-lac stays in the aqueous phase and may not influence the interaction of the peptide segment with phospholipid membrane.

Membrane perturbation

The influence of the melittin derivatives on the membrane structure was studied by using CF-encapsulated liposomes (Fig. 4). Melittin induced a remarkable CF leakage from the liposomes upon binding to phospholipid membrane. On the other hand, melittin-lac

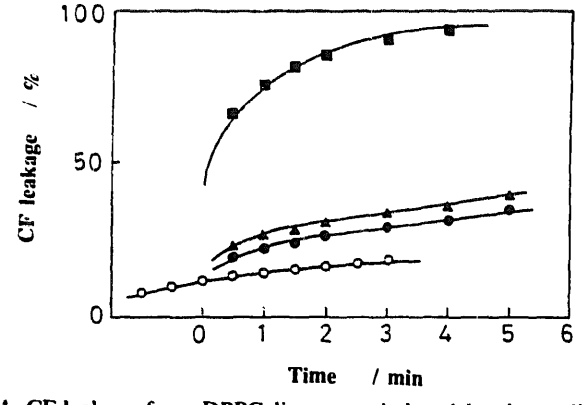

Fig. 4. CF-leakage from DPPC liposomes induced by the melittin derivatives. ■, [melittin] = 0.027 μM; ▲, [melittin 1–22] = 0.027 μM; ●, [melittin-lac] = 0.026 μM. [DPPC] = 27 μM. ○, without additives.

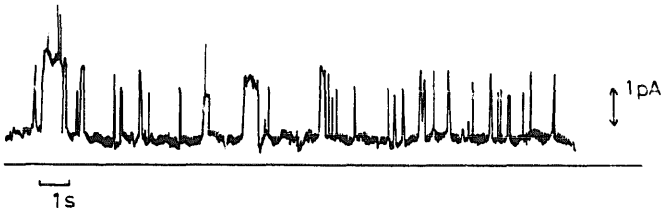

Fig. 5. Single-channel current fluctuation at constant applied voltage (60 mV) in planar bilayers modified by melittin 1–22 (1 nM).

and melittin 1–22 showed a lower degree of CF leakage than melittin, indicating that the basic residues at the C-terminal region of melittin are essential in lysis of phospholipid membrane. The result is consistent with the report by Schroder et al. that melittin 1–20 does not have a lysing activity on red blood cells [42].

The extent of CF leakage was lower with melittin-lac than with melittin 1–22, though melittin-lac possesses a higher membrane affinity than melittin 1–22. It has been pointed out that carbohydrates stabilize the membrane structure by covering the liposome surface [43]. The lactose fragment of melittin-lac might also stabilize phospholipid membrane by interacting with polar groups of the phospholipid membrane.

BLM measurement

Melittin has been reported to form a voltage-dependent ion-channel in BLM [4,5]. However, the relation of channel formation with hemolysis and phospholipase A_2 activation by melittin is unknown. Channel formation is explained in terms of a tetramer model [18], in which four melittin molecules associate with each other taking a trans-bilayer parallel orientation. In this model, the hydrophilic residues at the C-terminal region are not directly involved in the channel formation. Current-voltage response of BLM was examined in the presence of melittin 1–22 (Fig. 5). The stepwise current response against electric field should be ascribed to a single channel formation of melittin 1–22 in BLM, indicating that the hydrophilic residues at the C-terminal region of melittin are not indispensable for ion-channel formation. Since the lysis activity of melittin 1–22 is very low, the ion-channel formation is not directly related to the lysis activity.

Two succesive basic residues neighboring of a hydrophobic segment are considered to help the incorporation of the segment into the membrane and promote a perpendicular orientation of the peptide, which results in formation of an ion channel in the case of melittin. This finding is consistent with the binding mechanism of signal peptides to phospholipid membrane proposed by Inouye and Halegoua [29]. However, continuous four basic residues increase the membrane affinity of the neighboring segment, which is liable to cause membrane instability.

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