Molecular Basis for Membrane Selectivity of an Antimicrobial Peptide, Magainin 2

Katsumi Matsuzaki,* Ken-ichi Sugishita, Nobutaka Fujii, and Koichiro Miyajima

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

Received August 22, 1994; Revised Manuscript Received November 29, 1994[⊗]

ABSTRACT: Magainin peptides, isolated from *Xenopus* skin, kill bacteria by permeabilizing their cell membranes whereas they do not lyse erythrocytes. To elucidate the rationale for this membrane selectivity, we compared the effects of the membrane lipid composition and the transmembrane potential on the membrane-lytic power of magainin 2 with that of hemolytic melittin. The activity of magainin to zwitterionic phospholipids constituting the erythrocyte surface was extremely weak compared with that of melittin, and acidic phospholipids are necessary for effective action. The presence of sterols reduced the susceptibility of the membrane to magainin. The generation of an inside-negative transmembrane potential enhanced magainin-induced hemolysis. We can conclude that the absence of any acidic phospholipids on the outer monolayer and the abundant presence of cholesterol, combined with the lack of the transmembrane potential, contribute to the protection of erythrocytes from magainin's attack.

A number of antimicrobial peptides of 15−40 amino acid residues have been discovered in the animal kingdom, playing defensive or offensive roles, for example, defensins from mammalian neutrophiles (Ganz et al., 1990), magainins from frog skin (Sansom, 1991; Zasloff, 1987), cecropins from insects (Steiner et al., 1981), melittin from bee venom (Dempsy, 1990; Sansom, 1991), and tachyplesins from horseshoe crab hemolymph (Nakamura et al., 1988). Microorganisms are also a treasury of peptidic antibiotics, such as alamethicin (Sansom, 1991) and gramicidin S (Ovchinnikov & Ivanov, 1982). Figure 1 depicts the amino acid sequences of some of these peptides. The action mechanisms of these peptides are thought to enhance the permeability of biomembranes, although the detailed mechanisms are not yet fully revealed (Cornut et al., 1993; Saberwal & Nagaraj, 1994; Sansom, 1991). Some peptides such as magainins and cecropins preferentially act on bacterial cells (Steiner et al., 1981; Zasloff, 1987; Chen et al., 1988) whereas others such as melittin and gramicidin S interact with both bacteria and eukaryotic cells, e.g., erythrocytes (Katsu et al., 1989; Steiner et al., 1981). The former two classes of peptides can also discriminate between normal and tumor cells (Cruciani et al., 1991; Jaynes et al., 1989). Elucidation of the underlying principle of this "membrane selectivity" will make a great contribution to developing potent antimicrobial or antitumor drugs with less toxicity.

To this goal, we focused on two major differences between erythrocyte membranes and bacterial plasma membranes, i.e., lipid composition and the magnitude of the transmembrane potential. The outer leaflets of human erythrocyte bilayers are exclusively composed of zwitterionic (electrically neutral) phospholipids (Verkleij et al., 1973), mainly phosphatidylcholine and sphingomyelin. Acidic phospholipids, such as phosphatidylserine, are sequestered in the inner leaflets. On the other hand, bacterial membranes abundantly contain acidic phospholipids (Ratledge & Wilkinson, 1988), such as phosphatidylglycerol, a fraction of which is confirmed to be located in the outer monolayers of the bilayer membranes (Duckworth et al., 1974; Rothman & Kennedy, 1977). Cholesterol is very abundant in erythrocyte membranes (Turner & Rouser, 1970) but absent in bacterial cell membranes (Ratledge & Wilkinson, 1988). The transmembrane potential is well-known to affect peptide−lipid interactions (de Kroon et al., 1991; Sansom, 1991; Vaz Gomes et al., 1993), e.g., by facilitating ion channel formation by peptide self-assembly. Erythrocytes differ from energized bacterial cells in the magnitude of the transmembrane potential. The membrane potential of normal human erythrocytes in standard media is about −9 mV (Rink & Hladky, 1982) whereas respiring bacterial cells have inside-negative potentials of 100−150 mV (Laris & Pershadsingh, 1974; Zilberstein et al., 1979).

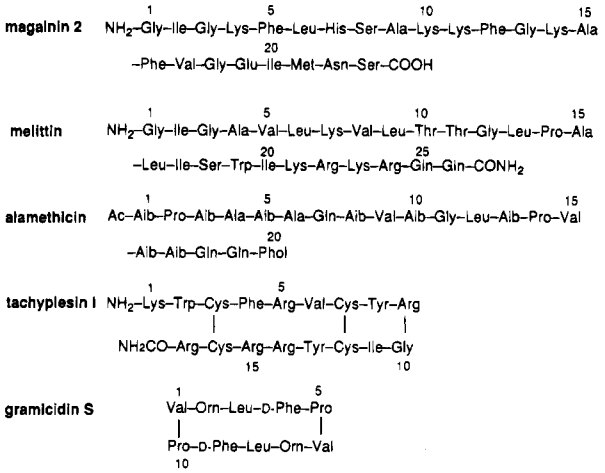

FIGURE 1: Amino acid sequences of five antimicrobial peptides used in this study. Alamethicin is a mixture of microheterogeneous analogs. The representative structure is shown.

Lipid vesicles (liposomes) are a model of biomembranes, useful for examining the effects of lipid composition on

* To whom correspondence should be addressed. Telephone: 81-75-753-4574. Fax: 81-75-761-2698.

[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1995.

0006-2960/95/0434-3423$09.00/0

peptide-induced lysis.[1] The influences of the transmembrane potential can be studied by artificially generating the potential across erythrocyte membranes by use of a potassium ion gradient combined with valinomycin, a potassium ionophore (Rink & Hladky, 1982). Thus, we compared lytic activities of magainin 2 against liposomes and human erythrocytes with those of melittin (Figure 1). We selected these two peptides because the former is practically nonhemolytic whereas the latter is strongly hemolytic (Chen et al., 1988) in spite of their similar molecular size (23 vs 26 amino acid residues), electric charge (+4 vs +6 at physiological pH), and intramembrane conformation (helix). We found that the selectivity appears to arise primarily from the fact that the eukaryotic cell surface is exclusively covered by zwitterionic phospholipids whereas acidic phospholipids are exposed on the bacterial cell surface, although the effects of cholesterol and the transmembrane potential also contribute to the membrane discrimination.

MATERIALS AND METHODS

Materials. Magainin 2, 12-Trp magainin 2, and tachyplesin I were synthesized by use of a Fmoc-based solid phase method and purified by reverse phase high-performance liquid chromatography and gel filtration, as reported previously (Matsuzaki et al., 1991a, 1994). The equipotency of magainin 2 and the Trp-substituted analog has been confirmed (Matsuzaki et al., 1994). Gramicidin S (Sigma, St. Louis, MO) was purified by gel filtration (Sephadex G-15, 2.5 × 35 cm column, 0.02 N HCl being used as an eluent). Alamethicin (Sigma) was used as received. Highly purified melittin was obtained from Boehringer Mannheim (Mannheim, Germany). The absence of phospholipid hydrolysis due to possible contamination of phospholipase A_2 was confirmed by a TLC/FID[2] Iatroscan MK-5 analyzer (Iatron Laboratories, Tokyo, Japan). The purity and concentration of the peptides were determined by quantitative amino acid analysis. The concentration of the Trp-containing peptides was routinely determined by the optical density at 280 nm. Egg yolk L-α-phosphatidylcholine (PC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), egg yolk L-α-phosphatidylethanolamine (PE), bovine brain sphingomyelin (SM), and bovine brain L-α-phosphatidyl-L-serine (PS) were obtained from Sigma. L-α-Phosphatidyl-DL-glycerol (PG) enzymatically converted from PC was a kind gift of Nippon Fine Chemical Co. (Takasago, Japan). Cholesterol (CH) and ergosterol (ER) were products of Sigma and Wako Chemical Co. (Tokyo, Japan), respectively. Sialidase from *Clostridium perfringens* (chromatographically purified), human albumin (essentially fatty acid free), and valinomycin were purchased from Sigma. Phospholipase D from *Streptomyces chromofuscus* was obtained from Asahi Chemical Industry (Sizuoka, Japan). Calcein and spectrograde organic solvents were supplied by Dojindo (Kumamoto, Japan). All other chemicals from Wako were of special grade. A Hepes−NaOH buffer (10 mM Hepes/150 mM NaCl/1 mM EDTA, pH 7.4) was prepared from water twice-distilled in a glass still.

Leakage. Large unilamellar vesicles (LUVs) were prepared and characterized, as described elsewhere (Matsuzaki et al., 1991a, 1994). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated with a 70 mM calcein solution (pH was adjusted to 7.4 with 1 N NaOH) and vortexed. The suspension was freeze-thawed for five cycles and then successively extruded through polycarbonate filters (a 0.6 μm pore size filter × 5 times, two stacked 0.1 μm pore size filters × 10 times). Calcein-entrapped vesicles were separated from free calcein on a Sephadex G-50 column. The lipid concentration was determined by phosphorus analysis (Bartlett, 1959). The release of calcein out of LUVs was monitored by fluorescence at an excitation wavelength of 490 nm and an emission wavelength of 520 nm on a Shimadzu RF-5000 spectrofluorometer, whose cuvette holder was thermostated at 37 ± 0.5 °C. The maximum fluorescence intensity corresponding to 100% leakage was determined by addition of 10% Triton X-100 (20 μL) to 2 mL of the sample. The lipid to peptide ratio at which 50% lysis was observed for 5 min after addition of the peptide, $(L/P)_{50}$, was determined by the linear regression of the dose−response data after the log−logit transformation. The value is expressed as the average ± SD for three independent experiments.

Erythrocyte. Human erythrocytes (blood type A) from a 25-year-old healthy male were freshly prepared prior to each experiment. The blood was centrifuged (800*g*, 10 min) and washed 3 times with the Hepes buffer to remove plasma and buffy coat. Erythrocyte specimens were always kept on ice.

Cholesterol Depletion. CH-depleted erythrocytes were prepared by incubating human erythrocytes (final concentration, 5% v/v) with serum albumin (final concentration, 10 mg/mL) and sonicated DPPC/CH (2:1, mol/mol) vesicles (final concentration, 2.5 mM) at 37 °C for 24 h (Cooper et al., 1975). Albumin-treated erythrocytes were used as the control. The erythrocyte lipids were extracted by the Bligh−Dyer method (New, 1990). The phospholipid and CH contents were determined in triplicate by phosphorus analysis and the TLC/FID analyzer, respectively. The CH to phospholipid ratio was decreased from 1.00 ± 0.07 (normal level) to 0.74 ± 0.03.

Phospholipase D Treatment. Phosphatidic acid (PA) was generated on the erythrocyte surface by phospholipase D treatment (Fujii & Tamura, 1979). Erythrocytes were reacted with the enzyme (80 units/mL of erythrocyte) in a Tris-buffered saline at 37 °C for 2 h. The reaction was stopped by addition of 10 volumes of saline containing 10 mM EDTA. The cells were washed 3 times with Hepes buffer.

Sialidase Treatment. Sialic acids were removed by sialidase treatment. Erythrocytes were incubated with the enzyme (278 units/mL of erythrocyte) in Hepes buffer at 37 °C for 2 h and washed 3 times with the buffer. Sialic acids

[1] In this paper, we use the term "lysis" in the meaning of not solubilization but membrane permeabilization. Westerhoff's group has clearly demonstrated that the magainin-induced permeabilization of mitochondrial (Westerhoff et al., 1989a,b) and liposomal (Juretić et al., 1994) membranes is not due to lysis (rupture or micellization) but reversible pore formation. Our ^{31}P-NMR experiments (Matsuzaki and Seelig, unpublished work) also support this conclusion. However, the other peptides could micellize lipid membranes at low lipid to peptide ratios (e.g., Matsuzaki et al., 1993). In the case of erythrocytes, peptide-induced membrane permeabilization will lead to hemolysis because of the colloid osmotic mechanism (Katsu et al., 1988). Furthermore, as for bacteria, permeabilization-triggered cell death will result in autolysis. In this context, we use for the sake of convenience the term "lysis" irrespective of the actual detailed mechanism.

[2] Abbreviations: TLC, thin-layer chromatography; FID, flame ionization detector; PC, egg yolk L-α-phosphatidylcholine; DPPC, dipalmitoyl-L-α-phosphatidylcholine; PE, egg yolk L-α-phosphatidylethanolamine; SM, bovine brain sphingomyelin; PS, bovine brain L-α-phosphatidyl-L-serine; PG, L-α-phosphatidyl-DL-glycerol enzymatically converted from PC; PA, phosphatidic acid; CH, cholesterol; ER, ergosterol; LUVs, large unilamellar vesicles.

Table 1: Effects of Lipid Composition on Liposome Lysis Induced by Magainin and Melittin[a]

lipid composition[b]	12-Trp magainin 2	melittin
PC	0.9 ± 0.2	101 ± 26
PG	59.9 ± 3.2	23.4 ± 3.1
PS	9.8 ± 0.5	11.9 ± 2.5
PS/PC	9.2 ± 1.7	19.6 ± 5.1
PS/SM	11.6 ± 0.8	24.3 ± 1.6
PS/CH	2.5 ± 0.6	24.0 ± 0.9
PS/PE	2.6 ± 0.3	11.6 ± 1.6
PS/ER	4.9 ± 0.4	18.9 ± 1.0

[a] The liposome lysis was determined on the basis of the leakage of calcein from LUVs of different lipid compositions. The lipid to peptide ratio at which 50% lysis was observed for 5 min after addition of the peptide, $(L/P)_{50}$, is listed as a function of the lipid composition. Mean ± SD (n = 3). [b] In the mixed lipid systems, the molar ratio of PS to the other component was always 2:1 to keep the membrane charge density similar.

were almost completely cleaved, as determined by the thiobarbituric acid assay (Warren, 1959).

Generation of Transmembrane Potential. Erythrocytes were suspended in a buffer [10 mM Hepes/150 mM (NaCl + KCl)/1 mM EDTA, pH 7.4] containing 97 mM (−9 mV) or 4 mM (−70 mV) K^+. The former potassium concentration, which corresponds to the resting potential, was determined by the "null-point method" (Rink & Hladky, 1982). The transmembrane potential was generated by addition of valinomycin (6.7 μM for 1% hematocrit). The establishment of the potential was confirmed by use of a potential-sensitive dye, 3,3′-dipropylthiodicarbocyanine.

Hemolysis. Each peptide solution at various concentrations was incubated with an erythrocyte suspension (final erythrocyte concentration, 1% v/v) for 1 h at 37 °C. The percent hemolysis was determined from the optical density at 540 nm of the supernatant after centrifugation (800g, 10 min), as described elsewhere (Matsuzaki et al., 1988). Hypotonically lysed erythrocytes were used as the standard for 100% hemolysis. The ED_{50} value (M) was calculated as the $(L/P)_{50}$ value.

Peptide Binding. The binding of 12-Trp magainin 2 to erythrocytes was determined under a nonhemolytic condition. A peptide solution (final concentration, 2 μM) was incubated with an erythrocyte suspension (final concentration, 1% v/v) for 10 min at 37 °C. The concentration of the free peptide was determined by measuring the Trp fluorescence of the supernatant after centrifugation (800g, 10 min). The fluorescence from a trace amount of contaminating hemoglobin was corrected.

RESULTS

Zwitterionic Phospholipids. During the first part of our work, we investigated the influences of lipid composition on the lysis of liposomes. Lysis was measured on the basis of leakage of a fluorescent dye, calcein, entrapped within LUVs composed of lipids occurring in erythrocyte or bacterial cell membranes (Matsuzaki et al., 1994). The lipid membranes used are in the fluid (liquid-crystalline) state, mimicking actual biomembranes. The results were expressed as $(L/P)_{50}$ values, the lipid to peptide molar ratio at which 50% leakage was observed for the initial 5 min (Table 1). A larger value implies a stronger activity. Magainin-induced permeabilization of PC bilayers was 2 orders of magnitude

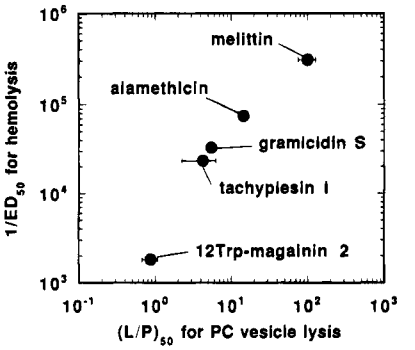

FIGURE 2: Relationship between peptides' lytic activities against human erythrocytes and PC liposomes. The hemolytic activity is expressed by the inverse of the ED_{50} value, the peptide concentration at which 50% hemolysis was observed. PC liposome-lytic activity was denoted by the $(L/P)_{50}$ value (see Table 1). The error bars represent the SD for three experiments.

Table 2: Effects of Phospholipase D Treatment on Binding of 12-Trp Magainin 2 to Erythrocytes

treatment	% bound[a]
none	5.9 ± 4.6
phospholipase D	19.0 ± 1.4[b]
chlorpromazine	5.9 ± 1.4

[a] A peptide solution (final concentration, 2 μM) was incubated with an erythrocyte suspension (final concentration, 1% v/v) for 10 min at 37 °C. The percent of the bound peptide is listed. Mean ± SD (n = 3). [b] Significant, $P < 0.05$.

weaker than that by melittin. The lytic activity against SM was similar to that against PC because the $(L/P)_{50}$ values against PS/PC (2:1, mol/mol) and PS/SM (2:1, mol/mol) vesicles were almost identical. Thus, the weak activity of magainin to zwitterionic lipids constituting the erythrocyte surface can be, at least partly, responsible for its low hemolytic activity. To confirm this hypothesis, the lytic activities of various peptides (Figure 1) against PC LUVs were compared with those against human erythrocytes. The latter was expressed with the inverse of the ED_{50} value (M^{-1}). A good correlation was found between both activities (Figure 2).

PE has some protective effects on the lysis induced by both peptides (Table 1); the $(L/P)_{50}$ values for PS/PE (2:1, mol/mol) were 2−4-fold smaller than those for PS/PC.

Acidic Phospholipids. Magainin lysed acidic phospholipids, PS and PG, as effectively as melittin (Table 1). Both peptides are reported to comparably enhance the respiration and permeability of cytochrome c oxidase liposomes composed of negatively charged asolectin (Juretić et al., 1994). Thus, both peptides are expected to kill bacteria by binding to exposed acidic phospholipids whereas they cannot react with erythrocyte PS which is sequestered in the inner leaflet. The absence of any acidic phospholipids on the erythrocyte surface appears to result in weak binding of magainin to the cell. Table 2 shows that only 6% of 12-Trp magainin 2 was bound, corresponding to ca. 1.5 mmol of bound peptide per mole of membrane lipid at the free peptide concentration of 1.9 μM. This weak binding is comparable to the binding to PC sonicated vesicles (Matsuzaki and Seelig, unpublished work). Generation of negatively charged PA on the erythrocyte surface by phospholipase D treatment enhanced the binding by 3-fold (Table 2). This enzyme treatment is known to cause a morphological change of the erythrocyte,

Table 3: Effects of Sialidase Treatment on Hemolysis Induced by Five Peptides[a]

peptide	control	sialidase treatment
melittin	307 ± 6	186 ± 2[b]
alamethicin	74.6 ± 0.5	40.3 ± 0.6[b]
gramicidin S	32.8 ± 0.3	31.8 ± 0.5
tachyplesin I	23.1 ± 0.3	23.1 ± 0.8
12-Trp magainin 2	1.82 ± 0.12	1.40 ± 0.05[c]

[a] The $1/ED_{50}$ value ($\times 10^3$ M^{-1}) for hemolysis of human erythrocytes. Mean ± SD ($n = 3$). [b] Significant, $P < 0.001$. [c] Significant, $P < 0.005$.

Table 4: Effects of Cholesterol and Transmembrane Potential on Hemolysis Induced by Magainin and Melittin[a]

factor	treatment	magainin 2 ($\times 10^3$ M^{-1})	melittin ($\times 10^6$ M^{-1})
cholesterol[b]	control	0.61 ± 0.05	4.29 ± 0.11
	CH-depleted	3.22 ± 0.41[d]	4.81 ± 0.41
potential[c]	−9 mV	1.36 ± 0.06	4.31 ± 0.12
	−70 mV	2.97 ± 0.10[d]	4.16 ± 0.25

[a] The $1/ED_{50}$ value for hemolysis of human erythrocytes. Mean ± SD ($n = 3$). [b] CH-depleted erythrocytes were prepared by incubating human erythrocytes with serum albumin and sonicated DPPC/CH (2:1, mol/mol) vesicles. Albumin-treated erythrocytes were used as the control. [c] Erythrocytes were suspended in a buffer [10 mM Hepes/150 mM (NaCl + KCl)/1 mM EDTA, pH 7.4] containing 97 mM (−9 mV) or 4 mM (−70 mV) K^+. The transmembrane potential was generated by addition of valinomycin. [d] Significant, $P < 0.001$.

i.e., stomatocyte formation (Fujii & Tamura, 1979). To examine the effect of this shape change on the binding, erythrocytes were treated for 5 min with 0.1 mM chlorpromazine, which also invaginates erythrocytes (Deuticke, 1968). The extents of invagination by both agents were similar, as examined by microscopy. Table 2 suggests that the shape change per se did not affect the binding of magainin to the membrane.

Sialic Acid. Negatively charged sialic acid moieties of sugar chains on the erythrocyte surface could also constitute binding sites for basic peptides to play some role in the peptide−erythrocyte interactions. Table 3 indicates that removal of sialic acid by sialidase treatment did not affect or slightly reduced the hemolytic activities of the five peptides. The binding of 12-Trp magainin 2 under non-hemolytic conditions was not significantly modified by the enzymatic treatment (data not shown).

Sterols. The incorporation of sterols in the membrane reduced the lytic activity of magainin. Table 1 shows that the presence of CH inhibited lysis of liposomes by magainin; the $(L/P)_{50}$ value for PS/CH (2:1, mol/mol) liposomes was about one-fourth of that for PS/PC. In contrast, CH showed no suppressive effects on melittin-induced lysis. We obtained parallel results on hemolysis induced by both peptides (Table 4); CH depletion enhanced the inverse of the ED_{50} value of magainin-induced hemolysis by 5-fold whereas it exerted no effect on melittin-mediated hemolysis. Table 1 shows that ER also possesses inhibitory effects on magainin-induced membrane lysis, although its efficacy is about half of that of CH.

Transmembrane Potential. Table 4 shows that an increase in the transmembrane potential from a physiological potential of ca. −9 mV to ca. −70 mV enhanced the hemolytic activity of magainin by about 2-fold. The potential did not affect melittin-induced hemolysis.

DISCUSSION

The membrane selectivity of vertebrate antimicrobial peptides was found for the first time in cecropins (Steiner et al., 1981) followed by magainins (Zasloff, 1987; Chen et al., 1988; Cruciani et al., 1991) and dermaseptins (Mor et al., 1991). The rationale for this has been discussed; however, a definite conclusion has not yet been reached. A number of observations have indicated that the primary target of these peptides is membranes; for example, magainins dissipate the membrane potential of *E. coli* (Westerhoff et al., 1989a; Juretić et al., 1989), hamster spermatozoa (de Waal et al., 1991), and rat liver mitochondria (Westerhoff et al., 1989a,b). In fact, the loss of the potential and the resultant uncoupled respiration are closely related to the loss of motility of the spermatozoa. Magainins also permeabilize lipid vesicles both with (Juretić et al., 1989, 1994) and without (Matsuzaki et al., 1989, 1991a, 1994; Vaz Gomes et al., 1993) proteins. Although factors other than membranes might also affect the membrane selectivity; e.g., the degradation of the peptides by endogenous proteases (Westerhoff et al., 1989a,b; Juretić et al., 1989) and the extent of dependence of the respiration on the transmembrane potential (Westerhoff et al., 1989a), we focused on membranes and used erythrocytes as a representative eukaryotic cell. The information obtained in this study will be also helpful for one to understand the interactions of these peptides with organelle membranes.

Christensen et al. (1988) suggested that the weak hemolytic activity of cecropin is ascribable to the presence of CH in erythrocytes whereas Steiner et al. (1988) rejected the involvement of lipid composition in membrane selectivity. The involvement of CH in magainin's membrane selectivity has also been hinted (Westerhoff et al., 1989a). On the other hand, it is inferred that acidic phospholipids in addition to CH also contribute to the membrane discrimination of magainin and related peptides (Jackson et al., 1992). The role of the transmembrane potential has also been argued (Steiner et al., 1988; Vaz Gomes et al., 1993). It is thus important to clarify the extent of contribution of each factor to membrane recognition by performing systematic studies. Therefore, we examined the effects of lipid composition and the transmembrane potential on magainin-induced membrane lysis and compared them with the effects on melittin-induced lysis.

Lipid Matrix. It is generally considered that membrane lipids constitute the main target of these peptides, or at least that peptide−lipid interactions are necessary for their activity. For example, all-D enantiomer peptides are equipotent in antimicrobial and ion channel-forming activities to the parent all-L peptides; they permeabilize both bacterial and artificial lipid membranes without specific interactions with chiral receptors or enzymes (Wade et al., 1990). Tosteson et al. (1985) concluded that the binding sites of melittin on erythrocytes are not specialized receptors but regions of the lipid bilayers that are relatively nonspecific, although this peptide has been reported to interact with membrane proteins (Clague & Cherry, 1989). Our results support the importance of the lipid matrix in peptide−erythrocyte interactions. First, the effects of CH on peptide-induced liposome lysis (Table 1) paralleled those on hemolysis (Table 4). Second, the weak binding of 12-Trp magainin 2 to erythrocytes is comparable with that to PC vesicles (Matsuzaki and Seelig, unpublished

work). Finally, the lytic activities of the five peptides against PC LUVs are correlated with those against human erythrocytes (Figure 2). It should be noted that the five peptides used here differ significantly from each other in size, electric charge, and secondary structure (Figure 1). The largest peptide, melittin, has 26 amino acid residues whereas the smallest, gramicidin S, is composed of only 10 amino acids. Alamethicin has −1 to 0 charge whereas the other peptides are positively charged (+2 to +7) at physiological pH. Tachyplesin I and gramicidin S conform to cyclic β-sheets while the others form helices.

What properties of peptides determine their activities against PC bilayers? The lytic power of a lysin is considered to be partly governed by the affinity of the lysin to membranes, which is related to lysin's hydrophobicity. The binding constants of the three helical peptides, i.e., magainin 2 (Matsuzaki and Seelig, unpublished work), alamethicin (Rizzo et al., 1987), and melittin (Beschiaschvili & Seelig, 1990), to PC sonicated vesicles have been reported to be on the order of 10^2, 10^3, and 10^4 M^{-1}, respectively, in keeping with the order of lytic activity (Figure 2). The mean hydrophobicities [a modified version of the normalized consensus scale of Eisenberg et al. (Sansom, 1991)] of magainin 2, alamethicin, and melittin (residues 1−20, the hydrophobic part) are 0.17, 0.54, and 0.62, respectively, coinciding with the order of membrane affinity. Tachyplesin I, having a rather low mean hydrophobicity (−0.4), reacts with the membranes more strongly than magainin (Figure 2). This is partly ascribable to a much smaller entropic loss in the binding free energy, because tachyplesin I takes a cyclic β-sheet structure both in aqueous (Tamamura et al., 1993) and in membranous (Matsuzaki et al., 1993) environments. We found no correlation between the peptide's hydrophobic moment and its lytic activity, although the former provides a quantitative measure to compare the lytic activity of the same series of analogs (Cornut et al., 1993).

Electrostatic Attractions. Electrostatic interactions between positively charged peptides and negatively charged lipids enhance the binding of these peptides to membranes (Batenburg et al., 1988; Matsuzaki et al., 1989, 1991b; Vaz Gomes et al., 1993). Indeed, the formation of PA on the erythrocyte surface enhanced the binding of 12-Trp magainin 2 (Table 2).[3] The binding appears to be governed by electrostatic interactions; in the mixed lipid systems in Table 1, the molar ratio of PS to the other component was always 2 to keep the membrane charge density similar. The binding isotherms of magainin 2 to these mixed lipid membranes were comparable to each other (Matsuzaki et al., unpublished work). However, the mode of action could differ among acidic phospholipids as well as between acidic and zwitterionic lipids; magainin and melittin more effectively lyse PG bilayers than PS membranes (Table 1). The lytic power of the latter peptide against acidic phospholipids is weaker than that against PC bilayers (Table 1; Dempsy et al., 1989) in spite of the stronger binding to the latter membrane (Batenburg et al., 1988). A deeper partitioning of the peptide into acidic phospholipid bilayers has been reported (Batenburg et al., 1987).

Sialic acids could modify the peptide−erythrocyte interactions. They might trap the peptides away from the membrane surface, preventing the membrane from peptide's attack. Alternatively, they might increase the local peptide concentration near the membrane surface through electrostatic interactions, enhancing hemolysis. Removal of sialic acid by sialidase treatment never increased hemolysis induced by the peptides (Table 3), arguing against the former possibility. The latter hypothesis, however, appears not to be valid. The sialidase treatment did not affect hemolysis induced by the most positively charged peptide, tachyplesin I, whereas it slightly weakened the lytic activity of alamethicin, which possesses no positive charge.[4] Thus, sialic acids do not appear to constitute the binding sites. A 1% v/v erythrocyte suspension contains ca. 5 μM sialic acid. Assuming a stoichiometric strong association of one peptide to four sialic acids, one can expect about 50% binding of 2 μM magainin. On the contrary, the observed binding percent was much smaller (6%, Table 2).

Inhibitory Lipids. The abundant presence of CH in erythrocytes has been suggested to be the rationale for the membrane selectivity. We found that CH has inhibitory effects on magainin-induced lysis of both liposomes (Table 1) and erythrocytes (Table 4), in keeping with the observation that CH inhibits magainin-induced disordering of phospholipid bilayers, as revealed by ^{2}H-NMR (Bechinger et al., 1992). PE also exhibits the lysis inhibitory effects (Table 1). However, this lipid does not help magainin to preferentially react with bacterial membranes; the extent of its exposure to the bacterial membrane surface is generally larger than that to the erythrocyte surface. The reasons for the inhibitory effects of sterols and PE are not clear at present, especially in that the former effects are specific for magainin.

Transmembrane Potential. An inside-negative transmembrane potential facilitated magainin-induced hemolysis (Table 4). Cruciani et al. (1991) reported that depolarization prevents cytotoxic activity of magainin peptides against tumor cells in accordance with our results. Furthermore, the potential-induced increase in magainin activity has been shown in asolectin sonicated liposomes (Vaz Gomes et al., 1993). The membrane permeabilization induced by other amphiphilic peptides, such as a mitochondrial presequence (Roise et al., 1986), is known to be enhanced by the presence of the potential. The latter peptide has recently been shown to translocate across phospholipid bilayers (Maduke & Roise, 1993). If magainin molecules also translocate into the inside of the cell across the membrane, as we have suggested (Matsuzaki et al., 1994), the inside-negative potential electrophoretically facilitates incorporation of the positively charged peptide. Melittin was insensitive to the transmembrane potential (Table 4) whereas Claque and Cherry (1988) reported that melittin-mediated hemolysis was moderately enhanced by the inside-negative potential. The major

[3] It is estimated that about one-third of PC was converted to PA, as judged from the reaction condition and the morphological change (Fujii & Tamura, 1979).

[4] The reason for the apparent reductions in the $1/ED_{50}$ value is not clear at present, although they are partly ascribable to the increase in the cell concentration. The concentration of erythrocytes was determined on the basis of the packed volume of the cell. The sialidase treatment increased the packing density by up to ca. 20%, as judged from the optical density corresponding to 100% hemolysis. The removal of sialic acid per se does not appear to affect the fragility of erythrocytes, because the hypotonic hemolysis occurred in the same salt concentration range irrespective of the enzyme treatment (data not shown).

difference between the two studies is the incubation time, i.e., 1 h in our study vs 3.5 min in the latter. The potential might modify the kinetics of hemolysis.

CONCLUSION

Our results show that the following three factors contribute to the protection of erythrocytes from magainin attack, although other factors, such as peptide−membrane protein interactions, might be involved: (1) the weak lytic activity of magainin to zwitterionic lipids constituting the erythrocyte surface and the absence of exposed acidic phospholipids in erythrocytes; (2) the abundant presence of cholesterol; and (3) the absence of a large transmembrane potential. These factors also appear to facilitate the magainin−mitochondria interactions (Westerhoff et al., 1989a,b); the inner membranes of rat liver mitochondria have abundant cardiolipin, poor CH, and the inside-negative potential (Daum, 1985). The first point appears to be the main cause for the membrane selectivity because the lytic activities of magainin against PC vesicles and erythrocytes are 2 orders of magnitude smaller than those of melittin (Figure 2) whereas sterols and the transmembrane potential only modify lytic activity by less than 1 order of magnitude. This hypothesis can also explain the membrane selectivity of cecropins (Steiner et al., 1981) and dermaseptins (Mor & Nicolas, 1994); they do not lyse erythrocytes but permeabilize bacterial membranes, and they practically do not interact with zwitterionic phospholipids (Mchaourab et al., 1994; Mor & Nicolas, 1994). It is also reported that cecropins scarcely bind to erythrocytes (Steiner et al., 1988). Furthermore, succinylation of melittin greatly reduced lysis of both PC vesicles (Stankowski et al., 1991) and erythrocytes (Dufton et al., 1984). As for the selective tumoricidal activity of magainins and cecropins, recent studies showed that tumorigenic cells express 3−8-fold more PS in their cell surface than do nontumorigenic cells (Connor et al., 1989; Utsugi et al., 1991). Jaynes et al. (1989) suggested that alterations in the cytoskeleton of transformed cells increase their sensitivity to the cytolytic activity exerted by cecropins. The involvement of the cytoskeleton in the maintenance of lipid asymmetry has been discussed (Devaux, 1991). In this connection, the spectrin−actin system of erythrocytes might play some role in the interactions with these peptides. Even in such a case the peptides should first bind to and then pass through the lipid matrix, the interactions with which will be crucial.

The suggestion that factors 2 and 3 do not play a decisive role is supported by the following observations. Magainin is active against yeasts and fungi (Zasloff, 1987; Zasloff et al., 1988) containing ER. Tumor cells have potentials (−5 to −40 mV) smaller than those (−40 to −90 mV) of normal nonproliferating cells (Binggeli & Weinstein, 1986).

Electrostatic attractions are necessary for effective binding of magainin to membranes. Negatively charged components, such as acidic phospholipids (Matsuzaki et al., 1989, 1991b; Vaz Gomes et al., 1993) and lipopolysaccharides (Rana et al., 1990), appear to play an important role in selective toxicity to bacterial and tumor cells. A structure−activity relationship study (Chen et al., 1988) has shown that the hemolytic activity of magainin analogs generally increases in parallel to the antimicrobial activity, suggesting that the cell affinity of the peptide is determined by (1) the intrinsic affinity mainly due to the hydrophobic effect (the binding constant to PC) and (2) the electrostatic part, and that the derivatization only modifying the former part will not improve the membrane selectivity.

ACKNOWLEDGMENT

We thank Prof. T. Fujii of the Kyoto College of Pharmacy for helpful discussions on phospholipase D treatment. We are also indebted to Nippon Fine Chemicals Co. for their kind gift of PG.

REFERENCES

Batenburg, A. M., Hibbeln, J. C. L., & De Kruijff, B. (1987) *Biochim. Biophys. Acta 903*, 155−165.
Batenburg, A. M., Van Esch, J. H., & De Kruijff, B. (1988) *Biochemistry 27*, 2324−2331.
Bechinger, B., Zasloff, M., & Opella, S. J. (1992) *Biophys. J. 62*, 12−14.
Beschiaschvili, G., & Seelig, J. (1990) *Biochemistry 29*, 52−58.
Binggeli, R., & Weinstein, R. C. (1986) *J. Theor. Biol. 123*, 377−401.
Chen, H.-C., Brown, J. H., Morell, J. L., & Huang, C. M. (1988) *FEBS Lett. 236*, 462−466.
Clague, M. J., & Cherry, R. J. (1988) *Biochem. J. 252*, 791−794.
Clague, M. J., & Cherry, R. J. (1989) *Biochim. Biophys. Acta 980*, 93−99.
Connor, J., Bucana, C., Fidler, I. J., & Schroit, A. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3184−3188.
Cooper, R. A., Arner, E. C., Wiley, J. S., & Shattil, S. (1975) *J. Clin. Invest. 55*, 115−126.
Cornut, I., Thiaudiére, E., & Dufourcq, J. (1993) in *The Amphipathic Helix* (Epand, R. M., Ed.) pp 173−220, CRC Press, Boca Raton, FL.
Cristensen, B., Fink, J., Merrifield, R. B., & Mauzerall, D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5072−5076.
Cruciani, R. A., Barker, J. L., Zasloff, M., Chen, H.-C., & Colamonici, O. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3792−3796.
Daum, G. (1985) *Biochim. Biophys. Acta 822*, 1−42.
De Kroon, A. I. P. M., De Gier, J., & De Kruijff, B. (1991) *Biochim. Biophys. Acta 1068*, 111−124.
Dempsy, C. E. (1990) *Biochim. Biophys. Acta 1031*, 143−161.
Dempsy, C., Bitbol, M., & Watts, A. (1989) *Biochemistry 28*, 6590−6596.
Deuticke, B. (1968) *Biochim. Biophys. Acta 163*, 494−500.
Devaux, P. F. (1991) *Biochemistry 30*, 1163−1173.
De Waal, A., Vaz Gomes, A., Mensink, A., Grootegoed, J. A., & Westerhoff, H. V. (1991) *FEBS Lett. 293*, 219−223.
Duckworth, D. H., Becers, E. M., Verkleij, A. J., Kamp, J. A. F. O. D., & Deenen, L. L. M. V. (1974) *Arch. Biochem. Biophys. 165*, 379−387.
Dufton, M. J., Hider, R. C., & Cherry, R. J. (1984) *Eur. Biophys. J. 11*, 17−24.
Fujii, T., & Tamura, A. (1979) *J. Biochem. 86*, 1345−1352.
Ganz, T., Selsted, M. E., & Lehrer, R. I. (1990) *Eur. J. Hematol. 44*, 1−8.
Jackson, M., Mantsch, H. H., & Spencer, J. (1992) *Biochemistry 31*, 7289−7293.
Jaynes, J. M., Julian, G. R., Jeffers, G. W., White, K. L., & Enright, F. M. (1989) *Peptide Res. 2*, 157−160.
Juretić, D., Chen, H.-C., Brown, J. H., Morell, J. L., Hendler, R. W., & Westerhoff, H. V. (1989) *FEBS Lett. 249*, 219−223.
Juretić, D., Hendler, R. W., Kamp, F., Caughey, W. S., Zasloff, M., & Westerhoff, H. V. (1994) *Biochemistry 33*, 4562−4570.
Katsu, T., Ninomiya, C., Kuroko, M., Kobayashi, H., Hirota, T., & Fujita, Y. (1988) *Biochim. Biophys. Acta 939*, 57−63.
Katsu, T., Kuroko, M., Morikawa, T., Sanchika, K., Fujita, Y., Yamamura, H., & Uda, M. (1989) *Biochim. Biophys. Acta 983*, 135−141.
Laris, P. C., & Pershadsingh, H. A. (1974) *Biochem. Biophys. Res. Commun. 57*, 620−626.
Maduke, M., & Roise, D. (1993) *Science 260*, 364−367.
Matsuzaki, K., Handa, T., Miyajima, K., Mikura, Y., Shimizu, H., & Toguchi, H. (1988) *Chem. Pharm. Bull. 36*, 4253−4260.

Matsuzaki, K., Harada, M., Handa, T., Funakoshi, S., Fujii, N., Yajima, H., & Miyajima, K. (1989) *Biochim. Biophys. Acta 981*, 130−134.
Matsuzaki, K., Fukui, M., Fujii, N., & Miyajima, K. (1991a) *Biochim. Biophys. Acta 1070*, 259−264.
Matsuzaki, K., Harada, M., Funakoshi, S., Fujii, N., & Miyajima, K. (1991b) *Biochim. Biophys. Acta 1063*, 162−170.
Matsuzaki, K., Nakayama, M., Fukui, M., Otaka, A., Funakoshi, S., Fujii, N., Bessho, K., & Miyajima, K. (1993) *Biochemistry 32*, 11704−11710.
Matsuzaki, K., Murase, O., Tokuda, H., Funakoshi, S., Fujii, N., & Miyajima, K. (1994) *Biochemistry 33*, 3342−3349.
Mchaourab, H. S., Hyde, J. S., & Feix, J. B. (1994) *Biochemistry 33*, 6691−6699.
Mor, A., & Nicolas, P. (1994) *J. Biol. Chem. 269*, 1934−1939.
Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., & Nicolas, P. (1991) *Biochemistry 30*, 8824−8830.
Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., & Shimonishi, Y. (1988) *J. Biol. Chem. 263*, 16709−16712.
Ovchinnikov, Y. A., & Ivanov, V. T. (1982) in *The Proteins* (Neurath, H., & Hill, R. L., Eds.) pp 391−398, Academic Press, New York.
Rana, F. R., Sultany, C. M., & Blazyk, J. (1990) *FEBS Lett. 261*, 464−467.
Rink, T. J., & Hladky, S. B. (1982) in *Red Cell Membranes—A Methodological Approach* (Ellory, J. C., & Young, J. D., Eds.) pp 321−334, Academic Press, London.
Rizzo, V., Stankowski, S., & Schwarz, G. (1987) *Biochemistry 26*, 2751−2759.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327−1334.
Rothman, J. E., & Kennedy, E. P. (1977) *J. Mol. Biol. 110*, 603−618.
Saberwal, G., & Nagaraj, R. (1994) *Biochim. Biophys. Acta 1197*, 109−131.
Sansom, M. S. P. (1991) *Prog. Biophys. Mol. Biol. 55*, 139−235.
Stankowski, S., Pawlak, M., Kaisheva, E., Robert, C. H., & Schwarz, G. (1991) *Biochim. Biophys. Acta 1069*, 77−86.
Steiner, H., Andreu, D., & Merrifield, R. B. (1988) *Biochim. Biophys. Acta 939*, 260−266.
Steiner, H., Hultmark, D., Engström, Å., Bennich, H., & Boman, H. G. (1981) *Nature 292*, 246−248.
Tamamura, H., Kuroda, M., Masuda, M., Otaka, A., Funakoshi, S., Nakashima, H., Yamamoto, N., Waki, M., Matsumoto, A., Lancelin, J. M., Kohda, D., Tate, S., Inagaki, F., & Fujii, N. (1993) *Biochim. Biophys. Acta 1163*, 209−216.
Tosteson, M. T., Holmes, S. J., Razin, M., & Tosteson, D. C. (1985) *J. Membr. Biol. 87*, 35−44.
Turner, J. D., & Rouser, G. (1970) *Anal. Biochem. 38*, 423−436.
Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., & Fidler, I. J. (1991) *Cancer Res. 51*, 3062−3066.
Vaz Gomes, A., de Waal, A., Berden, J. A., & Westerhoff, H. V. (1993) *Biochemistry 32*, 5365−5372.
Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & Van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178−193.
Wade, D., Boman, A., Wåhlin, B., Drain, C. M., Andreu, D., Boman, H. G., & Merrifield, R. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4761−4765.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971−1975.
Westerhoff, H. V., Juretić, D., Hendler, R. W., & Zasloff, M. (1989a) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6597−6601.
Westerhoff, H. V., Hendler, R. W., Zasloff, M., & Juretić, D. (1989b) *Biochim. Biophys. Acta 975*, 361−369.
Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5449−5453.
Zasloff, M., Martin, B., & Chen, H.-C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 910−913.
Zilberstein, D., Schuldiner, S., & Padan, E. (1979) *Biochemistry 18*, 669−673.

BI941937D