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Interaction of Mastoparan B and Its Ala-Substituted Analogs with Phospholipid Bilayers

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The interaction of mastoparan B, a tetradecapeptide toxin found in the hornet *Vespa basalis*, with phospholipid bilayers was investigated. Synthetic mastoparan B and its analogs, obtained by substituting one hydrophilic amino acid (2-Lys, 4-Lys, 5-Ser, 8-Ser, 11-Lys, or 12-Lys) in mastoparan B with Ala, were studied. Mastoparan B and its analogs were synthesized by the solid-phase method. As shown by circular dichroism spectra, mastoparan B and its analogs adopted an unordered structure in buffer solution. All peptides took an α-helical structure, and the α-helical content of its analogs increased in the presence of neutral and acidic liposomes as compared to that of mastoparan B. In the calcein leakage experiment, we observed that mastoparan B interacted more weakly with lipid bilayers in neutral and acidic media than its analogs. Mastoparan B also showed slightly lower antimicrobial activity and hemolytic activity towards human erythrocytes than its analogs. These results indicate that the greater hydrophobicity of the amphiphilic α-helix of mastoparan B by replacement with alamine residues results in the increased biological activity and helical content.

Introduction

Recently, mastoparan B (MP-B), an antimicrobial cationic tetradecapeptide amide, was isolated from the venom of the hornet *Vespa basalis*[1] (Figure 1). MP-B is the mastoparan homolog of vespid venoms. Mastoparan (MP) has shown various biological activities, such as activation of mast cell degradation histamine release,[2,3] phospholipase A_2[3,4] and C,[4,5] erythrocyte lysis, and binding to calmodulin.[6] In the case of G-protein-coupled receptors *in vitro*,[7] it is obvious that MP enhances the permeability of phospholipid bilayers[8] and activates GTP-binding regulatory proteins (G-proteins). Considering the structure-activity relationship in various natural and synthetic compounds, the amphiphilic α-helical structure with cationic amino acid residues on one side and hydrophobic residues on the other side is closely related to biological activity.[2,9] This structural feature is necessarily important but not itself sufficient to stimulate GTPase of G-protein.[10]

The amino acid sequence of the primary structure of MP-B is very distinct from those of other vespid mastoparans.[1] Compared to the common structure of vespid mastoparans (Lys at positions 4, 11 and 12), MP-B has a less hydrophobic sequence at several positions, such as an additional Lys at position 2 and a Trp at position 9. MP-B is a potent stimulator of histamine release from rat peritoneal mast cells, and shows more potent hemolytic activity than MP. MP-B was useful as a cardiovascular depressor[11] and an inhibitor of Gram-positive and -negative bacteria.[12] MP-B showed antimicrobial activity against bacteria and leakage ability.[13] This peptide revealed its amphiphilic properties as shown by helical wheels.[13] MP-B adopts an am-

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phiphilic α-helical structure which can be described as follows: one side contains with hydrophilic side chains (*i.e.* Lys-4, Ser-5, Ser-8, Lys-11, Lys-12) while the other side contains hydrophobic side chains (*i.e.* Leu-3, Ile-6, Trp-9, Ala-10, Val-13, Leu-14).[14] MP-B and its analogs have a random structure in buffer solution and an α-helical structure in the presence of phospholipid bilayers.[15] When the peptide forms an α-helical structure, positively charged groups (NH_2 groups of the N-terminus and three Lys residues) are on one side of the helix, while the other side is occupied by hydrophobic residues. This conformation in phospholipid bilayers is considered to play an important role in its toxic action.[16]

In a previous report, we also described the interaction of membranes with MP-B analogs substituted with individual Ala residues instead of hydrophobic amino acids in MP-B.[15] The interactions of MP-B and its analogs, substituted with individual Ala residues instead of hydrophilic amino acids in MP-B, with phospholipid bilayers have not been reported as yet. In this study, we investigated the detailed relationship between the hydrophilic amino acids in MP-B and its biological activity. Six MP-B analogs were prepared by replacing the hydrophilic amino acids in MP-B individually with Ala residues. Their structures and properties were studied by CD measurement, dye-leakage experiments, and hemolytic and antimicrobial activity assays.

Materials and Methods

General. Egg-yolk phosphatidylcholine (EYPC), egg-yolk phosphatidylglycerol (EYPG), and calcein were purchased from Sigma Chemical Co., St. Louis. Amino acids were purchased from Watanabe Chemical Industries, LTD., Hiroshima. Phospholipid concentration was determined by an assay using the phospholipids-test reagent from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were of the highest grade available.

Synthesis and purification of MP-B and its analogs. MP-B and its analogs were synthesized and purified as described previously.[15] Amino acid analysis was carried out using a JASCO HPLC amino acid analysis system equipped with an FP-210 spectrofluorometer as a detector after the hydrolysis of the peptide in 5.7 M HCl in a sealed tube at 110 °C for 24h. The amino acid analyses of the synthetic peptides were as follows: **2MP-B**; Ala 2.21 (2), Lys 3.01 (3), Leu 3.16 (3), Ser 1.86 (2), Val 1.87 (2), Ile 0.81 (1). **4MP-B**; Ala 2.17 (2), Lys 3.11 (3), Leu 3.07 (3), Ser 1.88 (2), Val 1.93 (2), Ile 0.87 (1). **5MP-B**; Ala 2.28 (2), Lys 4.17 (4), Leu 3.23 (3), Ser 1.02 (1), Val 1.74 (2), Ile 0.71 (1). **8MP-B**; Ala 2.20 (2), Lys 3.96 (4), Leu 3.08 (3), Ser 0.86 (1), Val 1.89 (2), Ile 0.82 (1). **11MP-B**; Ala 2.24 (2), Lys 3.11 (3), Leu 2.89 (3), Ser 2.01 (2), Val 1.84 (2), Ile 0.73 (1). **12MP-B**; Ala 2.12 (2), Lys 3.04 (3), Leu 2.87 (3), Ser 1.89 (2), Val 1.93 (2), Ile 0.89 (1). Numbers in parentheses are theoretical values.

Molecular weight was determined by fast atom bombardment mass spectrometry (FAB-MS) using a JEOL SX-102A. FAB-MS data of the synthetic peptides were as follows: **2MP-B**: base peak, 1553.6, calcd. for $C_{75}H_{131}O_{16}N_{19}$, 1555.3. **4MP-B**: base peak, 1553.7, calcd. for $C_{75}H_{131}O_{16}N_{19}$, 1555.3. **5MP-B**: base peak, 1595.5, calcd. for $C_{78}H_{138}O_{15}N_{20}$, 1596.4. **8MP-B**: base peak, 1595.5, calcd. for $C_{78}H_{138}O_{15}N_{20}$, 1596.4. **11MP-B**: base peak, 1553.8, calcd. for $C_{75}H_{131}O_{16}N_{19}$, 1555.3. **12MP-B**: base peak, 1553.8, calcd. for $C_{75}H_{131}O_{16}N_{19}$, 1555.3.

Peptide concentrations for MP-B and its analogs were determined from UV-absorbance of Trp in 8 M urea .

Preparation of liposomes. Small unilamellar vesicles (SUVs) were obtained from two lipids composed of EYPC and EYPC-EYPG (3 : 1) as neutral and acidic vesicles, respectively. Calcein trapped SUVs were prepared as described previously.[15] The obtained SUVs were used for the CD measurement.

Circular dichroism spectra. CD spectra were recorded on a JASCO J-600 spectropolarimeter using a quartz cell of 1 mm pathlength. Spectra of peptides were measured as described previously.[15] All measurements were performed at 25 °C and pH 7.4 of 5 mM Tes-buffer. The α-helical contents were calculated from θ_{222} by Ben-Efraim *et al.*[17]

Fluorescence spectra. Fluorescence spectra were recorded on a JASCO FP-550A spectrofluorophotometer. Leakage of liposome contents was determined using a minor modified fluorescence dye-release experiment.[18] All measurements were performed at 25 °C and pH 7.4 of 0.15 M NaCl/20 mM Tes-buffer (pH 7.4). The percentage of dye-release caused by the peptides was evaluated by the equation of $100\times(F-F_0)/(F_t-F_0)$, where F is the fluorescence intensity obtained by the peptides, and F_0 and F_t are the fluorescence intensities without the peptides and with Triton X-100 treatment, respectively.

Antimicrobial activity. The minimum inhibitory concentration (MIC) in the growth of microorganisms was determined by the standard agar dilution method using Muellar Hinton medium (Difco).

Hemolytic assay. Hemolytic activity of erythrocytes was determined as described previously.[15]

Results

CD study. The effect of hydrophilic amino acids in MP-B on biological activity and conformation was investigated. Several MP-B analogs containing individual Ala residues instead of hydrophilic amino acids (2-Lys, 4-Lys, 5-Ser, 8-Ser, 11-Lys, 12-Lys) were designed in this study. The primary structures of peptides used in this study are shown in Figure 1.

Previous study reported that MP-B and its analogs took a random structure in buffer solution and that most peptides adopted an α-helical structure in the presence of EYPC and

MP-B	Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2
2MP-B	Leu-Ala-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2
4MP-B	Leu-Lys-Leu-Ala-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2
5MP-B	Leu-Lys-Leu-Lys-Ala-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2
8MP-B	Leu-Lys-Leu-Lys-Ser-Ile-Val-Ala-Trp-Ala-Lys-Lys-Val-Leu-NH_2
11MP-B	Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Ala-Lys-Val-Leu-NH_2
12MP-B	Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Ala-Val-Leu-NH_2

Figure 1. Primary structure of mastoparan B and its analogs.

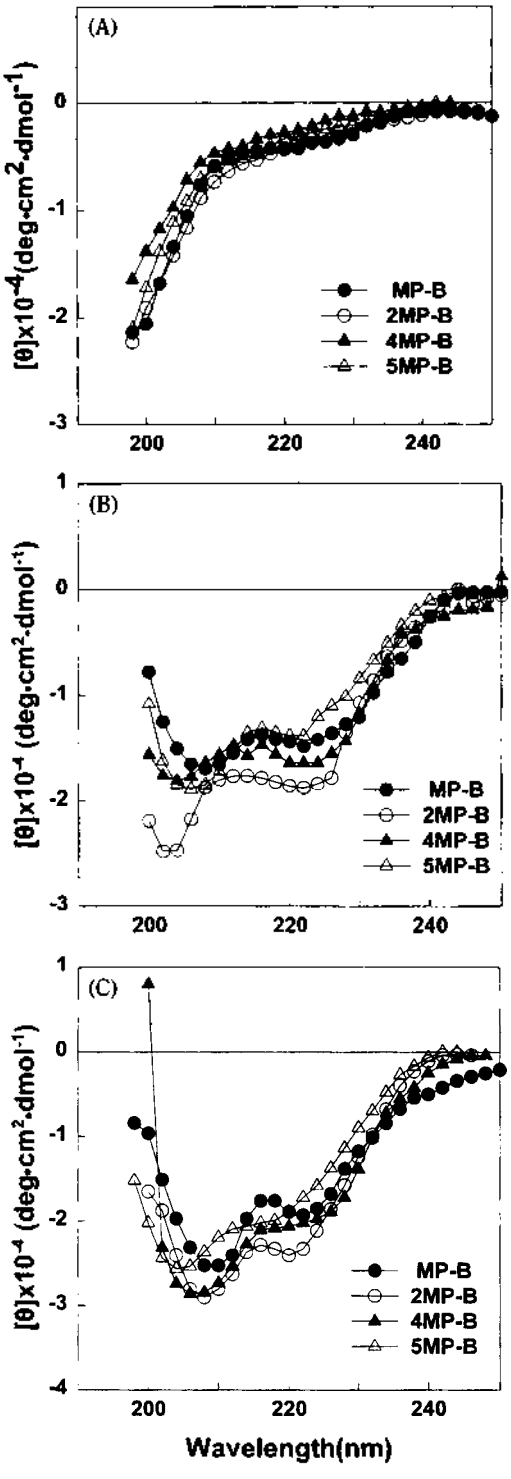

Figure 2. CD spectra of MP-B, 2MP-B, 4MP-B, and 5MP-B in TES buffer (A) in the presence of EYPC liposomes (B) and EYPC-EYPG (3 : 1) liposomes (C). MP-B (●), 2MP-B (○), 4MP-B (▲), and 5MP-B (△). Peptide and lipid concentrations are 0.1 and 1 mM, respectively.

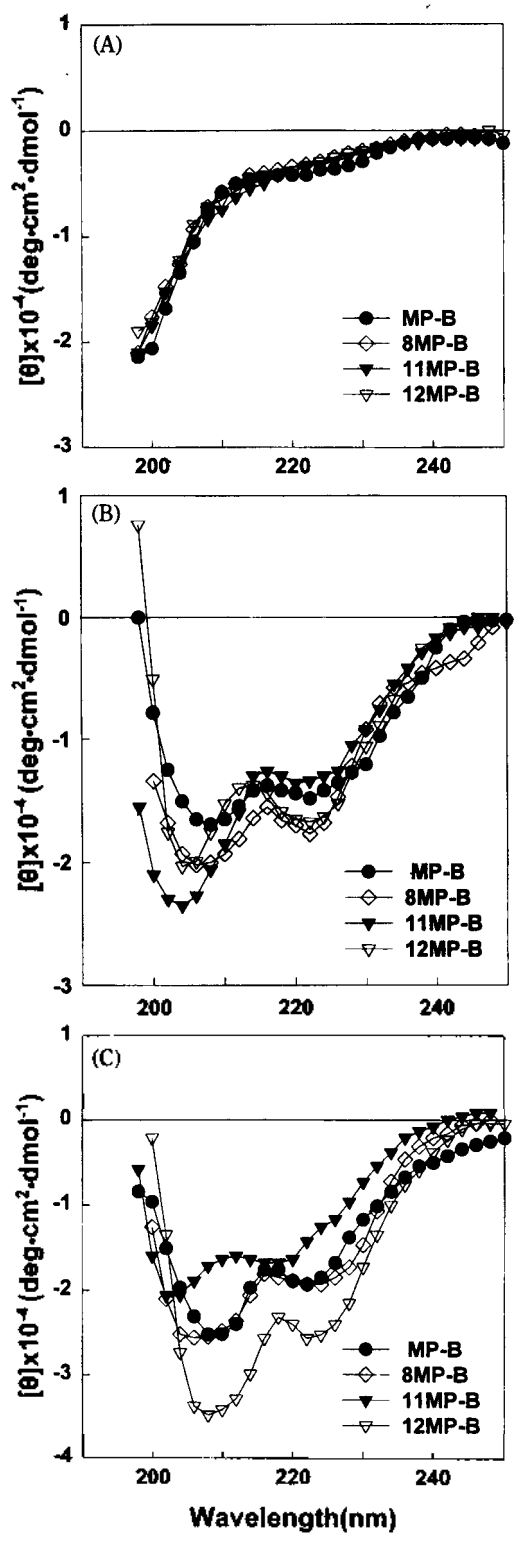

Figure 3. CD spectra of MP-B, 8MP-B, 11MP-B, and 12MP-B in TES buffer (A) in the presence of EYPC liposomes (B) and EYPC-EYPG (3 : 1) liposomes (C). MP-B (●), 8MP-B (◇), 11MP-B (▼), and 12MP-B (▽). Peptide and lipid concentrations are 0.1 and 1 mM, respectively.

EYPC-EYPG (3 : 1) bilayers.[15] In order to investigate the conformations of MP-B and its analogs, we obtained CD spectra in Tes-HCl buffer containing 100 mM NaCl and CD spectra in the presence of EYPC and EYPC-EYPG (3 : 1) liposomes. These spectra are shown in Figures 2 and 3.

MP-B and its analogs adopted mainly random structures in buffer solution (Figures 2A and 3A). In the presence of neutral liposomes (Figures 2B and 3B), MP-B exhibited two

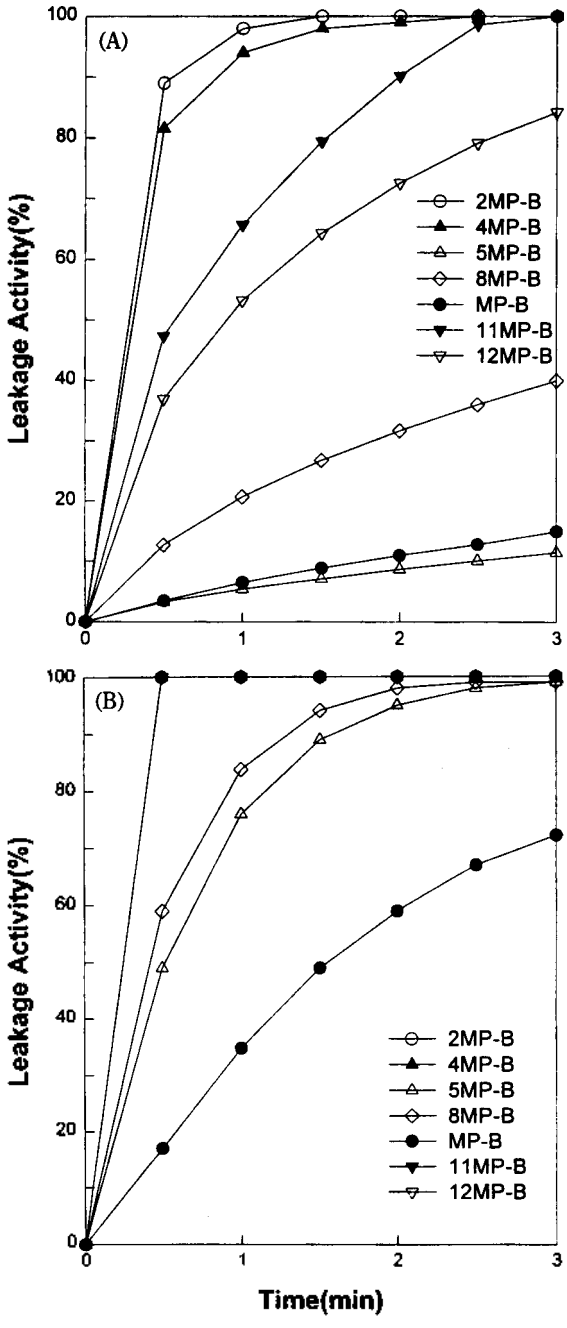

Figure 4. Time course of calcein release from EYPC (A) and EYPC-EYPG (3 : 1) (B) liposomes by MP-B and its analogs. Peptide concentrations are 50 μM (A) and 10 μM (B), respectively. MP-B (●), 2MP-B (○), 4MP-B (▲), 5MP-B (△), 8MP-B (◇), 11MP-B (▼), and 12MP-B (▽).

minimum peaks around 205 and 222 nm corresponding to an α-helix. The α-helical content decreased in the following order: 8MP-B (53%)> 2MP-B, 12MP-B (50%)> 4MP-B (48%)> MP-B, 5MP-B (43%) and 11MP-B (40%). Interestingly, 2MP-B, 4MP-B, 8MP-B, and 12MP-B had increased α-helical content as compared to MP-B. The helical content progressively increased in the presence of acidic liposomes (Figures 2C and 3C) and its order was as follows: 12MP-B (78%)> 2MP-B (66%)> 4MP-B (61%), 8MP-B, MP-B and 5MP-B (58%)> 11MP-B (52%). These results indicate that the increased hydrophobicity in MP-B is an important factor in the helical structure.

Leakage of liposome content. The ability of MP-B and its analogs to cause calcein leakage from liposomes was evaluated to examine the perturbation of lipid bilayers induced by lipid-peptide interaction. Profiles of the dye release from EYPC and EYPC-EYPG (3 : 1) liposomes are shown in Figure 4. The addition of 50 μM of peptides to calcein-containing EYPC liposomes induced dye release in relative amounts as follows: 2MP-B> 4MP-B> 11MP-B> 12MP-B> 8MP-B>> MP-B> 5MP-B (Figure 4A). Among these peptides, 2MP-B shows the highest dye release ability in neutral liposomes. The ability of MP-B to release dye was the lowest among the analog peptides. MP-B reached a plateau with about 15% dye-release at about 50 μM, and further release was not observed within the concentration range studied. When 10 μM of peptides were added to EYPC-EYPG (3 : 1) liposomes (Figure 4B), leakage ability was measured as follows; 2MP-B, 4MP-B, 11MP-B, and 12MP-B> 8MP-B> 5MP-B>> MP-B. In acidic liposomes, all peptides except for MP-B reached a plateau with about 100% dye-release at 10 μM, and their leakage abilities were significantly higher than those in neutral liposomes. MP-B did not reach complete leakages at 10 μM. These results suggest that the substitution of hydrophilic amino acid residues with Ala residues in the amphiphilic structure is very effective for membrane perturbation.

Antimicrobial activity. Antimicrobial activities of MP-B and synthetic peptides are shown in Table 1. MP-B showed considerable potential for inhibiting the growth of both Gram-positive bacteria such as *Staphylococcus aureus, S. epidermidies, and Bacillus subtilis*, and Gram-negative bacteria such as *Shigella flexneri* and *Pseudomonas aeruginosa*. On the other hand, the analogs of MP-B exhibited stronger activities against Gram-positive and -negative bacteria in comparision with MP-B. It is noted that the antimicrobial activity of the peptides is closely related to their helical content as well as leakage ability.

Interaction of the peptides with red blood cells.

Table 1. Antimicrobial activities of MP-B and its analogs[a]

Organism	Mastoparan	2MP-B	4MP-B	5MP-B	8MP-B	11MP-B	12MP-B
Staphylococcus aureus FDA 209P	50	25	25	25	50	25	25
S. epidermidie ATCC 12228	25	12.5	12.5	12.5	12.5	12.5	25
Bacillus subtills PCI 219	12.5	6.25	6.25	6.25	6.25	6.25	6.25
Shigella flexneri EW-10	25	50	25	12.5	25	25	25
Pseudomonas aeruginosa U-31	>100	>50	>50	>50	>50	>25	>25

[a] Method: Agar dilution method, Medium: Muellar Hinton agar (Difco), Inoculum size: 10^6 cells mL^{-1}

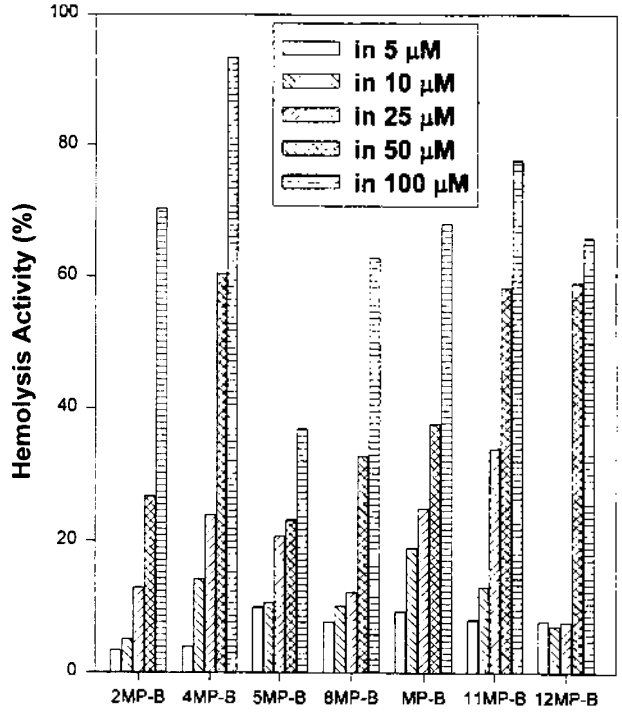

Figure 5. Erythrocyte lysis by MP-B and its analogs. An erythrocyte suspension was exposed to varying concentration of MP-B and its analogs. The amount of hemoglobin released was determined spectrophotometrically by measuring the absorbance of 540 nm. Complete lysis (100%) was obtained by the addition of Triton X-100 to the erythrocyte suspension.

Figure 5 shows the hemolysis of human erythrocytes as a function of the peptide concentration. All peptides lyse the erythrocyte very little up to 5 μM and considerable lyses are shown beyond 50 μM. 4MP-B, 11MP-B, and 12MP-B showed about 60% lysis at 50 μM and 70-90% lysis at 100 μM, but the hemolytic activity of 5MP-B was lower than that of other peptides. These results indicate that an hydrophobic regions in an amphiphilic helix critically affects the hemolytic activity of MP-B.

Discussion

CD and NMR studies have shown that MP-B has a strong affinity for phospholipid bilayers and takes an amphiphatic α-helical structure.[13,14] The overall structural feature of eight residues having different hydrophobicities is quite similar to the conformation of mastoparan-X (MP-X) in perdeuterated DPPC vesicles.[19] The model peptides, substituted with individual Ala residues instead of hydrophobic amino acids, also took an amphiphatic α-helical structure in the presence of neutral and acidic phospholipid bilayers.[15] Considering the peptide-lipid interaction, the hydrophilic side of the helix is present in the acidic moiety of the bilayer lipid head group and the hydrophobic side is immersed in the membranes in a horizontal manner for the surface of lipid bilayer. The amphiphilicity of a peptide comes from the hydrophobic moment.[20,21] Evaluating the hydrophobicity scales for amino acid residues offered by Eisenberg,[21] the hydrophobicities of MP-B, 2MP-B, 4MP-B, 5MP-B, 8MP-B, 11MP-B, and 12MP-B are −0.06, 0.03, 0.03, −0.08, −0.08, 0.03, and 0.03, respectively. Additionally, the hydrophobic moments of MP-B, 2MP-B, 4MP-B, 5MP-B, 8MP-B, 11MP-B, and 12MP-B are 0.27, 0.32, 0.22, 0.25, 0.23, 0.25, and 0.20, respectively. Based on these similar values of amphiphilicity, it is proposed that MP-B and its analogs interact with lipid bilayers.

Considerable difference between MP-B and its analogs, however, is observed for their α-helical content in the presence of neutral (Figures 2B and 3B) and acidic liposomes (Figures 2C and 3C). The helical content of MP-B and its analogs in neutral lipid bilayers are less than that in acidic liposomes. In a previous report, the helix-forming ability of MP-B in the presence of artificial membranes was much larger than that of its analogs.[15] In particular, 9MP-B showed a modest decrease in hemolytic activity. This finding is due to the hydrophobic regions consisting of almost the same amino acid residues as MP-B and its analogs. Therefore, the increase in hydrophobic regions in the amphiphilic helix as well as the existence of Trp at position 9 in MP-B plays an important role in stabilizing the α-helical structure.

MP-B can be readily associated with the gel state bilayers prepared from DPPC and DPPC-DPPG (3 : 1).[13] This association can be accompanied by penetrating the indole ring of the Trp residue into a less polar environment in the vicinity of aliphatic chains. The Trp residue in MP-B is in the hydrophobic area of the amphiphilic helices. It seems that MP-B and its analogs can interact with lipid bilayers as reported in a previous NMR study.[14]

In the dye-release experiment, the membrane perturbation effect of model peptides was larger than that of MP-B in both neutral and acidic liposomes. The replacement of cationic residues ($Lys^{2,4,11 \text{ or } 12}$) in MP-B by Ala was more effective for membrane perturbation than that of MP-B, and the replacemnt of the next hydrophilic residue ($Ser^{5,8}$) was also effective. The replacement of hydrophobic amino acid residues was ineffective for membrane perturbation[15]; one peptide, 6MP-B (Ile by Ala) had no ability. An increased hydrophilicity of MP-B might lead to such a difference. It should be noted that the ability of MP-B and its analogs to mediate dye release is much higher in acidic liposomes than in neutral liposomes. These results suggest that hydrophobic side-substitution in the amphiphilic structure is more effective for membrane perturbation than hydrophilic-side substitution. Similarly, the helical content of peptides is much greater in acidic liposomes than in neutral liposomes.

Amphiphilic structure have been found in biologically active peptide molecules.[22] In particular, the basic amphiphilic α-helical structure is considered to be one of the most important structural units for antimicrobial activity as found in naturally occurring peptides[23-26] and model peptides.[27-29] As shown in Table 1, MP-B and its analogs exhibited strong antimicrobial activity against Gram-positive and -negative bacteria. In both bacteria, antimicrobial activity generally decreased as 2MP-B, 4MP-B, 5MP-B, 8MP-B, 11MP-B, and 12MP-B> MP-B. The replacement of amino acid residues located on the hydrophilic side in the amphipathic helix by Ala exhibited stronger activity against both bacteria than MP-B itself. This is almost coincident with the releasing ability of encapsulated calcein from acidic liposomes, indicating that the antimicrobial activity of α-helical peptides against both bacteria can be represented by the binding af-

finity of the peptide to phospholipid bilayers.

According to a recent paper,[11] MP-B showed a marked hemolytic action in the red cells of several species of animals, such as the guinea pig and the rat. MP-B and model peptides showed a high lytic activity against human erythrocytes. However, in a previous report, the replacement of hydrophobic amino acid residues by Ala in MP-B showed less lytic activity against the human erythrocyte.[15] Such specificity of the peptides can be mainly confirmed by the hydrophobicity, conformation, and chemical nature of hydrophobic and cationic sites as proposed by Kini and Evans.[30] According to the experimental results described above, the helical contents of peptides seem to be closely connected with their antimicrobial activities and hemolysis as well as leakage ability. The hydrophobic interaction between the hydrophobic interior of phospholipid bilayers and the hydrophobic amino acid residues also plays an important factor in determining the helical structure. These results indicate that the existence of a tryptophan residue and the appropriate hydrophobicity, including the proper orientation of hydrophilic and hydrophobic groups in MP-B, strongly support an α-helical structure and a variety of biological activities. In addition, these results will be helpful to understand the structure-activity relationship of mastoparan toxins and to design peptides that selectively activate a variety of biological processes, and in understanding the specificity and generality of receptor-G-protein interactions.

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