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Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles

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Effects of magainin 1, a novel antimicrobial peptide, on the permeability of lipid vesicles were investigated by using calcein as a trapped fluorescent marker. Magainin 1 induces the leakage of calcein specifically out of negatively-charged vesicles. The peptide binds to bovine brain phosphatidylserine sonicated vesicles according to the Langmuir isotherm with a binding constant of $3.8 \cdot 10^5$ M^{-1} and a binding-site number of 0.10 per lipid molecule. The leakage seems to occur at a critical binding number of approx. 0.03 per lipid molecule. A circular dichroism study revealed that magainin 1 conforms mainly to an unordered structure both in an aqueous solution and in the presence of egg yolk phosphatidylcholine vesicles, whereas to an amphiphilic helix with the phosphatidylserine vesicles. In conclusion, magainin 1 interacts with acidic lipids through electrostatic interactions followed by hydrophobic interactions to form an amphiphilic helix, inducing the leakage.

Introduction

Lipid-peptide interactions have been extensively studied for elucidating the action mechanisms of bio-active peptides and for understanding the organization principles of biomembranes and lipoproteins composed of lipids and proteins. Recently, intriguing antimicrobial peptides, magainin 1 and 2, consisting of 23 amino acid residues were discovered from *Xenopus* skin [1,2]. The truly novel sequences of the peptides contain 6 or 7 regularly-arranged charged residues alternating with 2-4 consecutive hydrophobic ones: Gly-Ile-Gly-Lys-Phe^5-Leu-His-Ser-Ala-Gly^{10} (for magainin 1, Lys^{10} for 2)-Lys-Phe-Gly-Lys-Ala^{15}-Phe-Val-Gly-Glu-Ile^{20}-Met-Lys (for 1, Asn for 2)-Ser. These primary structures suggest the preference of an amphiphilic helical conformation and resultant surface activity [1], therefore membrane affinity. Indeed, a two-dimensional NMR study [3] and a circular dichroism (CD) study [4] revealed that the peptides adopt α-helical structures in a mixture of trifluoroethanol and water. Furthermore, analog peptides with high helical contents were found to exhibit increased antimicrobial activities [4]. The positive charges of the peptides also seem to be important for the activities [4]. These findings suggest that the peptides will fold in amphiphilic helices to interact preferentially with acidic lipids. Researches of the prospective interactions with lipid membranes may give us a clue to the veiled action mechanisms because the peptides have been suggested to perturb membrane functions responsible for osmotic balance in protozoas [1].

Thus we synthesized magainin 1 by *t*-butoxycarbonyl (Boc) based solid phase synthesis and investigated effects of the peptide on the permeability of charged and uncharged lipid bilayers. Additionally, we examined conformational changes of the peptide upon binding to the bilayers by using CD spectra. We will discuss the specific interactions of magainin 1 with negatively-charged lipids.

Materials and Methods

Materials. Magainin 1 was synthesized by *t*-Boc based solid phase synthesis by using an Applied Biosystems model 430A automated peptide synthesizer. The peptide was deprotected and cleaved from the resin by 1 M trimethylsilyl trifluoromethanesulfonate-thioanisole in trifluoroacetic acid [5] in the presence of 1,2-ethane-

Abbreviations: CD, circular dichroism; Boc, butoxycarbonyl; PC, egg yolk L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine; PA, L-α-dipalmitoylphosphatidic acid sodium salt; DCP, dicetyl phosphate; SA, stearylamine.

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dithiol and *m*-cresol at 0 °C for 2 h. The deprotected peptide thus obtained was purified by gel-filtration on Sephadex G-15, followed by HPLC on a Cosmosil $5C_{18}$ (yield: 71% from Boc-Ser (benzyl)-phenylacetamidmethyl-resin). The purity of synthetic magainin 1 was ascertained by amino acid analysis, after leucine aminopeptidase (Sigma, L-6007) digestion and analytical HPLC on a Cosmosil $5C_{18}$ST (retention time 52 min, on gradient elution with acetonitril (10–40%, 60 min) in aqueous trifluoroacetic acid (0.1%)). Amino acid ratios in the leucine aminopeptidase digest were: Ser, 2.00 (2); Glu, 1.06 (1); Gly, 4.88 (5); Ala, 2.14 (2); val, 1.07 (1); Met, 0.92 (1); Ile, 1.98 (2); Leu, 0.98 (1); Phe, 2.91 (3); Lys, 3.86 (4); His, 0.91 (1) (recovery of Ser 96%).

Egg yolk L-α-phosphatidylcholine (PC), bovine brain L-α-phosphatidyl-L-serine (PS), L-α-dipalmitoylphosphatidic acid sodium salt (PA), dicetyl phosphate (DCP), and stearylamine (SA) were purchased from Sigma Chemical Co. Calcein was obtained from Dojin (Kumamoto, Japan).

Preparation of vesicles. Ailquots of a lipid solution in chloroform/methanol were placed in a round-bottom 20-ml flask. After the evaporation of the solvent, the residual film was dried under vacuum over night. The lipid film was hydrated with a 70 mM calcein solution (pH 7.0). The suspension was vortexed, followed by sonication in ice water with nitrogen bubbling for 20 min by using a titanium tip sonifier (Tomy UD-200). The lipid concentration was 5 mM. Untrapped calcein was removed by gel filtration (Sephadex G-50, 2 cm × 35 cm column, 10 mM Tris/150 mM NaCl buffer (pH 7.0) as an eluent). The separated vesicular fraction was appropriately diluted with the buffer and then mixed with calcein-free vesicles for obtaining a desired lipid concentration. The lipid concentration was determined by phosphorus analysis [6].

Leakage measurement. The vesicular suspension was mixed with a magainin 1 buffer solution in a cuvette. The leakage of calcein out of the vesicles was monitored by measuring fluorescence intensity at 520 nm (excitation at 490 nm) on a Jasco FP-550 spectrofluorimeter [7]. The fluorescence intensity corresponding to 100% leakage was determined by adding a 10 v/v% Triton X-100 solution (0.1 ml) to 3 ml of the sample. The experiments were carried out at 30 °C.

CD measurements. The CD spectra of a 25 μM magainin 1 buffer solution in the absence and the presence of the sonicated vesicles (443 μM PC or 425 μM PS) were recorded on a Jasco J-600 instrument with a microcomputer (NEC PC-9801). A quartz cuvette of 2 mm-path length was thermostatted at 30 ± 0.5 °C. From the repeatedly-scanned (eight times) spectra, the corresponding blank spectra of the buffer or the vesicle suspension were subtracted. The instrumental outputs were calibrated by using non-hygroscopic ammonium *d*-camphor 10-sulfonate [8]. The contents of helix-, beta-, turn-, and unordered-structures were estimated by using a constrained least-square calculation [9] with a set of reference spectra by Chang et al. (a mean helical length of 10 residues was assumed.) [10].

Results

Leakage

Fig. 1 shows effects of lipid charge on the leakage rate. Addition of 50 μM magainin 1 to the PC vesicles containing 10 mol% of acidic lipids (PS, PA, and DCP) leads to the leakage of entrapped calcein. A more significant leakage was observed in the case of the PS vesicles at a peptide concentration of 15 μM. On the contrary, the neutral (PC) and the positive (PC/SA = 9 : 1) vesicles leak little calcein on addition of magainin 1 at a higher concentration (100 μM). The peptide contains four Lys, one His, and one Glu residues, bearing a net positive charge at pH 7.0. Accordingly, the peptide–lipid electrostatic interactions will play an important role in the observed permeability change of the lipid bilayers.

We further investigated magainin 1-induced leakage using the PS vesicles to evaluate (1) the affinity of the peptide to the vesicles and (2) the amount of membrane-bound magainin 1 necessary for the leakage. These factors can be estimated by analyzing both the peptide- and the lipid-concentration dependency of the leakage rate [11,12]. Fig. 2a shows dose-response curves for the leakage rate (expressed as % leakage for initial 1 min) at different lipid concentrations. A rise in the lipid concentration shifts the curve to the right, suggesting that the binding of magainin 1 to the vesicles is involved in the leakage process. Here we assume that the leakage rate is determined only by the amount of the membrane-bound peptide per lipid (r). The amount r can be

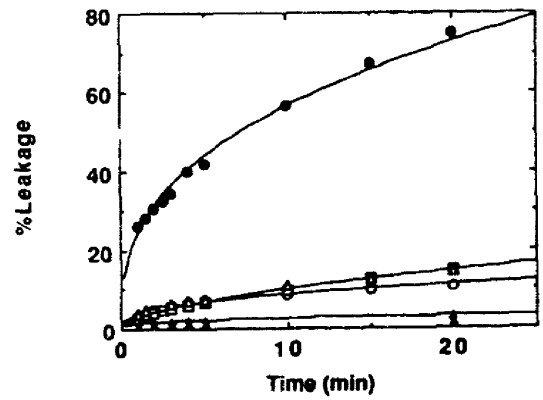

Fig. 1. Effects of lipid charge on the leakage of entrapped calcein out of sonicated vesicles. Percent leakage is plotted as a function of time. Magainin 1 concentration: 100 μM for PC (×) and PC/SA = 9 : 1 (+) vesicles; 50 μM for PC/PS = 9 : 1 (○), PC/PA = 9 : 1 (□), and PC/DCP = 9 : 1 (△) vesicles; 15 μM for PS (●) vesicles. The lipid concentrations are 200–220 μM.

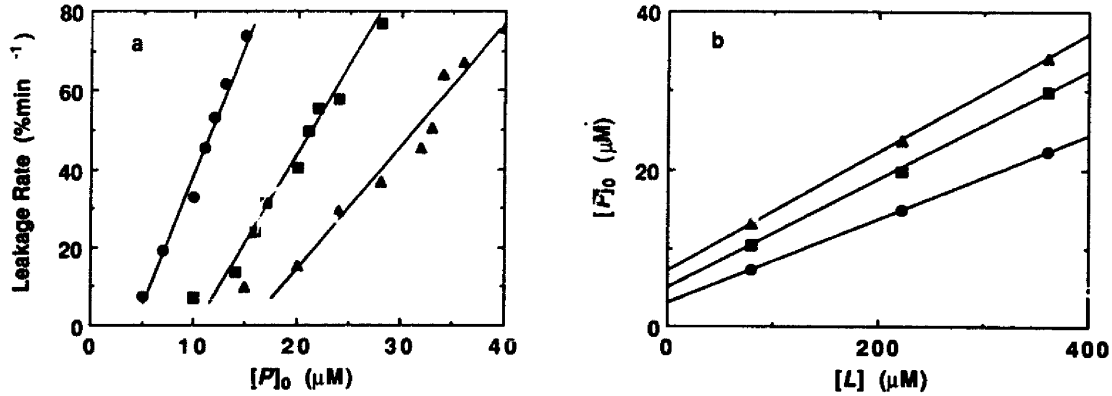

Fig. 2. (a) Dose-response curves for the magainin 1-induced leakage of entrapped calcein out of PS sonicated vesicles. The leakage rate expressed as % leakage for initial 1 min is shown as a function of the peptide concentration, $[P]_o$, at various lipid concentrations. The lipid concentration: ●, 79 μM; ■, 223 μM; ▲, 361 μM. (b) Determination of free and membrane-bound magainin 1 concentrations at several leakage rates. The leakage rate: ●, 20% · min^{-1}; ■, 40% · min^{-1}; ▲, 60% · min^{-1}.

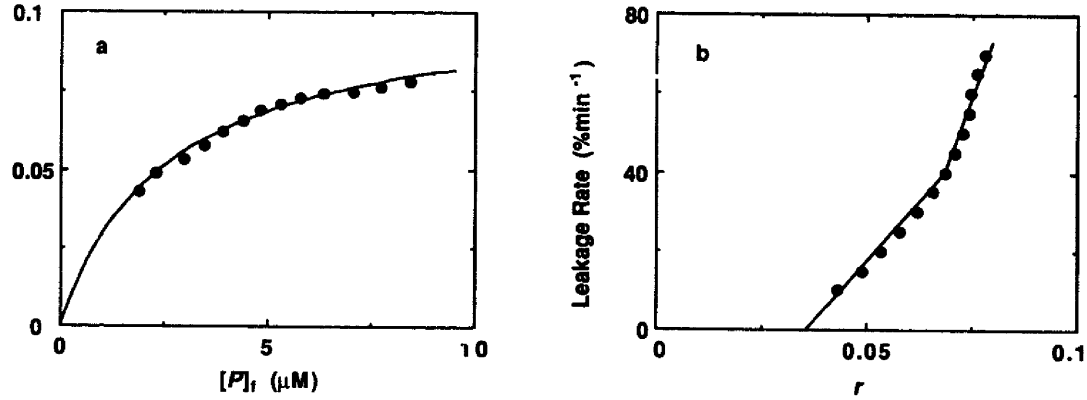

Fig. 3. (a) Binding isotherm of magainin 1 to PS sonicated vesicles at 30 °C. The solid line is a curve fit according to the Langmuir isotherm with a binding constant of $3.8 \cdot 10^5$ M^{-1} and a maximum site number of 0.10 per lipid molecule. (b) Relationship between the leakage rate and the amount of PS-bound magainin 1.

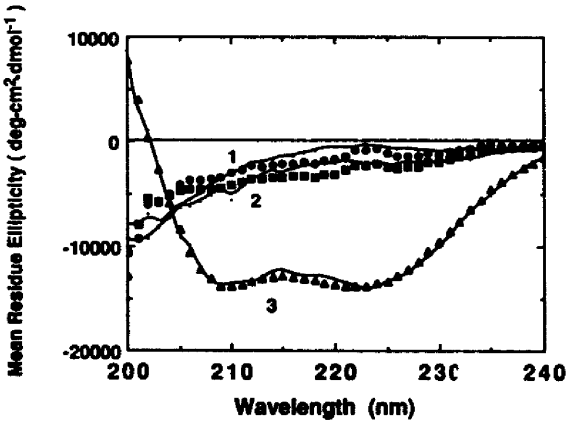

Fig. 4. CD spectra of magainin 1 in the absence and the presence of lipid vesicles. The peptide concentration was 25 μM. Observed spectra: curve 1, in the buffer; curve 2, in a 443 μM PC vesicle suspension; curve 3, in a 425 μM PS vesicle suspension. Calculated spectra are shown by ●, ■, and ▲, respectively (see Table I).

connected with the experimental conditions (the total peptide concentration, $[P]_o$ and the lipid concentration, [L]) through a material balance equation:

$$[P]_o = [P]_f + r[L]$$

where $[P]_f$ stands for the free peptide concentration. The r value and the corresponding $[P]_f$ value can be thus estimated with three sets of $[P]_o$ and [L], obtained from Fig. 2a, at which a given leakage rate is observed. A $[P]_o$ versus [L] plot at any given rate will give a linear relation from which we can estimate $[P]_f$ and r, as Fig. 2b shows. A binding isotherm from the evaluated $[P]_f$ and r values is illustrated in Fig. 3a (closed circles). The isotherm can be well explained on the basis of the Langmuir equation [13] by using an r_∞ (number of binding site per lipid) value of 0.10 and a K (binding constant) value of $3.8 \cdot 10^5$ M^{-1} (the solid line in Fig. 3a). Fig. 3b depicts the relationship between the leakage

TABLE I

Estimated secondary structures of magainin 1

The percent contents of each secondary structure were calculated from the observed CD spectra (Fig. 4) by a constrained least-squares analysis [9].

Condition	% Content			
	helix	beta	turn	unordered
In the buffer	0.0	17.5	31.5	51.0
In a 443 μM PC vesicle suspension	4.5	20.0	28.0	47.5
In a 425 μM PS vesicle suspension	50.5	0.0	21.5	28.0
PS vesicle-bound [a]	56.6	−2.1	20.3	25.2

[a] Calculated by using the binding isotherm (Fig. 3a).

rate and r, suggesting the existence of a critical r value for the leakage around 0.03.

Secondary structures

Fig. 4 illustrates the CD spectra of magainin 1 in the absence and the presence of the lipid vesicles. The contents of each secondary structure calculated by a constrained least-square analysis [9] are summarized in Table I. In the buffer, magainin 1 conforms mainly to an unordered structure with a 0% helical content, coinciding with the previous report [4]. No concentration dependency was observed in the peptide concentration range 5 to 50 μM. In the presence of the neutral PC vesicles, only a slight spectral change was detected with a little modification of the evaluated secondary structures. There are two possibilities: the peptide does not interact strongly or it does with its conformation unaltered. In contrast to the above cases, the presence of the acidic PS bilayers induces a significant change exhibiting a helical folding. The binding isotherm (Fig. 3a) enables us to calculate the secondary structure of the PS-bound peptide. The experimental condition in Fig. 4 corresponds to a bound percentage of 89%. Thus, the bound magainin 1 has an enhanced helical content of 57% at the expense of the reduced fractions of the other conformations (Table I).

Discussion

Magainin 1, consisting of both hydrophilic and hydrophobic residues, is amphiphilic, especially when the peptide folds in an α-helical conformation. In fact, our preliminary experiment suggests that the surface pressure of a foamy magainin 1 solution decreases at a concentration as low as several μM. The peptide, however, does not induce the leakage out of the PC vesicles. Many amphiphilic peptides interacting with lipid membranes tend to conform to helical structures [14–16]. Electrostatic repulsions between the closely-spaced positively-charged residues of magainin 1 will inhibit the helical formation. The CD data also support the weak peptide–PC interactions. On the contrary the peptide binding to acidic lipids, which is enhanced by preferential Coulomb interactions, leads to the charge neutralization, promoting the helical folding. The hydrophobic face formed on the amphiphilic helix can then interact with the acyl chains of the lipids through hydrophobic interactions. Such an acidic lipid-specific helical formation has been reported in the case of basic hormonal peptides, calcitonins [15]. An acidic synthetic peptide designed to form an amphiphilic helix has been shown to interact with PC bilayers only under charge-neutralizing conditions by reducing medium pH [16]. Our CD study confirms the negatively-charged lipid specific helical folding of magainin 1. Chen et al. synthesized several magainin analogs to clarify the structure-activity relationship [4]. The antimicrobial activity was found to be closely related to the helical content in a hydrophobic media, i.e. a mixture of trifluoroethanol and water. Our acidic-lipid vesicles seem to be more appropriate system for evaluating the helix-forming ability because of the suggested important role of the peptide–membrane interactions in the bio-activities [1].

We investigated the peptide-induced leakage out of the PS vesicles more quantitatively. The peptide is expected to change the permeability of the lipid membranes in two steps, i.e., the binding to the membrane and the following membrane perturbation. Accordingly, two determinants for the leakage are (1) the membrane affinity of the peptide and (2) its membrane-perturbing activity. The latter can be expressed as the amount of membrane-bound peptide necessary for the permeability change. We estimated the two factors by the indirect method [11,12]. This method has two advantages over the direct binding experiments: First, the troublesome bound/free separation procedure is omissible. Second, the binding isotherm can be directly correlated with the leakage rate. The binding isotherm (Fig. 3a) shows the saturation of r at the higher $[P]_f$ region. This implies that membrane-bound magainin 1 is present mainly as a monomer on the assumption that the monomeric peptide is the only species in the aqueous phase. The concentration independence of the CD spectra of magainin 1 buffer solutions is compatible with this assumption. The isotherm can be explained on the basis of the Langmuir equation [13], as in the case of salmon calcitonin-dimyristoylphosphatidylglycerol system [15]. The obtained K ($3.8 \cdot 10^5$ M^{-1}) and r_∞ (0.10) values are comparable with those of the above calcitonin system ($K = 1 \cdot 10^5$ M^{-1} and $r_\infty = 0.2$). The r_∞ value of 0.10 corresponds to an occupied area of approx. 700 $Å^2$ per bound magainin 1 molecule when the intramembrane uniform distribution of the peptide is supposed. A 30 Å-long helix bound to the membrane with the helical

axis parallel to the membrane surface would occupy a circular area of $15^2\pi$ (approx. 700) Å^2, coinciding with our result. However, the conformation and the orientation of the peptide in the lipid membranes should be further investigated to clarify what the r_∞ value means.

Fig. 3b demonstrates that the leakage occurs in the r range 0.04 to 0.08. This range, therefore the membrane-perturbing activity, is comparable with surfactant-induced leakage out of PC reversed-phase evaporation vesicles (Triton X-100, approx. 0.05; sodium cholate, approx. 0.1) [17]. The antimicrobial and protozoa-lysing effects may be understood on the basis of this fundamental leakage phenomenon. The leakage mechanism is veiled at the present stage. It may be the peptide-induced formation of lipid patches that produce leaky regions at the interfaces with surrounding lipids, or may be the partial solubilization of lipids by the peptide. Although our binding isotherm fails to suggest the extensive intramembrane peptide aggregation, a transmembrane channel mechanism [3] may not be ruled out because the large number of the bound peptide per vesicle (approx. 100–300) permits the coexistence of some aggregates which does not affect the shape of the isotherm.

We have demonstrated that magainin 1 folds in an amphiphilic helix to interact specifically with the acidic lipid bilayers, resulting in the permeability change. The vesicles of dipalmitoylphosphatidylglycerol, which is abundant in bacterial membranes, were found to have comparable susceptibility to magainin 1 (Matsuzaki, K., et al., unpublished work). We will further investigate magainin 1–lipid interactions by various spectroscopic and thermal techniques to reveal the whole features of the interactions, including the leakage mechanism.

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