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Parallel and Antiparallel Dimers of Magainin 2: Their Interaction with Phospholipid Membrane and Antibacterial Activity

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Abstract: Magainin 2 (M2) forms pores by associating with several other M2 molecules in lipid membranes and shows antibacterial activity. To examine the effect of M2 dimerization on biological activity and membrane interaction, parallel and antiparallel M2 dimers were prepared from two monomeric precursors. Antibacterial and haemolytic activities were enhanced by dimerization. CD measurements showed that both dimers and monomers have an α-helical structure in the presence of lipid vesicles. Tryptophan fluorescence shift and KI quenching studies showed that all the peptides were more deeply embedded in acidic liposomes than in neutral liposomes. Experiments on dye-leakage activity and membrane translocation of peptides suggest that dimers and monomers form pores through lipid membranes, although the pore formation may be accompanied by membrane disturbance. Although dimerization of M2 increased the interaction activity with lipid membranes, no appreciable difference between the activities of parallel and antiparallel M2 dimers was observed.

Keywords: magainin 2; parallel and antiparallel dimers; antibacterial activity; circular dichroism; membrane permeability

INTRODUCTION

In nature, there are a variety of amphiphilic α-helical peptides, many of which have antibiotic, self-defence, toxic and hormonal activities [1,2]. Structural studies have clarified the essential factors for activity and the functional mechanisms. The properties and behaviour of these peptides are generally governed by their hydrophobicity, helicity and amphiphilicity and are also influenced by the environment [3,4].

Magainin 2 (M2) is a cationic amphiphilic peptide in the skin of the African clawed frog (*Xenopus laevis*) that has antibacterial activity [5]. M2 can recognize specific biomembranes because it has antibacterial activity with a broad spectrum but shows little haemolysis against mammalian erythrocytes. Several M2s associate to form pores in bacterial cell membranes; the pores increase membrane permeability which then kills the cells [6,7]. M2 does not appreciably interact with neutral phospholipid but strongly interacts with acidic phospholipid [6]. Syntheses and properties of some parallel dimers and antiparallel dimers of the M2-related peptides have been reported [8–11]. An antiparallel disulphide-dimerized M2 analogue was found to form pores that lasted longer and that were slightly larger than those formed by the corresponding monomer [9]. Parallel heterodimers made up of M2 and PGLa analogues exhibited an order

Abbreviations: CD, circular dichroism; DNS-PE, *N*-dansyl-DL-3-phosphatidylethanolamine; DOPC, dioleoyl-DL-3-phosphatidylcholine; DOPG, dioleoyl-DL-3-phosphatidylglycerol; FITC, fluorescein isothiocyanate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MIC, minimum inhibitory concentration; MLV, multilamellar vesicle; M2, magainin 2; NBD-PE, *N*-(7-nitro-benz-2-oxa-1,3-diazole-4-yl)dipalmitoyl-3-phosphatidylethanolamine; RP-HPLC, reversed phase high-performance liquid chromatography; SUV, small unilamellar vesicle; TFE, 2,2,2-trifluoroethanol; TSB, tryptic soy broth.

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of magnitude higher membrane permeabilization than that of the monomeric species, although their biological activity was not examined [10]. A parallel M2 dimer containing β-cyclodextrin exhibited membrane permeability 10 times higher than a monomer-type derivative [11]. In this connection, an alamethicin dimer connected with a long linker was also found to form more stable channels than alamethicin itself [12]. In this study, to examine whether there are any differences between the properties of parallel and antiparallel dimers, we synthesized two M2 analogues, M2-C and M2-N, which are linked by a βAla-Cys bond at the *C*-terminus and a Cys-βAla bond at the *N*-terminus, respectively. Then, we prepared a parallel M2 dimer (M2-CC) from M2-C and an antiparallel M2 dimer (M2-CN) from M2-C and M2-N, and investigated their properties and antibacterial activities.

MATERIALS AND METHODS

Peptide Synthesis

M2-C and M2-N were synthesized by the solid-phase method using Fmoc-amino acids and 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy resin (Rink Amide resin). The products were purified by gel chromatography on a Sephadex G-10 column followed by RP-HPLC on a YMC-Pack ODS-AC18 column. M2-CC was prepared by air oxidation of M2-C in 0.1 M NH_4HCO_3 (pH 8.0) for 3 h at 25 °C. M2-CN was obtained by the S-S exchange reaction between M2-N and M2-C, which was activated with 2.2′-dithiodipyridine in water for 10 min at 25 °C. The dimers were also purified by gel chromatography followed by RP-HPLC. The purity of the final products was evaluated by analytical RP-HPLC and MALDI-TOF-MS, and was found to exceed 95%.

Antibacterial and Haemolytic Activity

The serial solution dilution method was used to determine the MIC values as described by Yoshida *et al.* [13]. The cell suspension was diluted with tryptic soy broth (TSB) medium (pH 7.4) to 10^4 cells/ml. Several concentrations of the peptide solution were placed in test tubes, made up to 20 µl with the medium and 180 µl of cell suspension was added. After incubation for 24 h at 37 °C, absorbance at 620 nm was measured. Haemolytic activity was assayed by the method of Yoshida *et al.* [13]. TSB medium (0.5 ml) was added to 0.5 ml of fresh rabbit blood. The resulting mixture was centrifuged at 2000 rpm for 3 min, and the precipitates were collected. After being washed with TSB medium three times, the obtained precipitates were suspended in 2 ml TSB solution. TSB medium (1 ml) was added to the rabbit erythrocyte solution (5 µl) followed by the peptides. The resulting suspension was incubated for 20 min at 37 °C, and then centrifuged. The supernatant was monitored at 413 nm. To measure the absorbance for 100% activity, 10% Triton X-100 (10 µl) instead of peptide was added to the erythrocyte solution.

Preparation of Phospholipid Vesicles

SUVs of DOPC and DOPC/DOPG (3 : 1) were prepared for the CD and spectrophotometric measurements. Phospholipid (5 µmol) was dissolved in $CHCl_3$/MeOH (2 : 1 v/v, 0.4 ml), then dried under a stream of N_2 gas. The dried lipid was hydrated in 20 mM Tris HCl buffer (pH 7.4, 5 ml) using a Branson bath-type sonicator. The suspension was sonicated for 20 min at 50 °C using a Titech Ultrasonic Processor Model VP-5T at an intensity of 10 W. The vesicles were allowed to stand for 30 min at 25 °C before the measurements were made. The lipid concentration was 1 mM Calcein-entrapped vesicles were similarly prepared. The dried lipid (27 µmol) was hydrated in 20 mM Tris HCl buffer (pH 7.4, 2.0 ml) containing 0.1 M calcein. The calcein-entrapped vesicles were separated from the free calcein by gel filtration using Sephadex G-75 (1 × 22 cm) with the same buffer (pH 7.4).

Circular Dichroism Measurements

CD spectra were recorded on a Jasco J-720 W spectropolarimeter with a thermostatted cell holder using a quartz cell of 1.0 mm path length. The peptides were dissolved in the buffer (pH 7.4), TFE and buffer containing 1 mM phospholipid vesicles. The concentrations of the monomer and dimer peptides were 20 µM and 10 µM, respectively. Measurements were done at 25 °C. The mean residue ellipticity is given in deg cm^2 $dmol^{-1}$ and the helical contents were calculated according to the method of Scholtz *et al.* [14].

Fluorescence Spectroscopy

The fluorescence values of Trp in the peptides were measured in Tris HCl buffer (pH 7.4) and in a dispersion of the vesicles. The concentrations

of the monomer and dimer peptides were 10 μM and 5 μM, respectively, and the ratio of lipid to peptide was varied from 0 to 40. The samples were excited at 280 nm and emission spectra were recorded from 300 to 400 nm on a Hitachi F-3010 spectrofluorometer at 25 °C. Fluorescence emission was expressed in arbitrary units. The quenching Trp fluorescence was measured in Tris HCl buffer (pH 7.4) and in vesicle dispersion according to the method of Mishra and Palgunachari [15]. The monomer peptide concentration was 10 μM and the ratio of lipid to peptide was 0 or 30. The dimer peptide concentration was 5 μM and the ratio of lipid to peptide was 0 or 30. After adding various amounts of a quencher, KI, the sample was excited at 280 nm and emission spectra were recorded at 350 nm at 25 °C. The degree of quenching was calculated according to the Stern-Volmer equation $(F_0/F) - 1 = \mathrm{Ksv}\ [Q]$, where F_0 and F are the fluorescence intensities observed without and with quencher, respectively, Ksv is the Stern-Volmer quenching constant and $[Q]$ is the concentration of quencher [16].

Calcein Leakage

The calcein leakage experiment was performed by the procedure of Lee *et al.* [17]. Twenty μl of the vesicles containing 0.1 M calcein (MW 623) was mixed with 2.0 ml Tris HCl buffer (pH 7.4) in a cuvette, resulting in a final phospholipid concentration of 70 μM. While the cuvette was in the holder of the spectrofluorometer at a temperature of 25 °C, 20 μl of the appropriate dilution of a peptide solution in buffer was added to the cuvette. The fluorescence intensities of calcein were monitored at 515 nm (excited at 495 nm) and measured 2 min after adding the peptides. To measure the fluorescence intensity corresponding to 100% calcein release, 10 μl of 10% Triton X-100 was added to dissolve the vesicles. The percentage of the calcein release was calculated by the equation $100 \times (F - F_0)/(F_t - F_0)$, where F is the fluorescence intensity obtained by adding a peptide solution, F_0 is the intensity observed by adding the buffer instead of a peptide solution, and F_t is the intensity observed after Triton X-100 treatment.

FITC-dextran Leakage

Leakage was also measured with FITC-dextran according to the method of Matsuzaki *et al.* [18]. SUVs of DOPC/DOPG/DNS-PE (3 : 1 : 0.06) entrapping 3 mM FITC-dextran (MW 4400, diameter 2.4 nm) were prepared as described for the preparation of phospholipid vesicles and incubated with peptide at pH 7.4 and 25 °C for 5 min. After the addition of a trypsin solution (5 mg/ml, 50 μl), the resulting mixture was chromatographed with Sephacryl S-200 to remove the FITC-dextran. The excitation spectrum of the vesicle fraction after solubilization with Triton X-100 was measured at an emission wavelength of 520 nm. The percent leakage was calculated according to the method of Matsuzaki *et al.* [18].

Membrane Translocation of Peptide

Translocation activity of the peptides though lipid membranes was measured by the method of Matsuzaki *et al.* [19]. Vesicles were prepared as described for the preparation of phospholipid vesicles using DOPC (3.75 μmol), DOPG (1.25 μmol) and NBD-PE (12.5 nmol) in 20 mM Tris HCl (pH 7.4, 5 ml) and were frozen-thawed five times to give a solution containing 1 mM DOPC/DOPG/NBD-PE (3 : 1 : 0.01) multilamellar vesicles (MLVs). A solution containing 1 mM DOPC/NBD-PE (4 : 0.01) MLVs was prepared similarly. A mixture of the buffer (800 μl, pH 7.4) containing various concentrations of peptide and 1 mM vesicle solution (100 μl) was placed in a quartz cell of 10 mm path length and kept for 1 min at 25 °C. One hundred μl of 100 mM $Na_2S_2O_4$ was added to the solution. The sample was excited at 470 nm and emission spectra were recorded at 530 nm for 4 min. The percentage of the quenched fluorescence of NBD was calculated by the equation $100 \times (F_0 - F)/(F_0 - F_t)$, where F, F_0 and F_t are as described above.

RESULTS AND DISCUSSION

The dimer peptides, M2-CC and M2-CN, contain two monomer-corresponding moieties. Therefore, the concentrations of the dimers are expressed in twofold values to make the concentrations of the monomer-corresponding moiety in the dimers and the monomers uniform, and evaluate the effect of dimerization on properties and biological activities of the peptides throughout the Results and Discussion and in the figures and table.

Peptide Design and Synthesis

In order to synthesize monomer and dimer peptides, we designed two M2 analogues, which have additional sequences of βAla-Cys at the *C*-terminus and

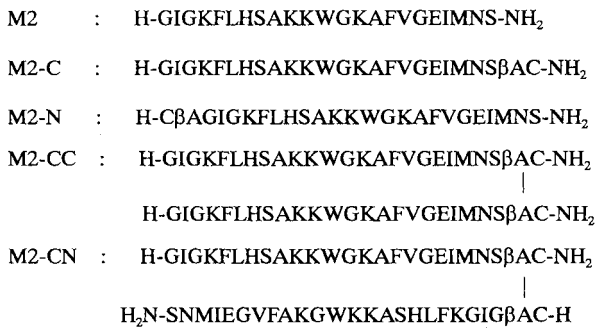

Figure 1 Amino acid sequences of M2, monomers and dimers. βA denotes βAla.

Cys-βAla at the *N*-terminus, respectively (Figure 1). The βAla residue was introduced for peptide flexibility and to maintain sufficient distance between the two helical peptides. The Phe residue at position 12 was replaced with a Trp residue to investigate the behaviour of peptides in lipid membranes by spectrometry [7]. Monomer peptides were synthesized by the usual SPS method. The parallel M2 dimer was obtained in a moderate yield (70%), whereas the yield of the antiparallel dimer was 31% (56% each in activation and in S-S exchange reaction). The MALDI-TOF-MS measurements showed that the observed molecular weights of the products agreed with their calculated molecular weights (Table 1).

Antibacterial and Haemolytic Activities

Antibacterial activity was examined by the serial solution dilution method using two Gram-positive bacteria (*S. aureus* and *S. subtilis*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). Gramicidin S, an antibacterial cyclic decapeptide, was used as a reference. The MICs of the peptides are listed in Table 1. All the peptides were active against both bacteria, and the antibacterial activities of all the peptides except gramicidin S were almost the same against Gram-positive and Gram-negative bacteria. Gramicidin S was mainly active against Gram-positive bacteria [20]. M2-N showed a much weaker antibacterial activity than natural M2. Modification of the *N*-terminal portion seemed to decrease the activity. On the other hand, M2-C had antibacterial activity similar to that of M2, indicating that modification of the *C*-terminus has little effect on the activity. Among the dimers, the antibacterial activity of M2-CC was slightly stronger than that of M2-CN.

As another evaluation of biological activity of peptides, haemolytic activity was measured using rabbit red blood cells (Figure 2). M2 and M2-N showed negligible haemolytic activity even at a concentration of 100 μM, whereas M2-C had an activity of 23%. M2-CC and M2-CN exhibited activities of 65% and 30%, respectively, at the same concentration. Because the blood cell membrane contains mainly neutral phospholipid, modification of the peptides with βAla-Cys or Cys-βAla seems to enhance the hydrophobic interaction of the peptides with neutral lipids. However, this can not explain why M2-N showed much weaker haemolytic activity than M2-C in spite of having the same hydrophobicity as that of M2-C. Other recent studies have also reported that an increase in hydrophobicity is not always accompanied by an increase in haemolytic activity [4,13,21].

Circular Dichroism

To examine the secondary structure of the synthetic peptides, CD was measured in 20 mM Tris

Table 1 Molecular Weights, Antibacterial Activities and α-Helical Contents of Peptides

	MALDI-TOF-MS		MIC [μM (μg/ml)]				α-Helical content (%)			
	Obs.	Calc. $[M+H]^+$	*S. aureus* IFO 12732	*B. subtilis* IFO 3134	*E. coli* IFO 12734	*P. aeruginosa* IFO 12582	Buffer (pH 7.4)	TFE	DOPC	DOPC/DOPG (3 : 1)
M2	2505.91	2505.96	8 (25)	8 (25)	4 (12)	8 (25)	<5	51	44	58
M2-C	2680.27	2679.22	8 (27)	8 (27)	8 (27)	4 (13)	<5	41	34	52
M2-N	2680.25	2679.22	16 (54)	32 (107)	32 (107)	32 (107)	5	47	40	56
M2-CC	5356.66	5356.42	4[a] (14)	4[a] (14)	4[a] (14)	2[a] (7)	6	45	37	50
M2-CN	5356.11	5356.42	8[a] (27)	8[a] (27)	8[a] (27)	4[a] (14)	6	52	47	61
Gramicidin S	—[b]	—[b]	4 (4)	2 (2)	16 (20)	16 (20)	—[b]	—[b]	—[b]	—[b]

[a] The concentrations (μM) of M2-CC and M2-CN are expressed in twofold values. [b] Not determined.

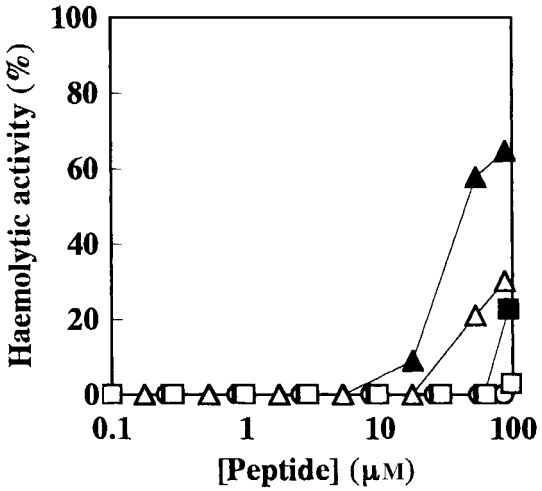

Figure 2 Haemolytic activities of the peptides. M2 (○), M2-C (■), M2-N (□), M2-CC (▲), M2-CN (Δ). The concentrations of the dimer peptides are expressed in twofold values.

HCl buffer (pH 7.4), TFE and in the presence of DOPC or DOPC/DOPG (3 : 1) vesicles. In Tris buffer (pH 7.4), all the peptides had almost completely random coil structures. In TFE, an α-helix-inducing solvent, they showed typical α-helix curves with double minima around 208 and 222 nm, corresponding to α-helical contents of 40%–50% (Table 1). The α-helical contents of the peptides were slightly lower in the presence of DOPC SUVs, whereas they were higher (50%–60%) in the presence of DOPC/DOPG (3 : 1) SUVs. These results suggest that the peptides interact more strongly with acidic phospholipid vesicles than with neutral ones. Although the α-helical contents of M2-C and M2-CC were slightly lower than those of M2-N, M2-CN and M2, all the peptides seemed to have a similar tendency to form α-helices. We found no clear relationship between structure and biological activity.

Fluorescence Spectroscopy

To determine the environment of the Trp residues of the peptides in the lipid membranes, the fluorescent properties of the Trp residue in M2 and its related peptides were examined in the presence or absence of DOPC or DOPC/DOPG (3 : 1) SUVs (Figure 3). In aqueous solution, the maximal fluorescence wavelength of the peptides was about 352 nm, indicating that the Trp residues are exposed to a hydrophilic environment. With an increasing [lipid]/[peptide] ratio, the maximal fluorescence wavelength was gradually shifted to shorter wavelengths (342–346 nm) in the presence of DOPC

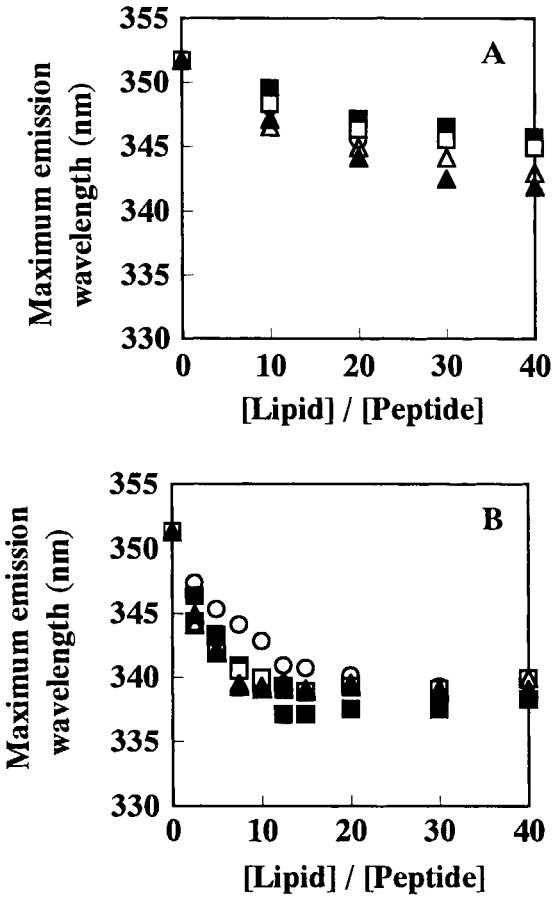

Figure 3 Trp titration curves of the peptides in DOPC SUVs (A) and DOPC/DOPG (3 : 1) SUVs (B). [Peptide] = 10 μM, λex = 280 nm, 25 °C. M2 (○), M2-C (■), M2-N (□), M2-CC (▲), M2-CN (Δ). Although the concentration of the dimer peptides is 5 μM, it is expressed in twofold values.

SUVs. In the presence of DOPC/DOPG (3 : 1) SUVs, the maximal fluorescence wavelength was rapidly shifted to shorter wavelengths (338–340 nm) and remained in this range at [lipid]/[peptide] ratios of 20 : 1 and higher. Because the maximal fluorescence wavelength is shifted to shorter wavelengths with increasing hydrophobicity of the environment [7, 13], the results show that the Trp residues of the peptides were more deeply embedded in the hydrophobic portion of DOPC/DOPG (3 : 1) SUVs than in the hydrophobic portion of DOPC SUVs.

Quenching of Trp fluorescence with KI as a quencher was also measured in the presence and absence of the lipid SUVs (data not shown). Trp was used as a reference. In aqueous solution, the value of

$(F_0/F) - 1$ increased with increasing quencher concentration and all the peptides gave similar quenching patterns. The quenching of Trp fluorescence was different from the quenching of the peptide fluorescence, which means that the Trp residues in the peptides were in a slightly hydrophobic environment. The degree of quenching was greater in the presence of DOPC/DOPG (3 : 1) SUVs than in the presence of DOPC SUVs. No appreciable difference between monomers and dimers was found, whereas Trp was highly quenched. These results also indicate that the Trp residues in the peptides are located in the hydrophobic portion of acidic lipid membranes.

Membrane Permeabilization

Membrane permeability of the peptides was evaluated by calcein leakage and FITC-dextran leakage experiments [9, 17]. The dye-leakage activity of the peptides was assayed by measuring the leakage of calcein (MW 623, diameter 1.3 nm) from calcein-containing vesicles at 25 °C (Figure 4). The vesicles were DOPC SUVs at a concentration corresponding to 70 μM phospholipid and in each case, the peptide concentration was 10 μM. The amounts of calcein that leaked out in 2 min were 31%, 26%, 15%, 13% and 5%, when M2-CC, M2-CN, M2-N, M2-C and M2 were used, respectively. The strong leakage activities of dimers and monomers might be due to the increase in the hydrophobicity of the peptides by modification of M2 with the hydrophobic linker. The higher permeability of dimers than monomers might be due to the formation of large pores or a greater disturbance of the lipid membrane by the bulky structure of the dimers. When DOPC/DOPG (3 : 1) SUVs at a concentration of 70 μM were used, the dye-leakage activities of M2-CC and M2-CN (10 μM) were both 91%. The dye-leakage activities of M2-C and M2-N (64%–68%) were also fairly strong. These results mean that the charge interaction between the peptide and the acidic phospholipid was more effective for increasing membrane permeability than the hydrophobic interaction. Furthermore, a clear difference was found between the dye-leakage activities of the dimer and the monomer. Hara *et al.* [9] found that an antiparallel M2 analogue connected by two disulphide bridges had a weaker leakage activity than the corresponding monomer at high [peptide]/[lipid] ratios. Because the disulphide-bridged analogue has a rigid conformation, the difference in the present results and their results may be due to a difference of the flexibilities of the dimers. Study on aggregational states of M2 gave a result that M2 formed an antiparallel dimer in phospholipid bilayers [7]. However, in the synthesis of M2-PGLa heterodimers, a parallel heterodimer was preferentially formed and exhibited stronger membrane permeability activity compared with an M2-PGLa antiparallel dimer [10]. These results indicate that subtle differences in amino acid compositions of the peptides, the linkers and compositions of lipid membranes may greatly affect the properties and biological activities of the peptides. It is notable, in this study, that there was no appreciable difference between the properties of the parallel and antiparallel dimers, which raises the possibility that M2 forms parallel and antiparallel mixed structures in lipid membranes.

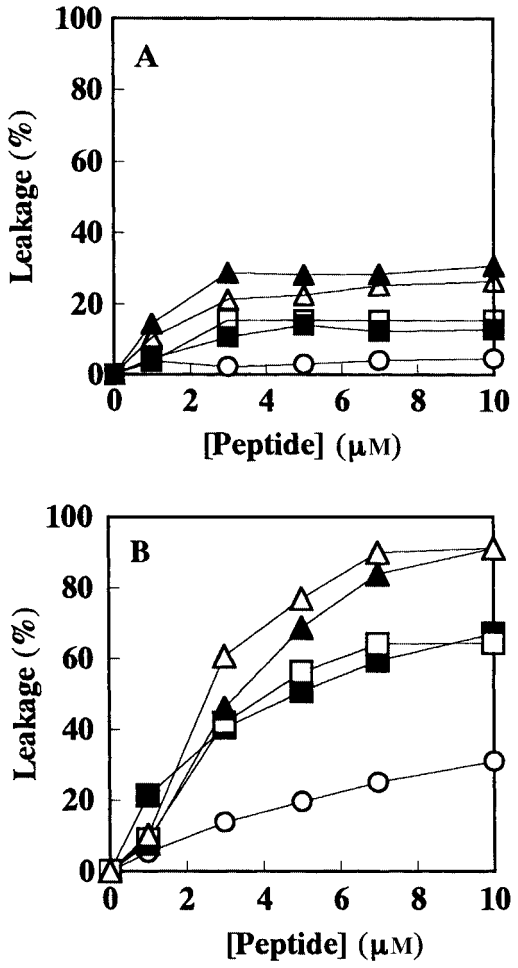

Figure 4 Calcein leakage activities of the peptides for DOPC SUVs (A) and DOPC/DOPG (3 : 1) SUVs (B). [Lipid] = 70 μM, 25 °C. M2 (○), M2-C (■), M2-N (□), M2-CC (▲), M2-CN (Δ). The concentrations of the dimer peptides are expressed in twofold values.

The FITC-dextran leakage experiment was conducted at a peptide concentration of 10 μM at 25 °C. For DOPC/DOPG/DNS-PE (3 : 1:0.06) SUVs entrapping 3 mM FITC-dextran, the leakage activities of M2-CC and M2-CN were 15% and 13%, respectively, whereas those of M2-C, M2-N and M2 were about 8%. This and the above results suggest that those peptides form pores across the phospholipid membrane. However, the fact that M2-CC and M2-CN had higher activities than M2 and the fact that they also have haemolytic activity as described above raises the possibility that these dimers have some membrane-perturbing activity.

Peptide Translocation

For DOPC MLVs, all the peptides exhibited weak translocation activity (data not shown). This result is reasonable because these peptides were shallowly embedded in a neutral lipid membrane and their calcein-leakage activity was rather weak. For DOPC/DOPG (3 : 1) MLVs, the order of translocation activity was dimers > monomers > M2 (Figure 5) and is consistent with the results in the membrane permeability experiment. It is likely that dimers and monomers, like M2, have translocation activity, although translocation of the peptides may be accompanied by some membrane perturbation.

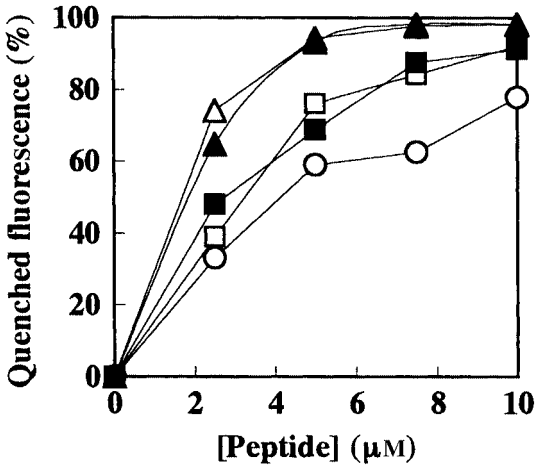

Figure 5 Membrane translocation activities of the peptides for DOPC/DOPG/NBD-PE (3 : 1:0.01). [Lipid] = 100 μM, 25 °C. M2 (○), M2-C (■), M2-N (□), M2-CC (▲), M2-CN (Δ). The concentrations of the dimer peptides are expressed in twofold values.

CONCLUSIONS

Magainin 2 (M2) forms pores through phospholipid membranes by association of several peptide molecules. To gain insight into how the molecules are arranged in a parallel or antiparallel state in the bilayer, parallel and antiparallel M2 dimers were synthesized. Both dimers had a greater biological activity and a greater ability to interact with membranes than the monomeric precursors. However, there was no appreciable difference between the properties of parallel and antiparallel M2 dimers, suggesting that M2 exists in both states when associated in lipid membranes, although the present result is not compatible with the reported result [10]. The increase in biological and membrane-perturbing activities of dimers and monomers may be due to modification of M2 with a hydrophobic linker. Several parallel and antiparallel M2 analogues have been synthesized and their behaviour on and in phospholipid membranes also have been investigated in detail [9–11]. However, until now, there have been no studies on their antibacterial and haemolytic activities. The present results should open the way to further studies on the structure–activity relationship of these peptides.

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