Peptide Hydrophobicity Controls the Activity and Selectivity of Magainin 2 Amide in Interaction with Membranes

Torsten Wieprecht,[‡] Margitta Dathe,*[,‡] Michael Beyermann,[‡] Eberhard Krause,[‡] W. Lee Maloy,[§] Dorothy L. MacDonald,[§] and Michael Bienert[‡]

Forschungsinstitut für Molekulare Pharmakologie, Alfred Kowalke Strasse 4, D-10315 Berlin, Germany, and Magainin Pharmaceuticals, Inc., 5110 Campus Drive, Plymouth Meeting, Pennsylvania 19462

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ABSTRACT: The magainins are antibacterial peptides from the skin of *Xenopus laevis*. They show a broad range of activity against prokaryotic cells but lyse eukaryotic cells poorly. To elucidate the influence of peptide hydrophobicity on membrane activity and selectivity, we designed and synthesized analogs of magainin 2 amide with slightly varying hydrophobicities but retained hydrophobic moment, peptide charge, and angle subtended by the hydrophilic helix region. Circular dichroism investigations of the peptides revealed that all peptides investigated adopt an α-helical conformation when bound to phospholipid vesicles. Dye-releasing experiments from vesicles of phosphatidylglycerol (PG) showed that the membrane-permeabilizing activity of the analogs is not influenced by peptide hydrophobicity. In contrast, the permeability-enhancing activity on vesicles bearing high amounts of phosphatidylcholine (PC) increases drastically with enhanced peptide hydrophobicity, resulting in a reduced selectivity of more hydrophobic analogs for negatively charged membranes. Likewise, the peptide affinity to PC-rich membranes increases in the order of hydrophobicity. Correlation of peptide binding and membrane permeabilization of PC/PG (3:1) vesicles revealed that the observed differences in peptide activity on membranes of low negative surface charge are mainly caused by the different binding affinities. The antibacterial and hemolytic activity of the peptides increases with enhanced hydrophobicity. A strong correlation was found between the hemolytic effect and the bilayer-permeabilizing activity against PC-rich vesicles. Whereas the antibacterial specificity of the more hydrophobic analogs is retained for *Escherichia coli*, the specificity for *Pseudomonas aeruginosa* decreases with increasing hydrophobicity.

Magainins are a class of antimicrobial peptides isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff, 1987). They have been shown to inhibit the growth of a wide range of bacteria, fungi, protozoa, and some kinds of tumor cells but are not hemolytic (Zasloff, 1987; Zasloff et al., 1988; Soravia et al., 1988; Cruciani et al., 1991; Baker et al., 1993). Peptides in this group have a net positive charge and the potential to adopt an amphiphilic α-helical conformation (Zasloff, 1987; Zasloff et al., 1988). Magainins have been shown to act by enhancing the permeability of biological membranes (Westerhoff et al., 1989; Matsuzaki et al., 1989, 1991; Juretic et al., 1994). Although the molecular mechanism of their action is not yet fully understood, there is consensus that in the first step of the membrane-disturbing process the peptides fold into amphiphilic α-helices when binding to the lipid membrane. In the following step, the peptides are thought to permeabilize the bilayer either by the formation of pores consisting of associated peptide helices or by fluctuating destabilization of the lipid bilayer caused by a disruption of the packing of lipid acyl chains (Duclohier et al., 1989; Cruciani et al., 1992; Grant et al., 1992; Matsuzaki et al., 1995a).

Investigations of the interaction of magainin 2 with biological and model membranes revealed that the peptide preferentially interacts with membranes containing a high amount of acidic phospholipids and a small amount of cholesterol and possessing an inside-negative transmembrane potential (Matsuzaki et al., 1991, 1995b; Vaz Gomes et al., 1993; Tytler et al., 1995). These findings led to the suggestion that the antibacterial specificity of magainin is caused by differences in the lipid composition of prokaryotic and eukaryotic cell membranes. Prokaryotic cell membranes are in contrast to eukaryotic membranes generally characterized by a high content of negatively charged phospholipids, a small amount of cholesterol, and a high, inside-negative transmembrane potential (Gennis, 1989; Lugtenberg & van Alphen, 1983; Zilberstein et al., 1979) and are hence expected to be highly susceptible to magainins.

Structure−activity studies of magainin analogs revealed that the enhancement of helicity and of the cationic charge leads to a higher antibacterial activity (Chen et al., 1988; Pathak et al., 1995; Bessalle et al., 1992; Maloy & Kari, 1995). But mostly, sequence modifications causing enhanced helicity resulted also in higher hemolytic activity. Other changes in the primary structure of magainin described so far affect the angle subtended by the hydrophobic helix face, the hydrophobicity, and the hydrophobic moment. Several modifications of the peptide led to more active antibacterial analogs, sometimes with and sometimes without an increase in hemolytic activity (Chen et al., 1988; Bessalle et al., 1992; Maloy & Kari, 1995; Pathak et al., 1995). Until now, the effect of such amino acid substitutions on activity and selectivity is poorly predictable. The main reason is that the modifications often change more than one of the

* Author to whom correspondence should be addressed at Forschunginstitut für Molekulare Pharmakologie.
[‡] Forschungsinstitut für Molekulare Pharmakologie.
[§] Magainin Pharmaceuticals.
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biophysical properties which are thought to influence peptide−lipid interactions. Additionally, the knowledge about the effect of each of these properties on membrane activity is very limited.

This study systematically investigates the influence of slight variations in peptide hydrophobicity on the ability of magainin 2 amide (M2a)[1] to interact with phospholipid vesicles as well as bacterial and red blood cell membranes. To elucidate the influence of peptide hydrophobicity on membrane activity and selectivity, we employed a new approach of minimal sequence modification, which allowed the other structural parameters such as peptide charge, charge distribution, hydrophobic moment, α-helix propensity, and angle subtended by the hydrophilic helix domain to be kept largely constant. We investigated the conformation of the peptides in the vesicle-bound state by circular dichroism (CD) spectroscopy, studied the abilities of the analogs to induce dye release from vesicles of different surface charge, and investigated the antibacterial and hemolytic activity. Furthermore, peptide binding at vesicles has been compared with the membrane-disturbing activity of the bound peptide.

The studies lead to the conclusion that slight modifications of peptide hydrophobicity effectively modulate the activity of M2a against membranes bearing a high amount of zwitterionic phospholipid by causing changes in peptide affinity. The structural changes influence peptide specificity for negatively charged lipid bilayers and can lead to alterations in peptide selectivity for Gram-negative bacterial membranes.

EXPERIMENTAL PROCEDURES

Materials. The lipids 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) were purchased from Avanti Polar Lipids, Inc., Alabaster, AL. Calcein was obtained from Fluka Chemie (Neu-Ulm, Germany), tris(hydroxymethyl)aminomethane (Tris) from Merck KGaA (Darmstadt, Germany), 2,2,2-trifluoroethanol (TFE) from Aldrich-Chemie (Steinheim, Germany), and the Fmoc amino acids from Novabiochem (Bad Soden, Germany). All other chemicals were of reagent grade.

Peptide Synthesis. Peptides were synthesized automatically by solid-phase methods using standard Fmoc chemistry on Tenta Gel S RAM resin (0.21 mmol/g; RAPP Polymere, Tübingen, Germany) in the continuous-flow mode on a MilliGen 9050 (Millipore, MA) peptide synthesizer (Beyermann et al., 1992). Purification was carried out by preparative HPLC on PolyEncap A300, 10 μm (250 × 20 mm i.d.) (Bischoff Analysentechnik GmbH, Leonberg, Germany), to give final products >95% pure by high-performance liquid chromatography (HPLC) analysis. All peptides were characterized by matrix-assisted laser desorption ionization mass spectrometry (MALDI II; Kratos, Manchester, U.K.) with the peptide content of lyophilized samples being determined by quantitative amino acid analysis (LC 3000, Biotronik-Eppendorf, Germany).

[1] Abbreviations: M2a, magainin 2 amide; CD, circular dichroism; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol; Tris, tris(hydroxymethyl)aminomethane; TFE, 2,2,2-trifluoroethanol; HPLC, high-performance liquid chromatography; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles.

Reversed-Phase HPLC. Chromatographic measurements were performed on a Shimadzu LC-10A gradient HPLC system consisting of two LC-10AD pumps, a SIL-10A autoinjector, a SPD-M10A diode array detector operating at 215 nm, and a CLASS-LC10 software package. Runs were carried out on a PolyEncap A300 column (250 × 4.6 mm i.d., 5 μm; Bischoff Analysentechnik GmbH, Leonberg, Germany).

The sample concentration was 1 mg/mL peptide in 0.1% trifluoroacetic acid (eluent A) with an injection volume of 20 μL. Separations were performed at 22 °C (thermostated system) and at an eluent flow rate of 1 mL/min. The precision of the retention time was ±0.1 min. Mobile phase A was 0.1% trifluoroacetic acid in water, and B was 0.1% trifluoroacetic acid in 50% acetonitrile/50% water (v/v). The retention times of the peptides were determined using a linear gradient 1−95% B in 40 min.

Small Unilamellar Vesicle Preparation. A lipid film was dried overnight under high vacuum and then suspended by vortex mixing in buffer [10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)] to give a final lipid concentration between 20 and 40 mM. The suspension was sonicated (under nitrogen, in ice/water) for 25 min using a titanium tip ultrasonicator. Titanium debris was removed by centrifugation. Dynamic light scattering experiments confirmed the existence of a main population of vesicles (more than 95% mass content) with a mean diameter of 41 ± 1 nm (polydispersity index: 0.3). For preparation of calcein-containing small unilamellar vesicles (SUVs), 70 mM calcein buffer solution was added to the dried lipid. Untrapped calcein was removed from the vesicles by gel filtration on a Sephadex G75 column [eluent: buffer containing 10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)].

Large Unilamellar Vesicle Preparation. After vortexing the dried lipid in calcein buffer solution [70 mM calcein, 154 mM NaCl, 10 mM Tris, 0.1 mM EDTA (pH 7.4)], the suspension was freeze-thawed in liquid nitrogen for seven cycles and extruded through polycarbonate filters (Hope et al., 1985) (6 times through two stacked 0.4 μm pore size filters followed by 8 times through two stacked 0.1 μm pore size filters). Untrapped calcein was removed from the large unilamellar vesicles (LUVs) by gel filtration on a Sephadex G75 column [eluent: buffer containing 10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)]. Lipid concentration was determined by phosphorus analysis (Böttcher et al., 1961).

Circular Dichroism Measurements. CD measurements of the peptide solutions were carried out on a Jasco 720 spectrometer between 190 and 260 nm in TFE and between 200 and 260 nm in vesicle suspension at 23°C. Minor contributions of circular dichroism and circular differential scattering of the SUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free suspensions. The helicity of the peptides was determined from the mean residue ellipticity [Θ] at 222 nm (Chen et al., 1972). All data are the mean of two independent measurements which do not deviate more than 5%.

Binding Isotherms. CD-derived binding isotherms were determined from the changes of the CD of peptide solutions (three different concentrations between 5×10^{-5} M and 5×10^{-6} M) after adding different amounts of SUVs. For determination of binding isotherms, the following relations were used: (a) $F = \Theta_{222}(0) - \Theta_{222}$ where F is the relative

signal, $\Theta_{222}(0)$ the ellipticity in the absence of lipid, and Θ_{222} the measured ellipticity in the presence of lipid; and (b) $F = F_\infty(c_B/c_P) = F_\infty(c_L/c_P)r$ where F_∞ is F of the completely bound peptide, c_L the lipid concentration, c_P the total peptide concentration, c_B the lipid-bound peptide concentration, and $r = c_B/c_L$. From these equations and the mass conservation equation, the binding isotherm can be evaluated [for a detailed description, see Schwarz and Beschiaschvili (1989)].

Calcein Release Assay. Aliquots of the vesicular suspension (10−20 μL) were injected into a cuvette containing 2.5 mL of a stirred peptide solution at 23 °C. Calcein release from vesicles was determined fluorometrically by measuring the decrease in self-quenching (excitation at 490 nm, emission at 520 nm) on a Perkin-Elmer LS 50B spectrofluorometer. The fluorescence intensity corresponding to 100% release was determined by addition of Triton X-100 (100 μL, 10% v/v in water).

Hemolytic Assay. The hemolytic activity of the peptides was determined using human red blood cells as described previously (Dathe et al., 1996). In brief, the suspensions containing the peptide and 1.8×10^8 cells/mL were incubated for 30 min at 37 °C. After cooling in ice/water and centrifugation, an aliquot of the supernatant was dissolved with 0.5% NH_4OH, and the optical density was measured at 540 nm. Peptide concentrations causing 50% hemolysis (EC_{50}) were derived from the dose−response curves. Values determined in repeat experiments differed by less than 5%.

Antibacterial Studies. Antimicrobial susceptibility testing was performed using a modification of the National Committee for Clinical Laboratory Standards (NCCLS) microdilution broth assay (1993). Mueller Hinton broth (BBL, Cockeysville, MD) was used for diluting the peptide stock solution and for dilution of the bacterial inoculum. The inoculum was prepared from mid-logarithmic phase cultures. The final concentration of bacteria in the wells was $(1-5) \times 10^5$ CFUs/mL. The final concentration of peptide solution ranged from 0.25 to 256 μg/mL in 2-fold dilution. Peptides were tested in duplicate. The microtiter plates were incubated overnight at 37 °C, and the absorbance was red at 630 nm. The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of peptide that completely inhibits growth.

RESULTS

Peptide Design. Amino acid substitutions in amphiphilic helical peptides may generally alter their structural parameters such as helical propensity, peptide charge, angle subtended by the hydrophobic helix domain, hydrophobic moment, and hydrophobicity. Since all these structural features are expected to modulate the interaction of peptides with biological as well as model membranes, we employed an approach of minimal sequence modification to design magainin analogs of different peptide hydrophobicity but largely conserved other structural properties. Starting from the C-terminal amidated magainin 2 (M2a), up to four amino acids were substituted under the following rules: (i) The four glycine residues of the peptide chain were not replaced because substitution of Gly by most of the other amino acids is expected to enhance helical propensity. (ii) The number and positions of the charged amino acids were maintained to avoid modifications of the electrostatic interaction properties. (iii) If possible, hydrophobic amino acids ($H > 0$; Eisenberg, 1984) were substituted by hydrophobic ones, and hydrophilic residues ($H < 0$) were replaced by hydrophilic amino acids. Thus, the angles of the hydrophobic and hydrophilic helix domains remained constant. (iv) The hydrophobic moment of M2a was preserved in the analogs by simultaneous amino acid substitutions in the hydrophobic and hydrophilic cores of the amphiphilic helix.

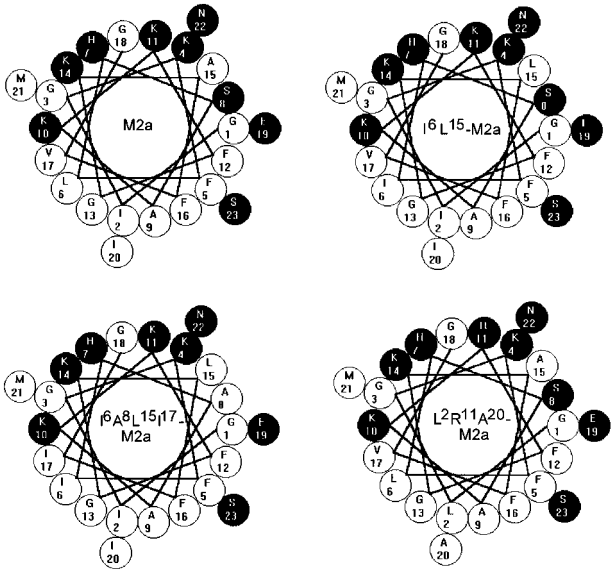

FIGURE 1: Helical wheel projection of M2a and the hydrophobicity-modified analogs. Black circles refer to charged or hydrophilic amino acids and white circles to hydrophobic residues.

Table 1: Mean Residue Hydrophobicities (H), Hydrophobic Moments (M), and HPLC Retention Times (t_R) of the Peptides[a]

peptide	H	M	t_R (min)
$L^2R^{11}A^{20}$-M2a	−0.0957	0.287	24.4
M2a	−0.0357	0.286	26.6
I^6L^{15}-M2a	−0.0148	0.284	27.7
$I^6A^8L^{15}I^{17}$-M2a	0.0157	0.280	30.3

[a] Mean residue hydrophobicities and hydrophobic moments per residue were calculated using the Eisenberg consensus scale of hydrophobicity (Eisenberg, 1984). Retention times were determined on a PolyEncap A300 column according to Experimental Procedures.

The helical wheel presentations of M2a and the derived analogs are given in Figure 1. The mean residue hydrophobicities of the peptides have been calculated to range between +0.0157 and −0.0957 using the Eisenberg consensus scale of hydrophobicity (Eisenberg, 1984) and follow the order $I^6A^8L^{15}I^{17}$-M2a > I^6L^{15}-M2a > M2a > $L^2R^{11}A^{20}$-M2a (Table 1). All peptides have an approximately constant hydrophobic moment of 0.283 ± 0.004.

To verify the differences in peptide hydrophobicity based on the Eisenberg consensus scale experimentally, we determined the reversed-phase HPLC retention times of the analogs. Since the chain length and the hydrophobic moment of all analogs are identical, the retention times of the analogs should increase with increasing peptide hydrophobicity. The retention times correlate well with the calculated peptide hydrophobicities, confirming the expected order of peptide hydrophobicity (Table 1).

Conformational Investigations. CD spectroscopic investigations of M2a and the analogs in Tris-buffered saline confirm an unordered peptide conformation (data not shown). The CD spectra of the peptides in the presence of TFE, a solvent suitable to investigate the helical propensity of

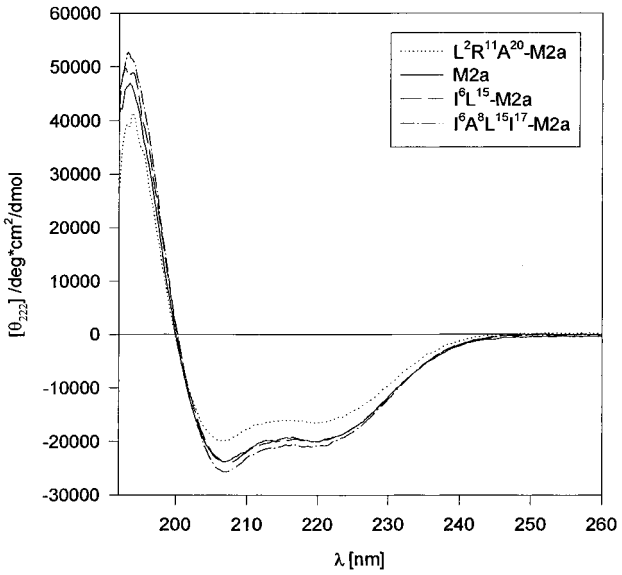

FIGURE 2: CD spectra of M2a and the hydrophobicity-modified analogs in 1:1 TFE/buffer (v/v). Peptide concentration was 50 μM in buffer [10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)].

Table 2: α-Helicity of M2a and Modified Analogs in Different Environments[a]

	solvent/lipid			
peptide	1:1 TFE/buffer (v/v) (%)	POPG SUV (%)	POPC/POPG (3:1) SUV (%)	POPC SUV (%)
$L^2R^{11}A^{20}$-M2a	45	58	56	18
M2a	57	77	77	51
I^6L^{15}-M2a	57	74	73	52
$I^6A^8L^{15}I^{17}$-M2a	61	76	80	73

[a] Helical content was evaluated according to Chen et al. (1972). The peptide concentration was 50 μM and the lipid concentration 8.3 mM in buffer [10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)]. All data are the mean of two independent measurements which do not deviate by more than 5%.

peptides (Lehrman et al., 1990), are characteristic of helical conformation (Figure 2). The helicity of the peptides in 1:1 TFE/buffer (v/v) ranges between 45% and 61% (Table 2). Whereas the helical contents of $I^6A^8L^{15}I^{17}$-M2a, I^6L^{15}-M2a, and M2a are quite similar, the helicity of the most hydrophilic analog $L^2R^{11}A^{20}$-M2a is reduced by more than 10%.

Likewise, in vesicle suspensions, the peptides fold into an α-helical conformation. In the presence of negatively charged POPG SUVs and POPC/POPG (3:1) SUVs (lipid:peptide ratio of 166), M2a and the analogs were found to be completely vesicle-bound as verified by titration experiments of peptide solutions with vesicle suspensions (data not shown). Hence, the calculated helicities represent the helicities of the completely lipid-bound peptides (Table 2). While the helicities of $I^6A^8L^{15}I^{17}$-M2a, I^6L^{15}-M2a and M2a range between 73% and 80%, the helicity of $L^2R^{11}A^{20}$-M2a was calculated to be 57%. Larger differences in helicity have been found in a POPC SUV suspension (lipid:peptide ratio of 166). The helicity of the peptides increases in the order $L^2R^{11}A^{20}$-M2a (18%) < M2a (51%) ≤ I^6L^{15}-M2a (52%) < $I^6A^8L^{15}I^{17}$-M2a (73%). Recent investigations revealed that M2a exhibits a helical content of about 75% in the completely POPC-bound state (Wieprecht et al., 1996). Thus, the observed helicity of M2a of only 51% should result from incomplete peptide binding under the conditions used. With respect to the little differentiated helical propensity of M2a and the analogs (Table 2, columns 2−4), the highly different helicity of the peptides in the presence of POPC liposomes should reflect differences in the binding affinity rather than differences in the conformation of the lipid-bound peptides. Conclusively, the affinity of the peptides to zwitterionic POPC vesicles increases with increasing peptide hydrophobicity.

Table 3: EC_{50} Values of the Initial Rate of Calcein Release from LUVs of Different Surface Charge Density[a]

	EC_{50} (μM)			
peptide	POPG LUV	POPC/POPG (1:1) LUV	POPC/POPG (3:1) LUV	POPC LUV
$L^2R^{11}A^{20}$-M2a	0.16 ± 0.01	0.52 ± 0.04	7.45 ± 0.05	65 ± 1
M2a	0.18 ± 0.01	0.29 ± 0.02	1.20 ± 0.10	5.8 ± 0.2
I^6L^{15}-M2a	0.11 ± 0.01	0.17 ± 0.02	0.52 ± 0.04	2.2 ± 0.2
$I^6A^8L^{15}I^{17}$-M2a	0.22 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.22 ± 0.03

[a] E_{50} values are the peptide concentrations inducing 50% dye-release after 1 minute. Values are the mean of two independent measures ± error. The lipid concentrations were 25 μM in buffer [10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)].

Permeabilization of Vesicles. The membrane-permeabilizing abilities of M2a and the analogs have been investigated by dye release from LUVs of different surface charge density. In addition to the highly negatively charged POPG LUVs and the electrically neutral POPC LUVs, we employed mixed vesicles of different POPG contents, POPC/POPG (1:1) and POPC/POPG (3:1). The EC_{50} values of initial calcein leakage (50% dye release after the initial 1 min) have been used as a measure for the membrane permeability-enhancing effect of the peptides (Table 3). Comparing the EC_{50} values, all peptides were found to permeabilize highly negatively charged POPG and POPC/POPG (1:1) LUVs in a narrow, submicromolar concentration range. No correlation between peptide hydrophobicity and leakage was observed for POPG LUVs (Figure 3A; Table 3, column 2), whereas the dye-releasing activity on POPC/POPG (1:1) LUVs increases slightly with increasing hydrophobicity in the order $L^2R^{11}A^{20}$-M2a < M2a < I^6L^{15}-M2a < $I^6A^8L^{15}I^{17}$-M2a (Table 3, column 3).

A substantial differentiation in the permeabilization ability of the peptides was found for vesicles bearing high amounts of zwitterionic lipid [POPC/POPG (3:1) and POPC vesicles]. The EC_{50} of the most hydrophilic analog, $L^2R^{11}A^{20}$-M2a, on POPC/POPG (3:1) LUVs was 7.45 μM, while a 62-fold lower concentration of the most hydrophobic peptide, $I^6A^8L^{15}I^{17}$-M2a, caused the same releasing effect (EC_{50} = 0.12 μM) (Table 3, column 4). The membrane-permeabilizing activity strongly increases with enhancing peptide hydrophobicity. The same order of membrane activity has been found for the interaction of M2a and the analogs with electrically neutral POPC vesicles (Figure 3B; Table 3, column 5). However, the differences in the permeabilizing ability are more pronounced compared to those on POPC/POPG (3:1) LUVs. The most hydrophilic peptide, $L^2R^{11}A^{20}$-M2a, is almost inactive on POPC vesicles (EC_{50} = 65 μM), while the EC_{50} of the most hydrophobic $I^6A^8L^{15}I^{17}$-M2a is 0.22 μM (295 times more active).

Comparing the membrane selectivity of the analogs, it can be concluded that the membrane selectivity of the peptides for negatively charged model membranes decreases with increasing peptide hydrophobicity. The activity of the most hydrophobic analog, $I^6A^8L^{15}I^{17}$-M2a is almost independent

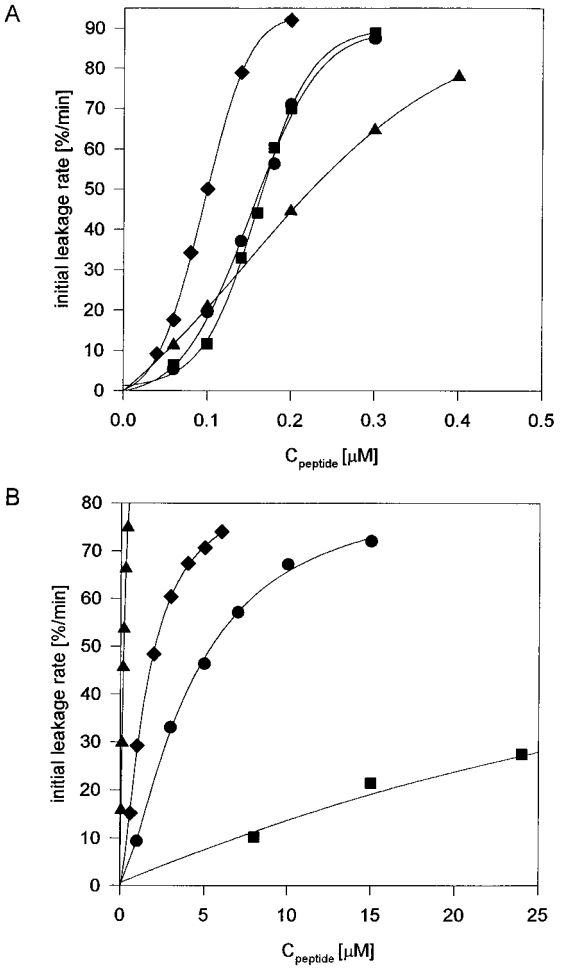

FIGURE 3: Initial calcein leakage rate as a function of total peptide concentration. Initial calcein leakage rate is defined as the percent leakage after 1 min for a lipid concentration of 25 μM: (A) POPG LUVs; (B) POPC LUVs. Symbols: $I^6A^8L^{15}I^{17}$-M2a (triangles); I^6L^{15}-M2a (diamonds); M2a (circles); and $L^2R^{11}A^{20}$-M2a (squares).

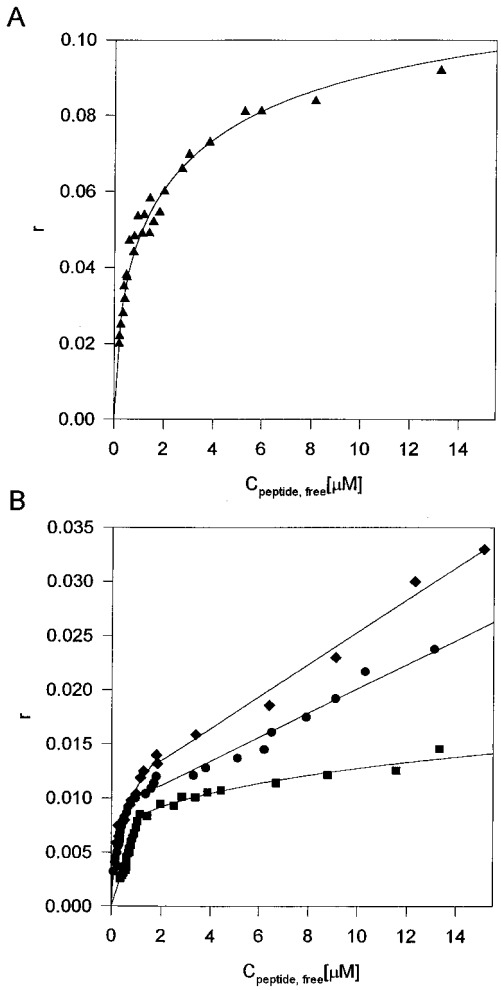

FIGURE 4: Binding isotherms for the interactions of (A) $I^6A^8L^{15}I^{17}$-M2a (triangles), (B) I^6L^{15}-M2a (diamonds), M2a (circles), and $L^2R^{11}A^{20}$-M2a (squares) with POPC/POPG (3:1) SUVs. Isotherms were derived by titration of three different peptide concentrations with vesicle suspensions.

of membrane composition, and the peptide permeabilizes all vesicle systems in a comparable, submicromolar concentration range.

Membrane-Binding and -Disturbing Activity. The different permeabilization activity of the peptides on vesicles with a high amount of zwitterionic lipids [POPC, POPC/POPG (3:1)] may be caused by differences in the binding affinity of the peptides and/or by differences in the membrane-disturbing activity of the bound peptide fraction. In order to determine the reason for the different peptide activity, we derived binding isotherms of all peptides on POPC/POPG (3:1) SUVs by titration of peptide solutions with vesicle suspensions using CD spectroscopy (Figure 4). The binding isotherms clearly show that the affinity of the peptides for the vesicles decreases with reduction of peptide hydrophobicity in the order $I^6A^8L^{15}I^{17}$-M2a > I^6L^{15}-M2a > M2a > $L^2R^{11}A^{20}$-M2a. The apparent binding constants, K_{app} (interpolated for $c_{peptide,free} = 0$ M) (Table 4), reveal that the most hydrophobic analog $I^6A^8L^{15}I^{17}$-M2a has a 14-fold higher apparent binding affinity than the most hydrophilic peptide $L^2R^{11}A^{20}$-M2a.

Calcein-releasing experiments from POPC/POPG (3:1) SUVs were performed in order to correlate peptide binding and membrane permeabilization (Figure 5A). The peptide concentrations inducing 50% calcein release were found to be 0.34 μM for $I^6A^8L^{15}I^{17}$-M2a, 0.66 μM for I^6L^{15}-M2a, 1.25 μM for M2a, and 7.3 μM for $L^2R^{11}A^{20}$-M2a (Table 4). From the total peptide and lipid concentrations (Figure 5A) and the binding isotherms (Figure 4), the ratios of bound peptide per lipid (r) corresponding to a given calcein release were calculated (Figure 5B). From figure 5B, it is seen that peptides of different hydrophobicitiy exhibit only slight differences in the amount of bound peptide per lipid necessary for the induction of 50% dye release (Table 4). The peptides significantly enhance membrane permeability

Table 4: Characterization of Binding to and Permeabilization of POPC/POPG (3:1) SUVs: Apparent Binding Constants (K_{app}),[a] EC_{50} Values of Calcein Release, Concentration of Free Peptide ($c_{peptide,free}$), and Ratio of Bound Peptide per Lipid (r) at Half-Maximal Dye Release[b]

peptide	K_{app} (M^{-1})	EC_{50} (μM)	$c_{peptide,free}$ (μM)	r
$L^2R^{11}A^{20}$-M2a	7400	7.3	7.0	0.0118
M2a	20000	1.25	1.00	0.0102
I^6L^{15}-M2a	25000	0.66	0.46	0.0085
$I^6A^8L^{15}I^{17}$-M2a	105000	0.34	0.095	0.0098

[a] Apparent binding constants on POPC/POPG (3:1) SUVs were calculated from the initial slope of the binding isotherms (Figure 5A). [b] EC_{50} values are the peptide concentrations inducing 50% calcein release from POPC/POPG (3:1) SUVs (25 μM) in buffer [10 mM Tris, 154 mM NaCl, 0.1 mM EDTA (pH 7.4)] after 5 min, when calcein release has reached almost a constant level.

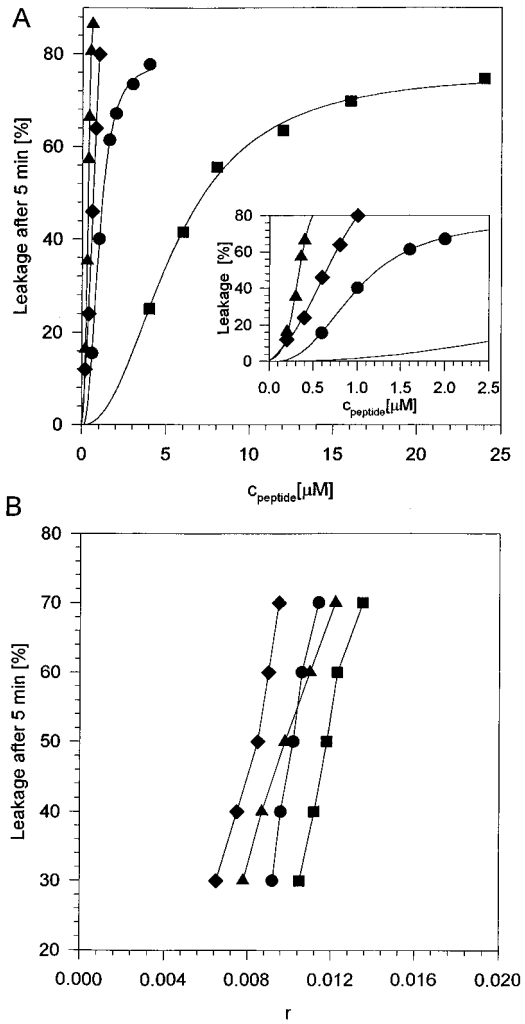

FIGURE 5: (A) Dependence of calcein leakage from POPC/POPG (3:1) SUVs on total peptide concentration. Calcein leakage is defined as the percent leakage after 5 min at a lipid concentration of 25 μM. The inset is a magnification for low total peptide concentrations. (B) Relationship between the percent calcein leakage and the molar ratio of bound peptide per lipid. Symbols: $I^6A^8L^{15}I^{17}$-M2a (triangles); I^6L^{15}-M2a (diamonds); M2a (circles); and $L^2R^{11}A^{20}$-M2a (squares).

Table 5: Antibacterial and Hemolytic Activity of the Peptides [a]

	antibacterial activity MIC (μM)		hemolytic activity	
peptide	*E. coli*	*P. aeruginosa*	ED_{50} (μM)	*L* (%)
$L^2R^{11}A^{20}$-M2a	75	>75	>1000	12
M2a	38	76	430	30
I^6L^{15}-M2a	19−38	76	260	40
$I^6A^8L^{15}I^{17}$-M2a	2.4	19−38	32	98

[a] MIC is the minimum inhibitory concentration of bacterial growth and EC_{50} the concentration of half-maximal lysis of erythrocytes. *L* (%) gives the percent lysis at 150 μM peptide concentration. The values determined in two experiments differed by less than 5%.

when about 10 peptide molecules are bound per 1000 lipid molecules.

Biological Activity. Table 5 shows the antibacterial activity of the peptides against the Gram-negative bacterial strains *Escherichia coli* and *Pseudomonas aeruginosa* as well as the effect on red blood cells. The minimum inhibitory concentration of magainin 2 amide against bacteria is considerably lower than the EC_{50} of the hemolytic effect, thus revealing higher activity against bacteria than erythrocytes. Enhancement of hydrophobicity increases both the hemolytic and the antibacterial effect. A drastic increase in biological activity has been observed by increasing the hydrophobicity from −0.0148 (I^6L^{15}-M2a) to 0.0157 ($I^6A^8L^{15}I^{17}$-M2a). In contrast, reduction of hydrophobicity ($L^2R^{11}A^{20}$-M2a vs M2a) decreases biological activity.

Enhancement of hydrophobicity increases activity against *Escherichia coli* and erythrocytes by approximately the same factor, thus conserving the antibacterial selectivity. But, whereas the hemolytic activity of $I^6A^8L^{15}I^{17}$ M2a compared to M2a increased by a factor of about 13, the activity against *Pseudomonas aeruginosa* is only 2−4 times higher. Consequently, an increase of hydrophobicity results in a reduced peptide specificity for *Pseudomonas aeruginosa*.

DISCUSSION

Several structure−activity studies about the magainins and other antibacterial peptides have been published in recent years (Maloy & Kari, 1995; Zhong et al., 1995; Perez-Paya et al., 1995; Sitaram et al., 1995; Pathak et al., 1995; Javadpour et al., 1996). It is well documented that biophysical properties such as secondary structure, overall charge, hydrophobicity, and hydrophobic moment influence the interaction of antibiotic peptides with model membranes and biological cells. Most of the modifications in the primary structure of amphiphilic peptides result in changes of more than one of these structural parameters. For example, enhancement of the peptide overall cationic charge may additionally change the hydrophobicity, the hydrophobic moment, and the angle subtended by the hydrophilic helix region, and introduction of highly hydrophobic residues in a peptide sequence generally affects both the hydrophobic moment and the hydrophobicity of the peptide. Therefore, it is often difficult to determine the biophysical parameter which is responsible for the observed effect. Furthermore, the membrane activity of peptides can generally be influenced by modulation of the two steps of the peptide−membrane interaction: (i) peptide binding to the membrane and (ii) membrane-disturbing activity of the bound molecules. Knowledge of the influence of the biophysical parameters on both steps of the permeabilization process will give insight in the driving forces of peptide binding and membrane disturbance.

The basis for this study was a set of carefully designed peptides of varying hydrophobicities. Minimal peptide sequence modifications led to M2a analogs of different hydrophobicities whereas other peptide properties such as peptide charge, charge distribution, hydrophobic moment, α-helix propensity, and angle subtended by the hydrophilic helix domain were kept almost constant.

CD spectroscopic investigations of M2a and the hydrophobicity-modified analogs in a TFE/buffer (1:1) mixture and in SUV suspensions revealed that all M2a analogs retain the ability to assume an α-helical conformation (Figure 2, Table 2). Whereas $I^6A^8L^{15}I^{17}$-M2a, I^6L^{15}-M2a, and M2a exhibit a similar helical content in TFE/buffer (between 57% and 61%) and when bound at negatively charged phospholipid vesicles (between 73% and 80%), the helicity of the most hydrophilic analog $L^2R^{11}A^{20}$-M2a is slightly reduced under all conditions. Since hydrophobic interactions between the hydrophobic side chains and the hydrophobic environment make a large contribution to the stabilization of helical conformations, it can be deduced that the reduced helicity

of $L^2R^{11}A^{20}$-M2a is related to less effective hydrophobic interactions. This is in agreement with the observations of Deber and Li (1995), who reported that the helicity of model peptides in a membrane environment correlates well with peptide hydrophobicity. M2a was described to be helical over the entire peptide chain in TFE/buffer and when bound to phospholipid vesicles (Bechinger et al., 1993; Wieprecht et al., 1996). Therefore, the change in helicity observed for $L^2R^{11}A^{20}$-M2a should result from an overall helix destabilization rather than from the formation of a nonhelical region within the peptide chain.

The dye-releasing experiments clearly demonstrate that the membrane-permeabilizing effect of the peptides on membranes bearing a high content of zwitterionic phosphatidylcholine [POPC/POPG (3:1), POPC] strongly correlates with peptide hydrophobicity (Table 3, columns 4 and 5). Increasing peptide hydrophobicity is associated with an enhanced membrane-permeabilizing activity. In these bilayer systems, hydrophobic interactions predominate the peptide-membrane interactions. Consequently, alteration of peptide hydrophobicity significantly affects the membrane-permeabilizing effect. A similar correlation between peptide hydrophobicity and permeabilization of phosphatidylcholine membranes has recently been reported comparing the activity of magainin 2, alamethicin, and melittin (Matsuzaki et al., 1995b). However, these three peptides exhibit quite different overall peptide charges, charge distributions, and helicities which are all known to be effective modulators of activity.

The cell membrane of human erythrocytes is characterized by a high content of zwitterionic phospholipids (e.g., phosphatidylcholine, sphingomyelin and phosphatidylethanolamine) and the absence of negatively charged lipids in the outer leaflet of the membrane (Verkleij et al., 1973).

A comparison of the permeabilizing activity of the peptides on POPC-rich vesicles and the hemolytic effect reveals strong correlation. The observation leads to the conclusion that the hemolytic effect is mainly determined by the interaction of the peptides with the electrically neutral lipid matrix of the red blood cells and therefore dictated by hydrophobic interactions. These results are in agreement with our previous investigations of a set of KLAL model peptides with different helicity and hydrophobicity (Dathe et al., 1996).

The specificity of magainins for prokaryotic membranes has been suggested to be caused by differences in the lipid composition of prokaryotic and eukaryotic cell membranes, particularly by the high content of negatively charged groups in the outer bacterial membrane and the lack of negatively charged phospholipids in the outer leaflet of most eukaryotic cell membranes (Matsuzaki et al., 1995b).

Our studies show that all investigated magainin peptides permeabilize highly negatively charged POPG and POPC/POPG (1:1) LUVs in a narrow, submicromolar concentration range (Table 3, columns 2 and 3). Furthermore, differences in the activity on POPG LUVs do not correlate with peptide hydrophobicity. Obviously, hydrophobic interactions play a secondary role, and the activity of the peptides on membranes with a high content of acidic phospholipids is determined by electrostatic interactions between the oppositely charged amino acid side chains and lipid head groups. This confirms previous investigations which reported a dominating electrostatic contribution in the interaction of magainins with negatively charged lipid bilayers (Matsuzaki et al., 1989; Vaz Gomes et al., 1993; Wieprecht et al., 1996).

Sequence modifications, which alter peptide activity on membranes mainly composed of zwitterionic lipids (Table 3, columns 4 and 5) but conserve their permeabilizing ability on highly negatively charged membranes (Table 3, columns 2 and 3), lead consequently to variations in membrane selectivity. Thus, the similar EC_{50} values of $I^6A^8L^{15}I^{17}$-M2a for all investigated lipid systems show that increasing the hydrophobicity of M2a by substitution of four amino acids ($I^6A^8L^{15}I^{17}$) eliminates selectivity for negatively charged model membranes.

Gram-negative bacterial envelops are composed of an outer and an inner membrane. The outer membrane contains a high amount of negatively charged lipopolysaccharides whereas the inner membrane is comparable to that of mammalian cells (Christensen et al., 1988). The lipopolysaccharide membrane may be expected to bear an even higher negative surface charge density than a pure POPG membrane (Dathe et al., 1996). Because of the dominance of electrostatic interactions, it is reasonable to assume that the peptides display similar activity in permeabilizing the outer bacterial membrane. After overcoming the outer barrier, the peptides bind to and permeabilize the inner membrane in the order of their hydrophobicity. These different interactions at the two different neighboring membranes of the bacterial cell wall might explain the higher antibacterial than hemolytic activity of the peptides (Table 5).

Regarding the influence of peptide hydrophobicity on antibacterial specificity, the picture is equivocal. Increasing hydrophobicity enhances the activity against *Escherichia coli* and the hemolytic activity almost by the same factor, thus retaining antibacterial selectivity. In contrast, the activity against *Pseudomonas aeruginosa* increases from M2a to $I^6A^8L^{15}I^{17}$-M2a only 2−4 times whereas the hemolytic activity increases by a factor of about 13, revealing a lower antibacterial selectivity for more hydrophobic peptides. It seems that electrostatic interactions are still more important for the overall process of membrane permeabilization of *Pseudomonas aeruginosa* than for *Escherichia coli.* The finding that an enhanced hydrophobicity may result in a decreased antibacterial specificity is in agreement with structure−activity studies of Prasad et al. (1992). They found that an increase in peptide hydrophobicity results in enhanced hemolysis and decreased specificity.

The different abilities of M2a and the analogs to permeabilize vesicles with high amounts of zwitterionic lipids may be caused by different lipid-binding and/or by different membrane-disturbing activities of the bound peptide. Correlation of the calcein-releasing experiments from POPC/POPG (3:1) SUVs with binding isotherms revealed that all analogs significantly enhance the permeability of POPC/POPG (3:1) membranes when approximately 10 peptide molecules are bound per 1000 lipid molecules (Figure 5B). Comparing the significant differences in peptide binding (binding isotherms, Figure 4; apparent binding constants, Table 4) and lytic activity (Figure 5A) with the almost identical number of bound peptide per lipid (r) inducing membrane permeabilization (Figure 5B), we conclude that the different abilities of the peptides to permeabilize POPC/POPG (3:1) vesicles are mainly caused by differences in the lipid-binding affinity of the peptides. The slight differences in the r values are of subordinate importance.

Our strategy of minimal sequence modifications allows the recognition of how sensitive membrane activity depends on peptide hydrophobicity. The benefit of our method becomes particularly clear when relating to a recent study of Pathak et al. (1995). They compared the influence of hydrophobicity, the hydrophobic moment, and helicity on antibacterial activity for a variety of peptides derived from the antisense peptide of magainin 2. Using multiple linear regression, Pathak et al. (1995) reported that the hydrophobic moment was the most important factor governing antimicrobial activity. They found that by including either hydrophobic moment or hydrophobicity in the regression equation, they obtained values of 0.91 and 0.46, respectively, for the square of the correlation coefficient. Consideration of both parameters in the regression scarcely improved the square of the correlation coefficient ($r^2 = 0.93$). The poor correlation between hydrophobicity and activity ($r^2 = 0.46$) and the only slight improvement of the quality of the regression by considering hydrophobicity in addition to the hydrophobic moment imply a less important and scarcely predictable influence of hydrophobicity on activity. However, the peptides investigated in their study have a quite different overall charge and angle subtended by the positively charged helix face. Especially, the enhancement of peptide charge by introduction of positively charged amino acids in the polar helix face strengthens the hydrophobic moment but generally decreases hydrophobicity. Such an enhancement of positive peptide charge has been shown to dramatically increase antibacterial activity (Bessalle et al., 1992; Maloy & Kari, 1995) and can be explained by charge-dominated peptide accumulation at the bacterial membrane. Substitution of cationic amino acids by negatively charged residues does not significantly affect hydrophobicity and the hydrophobic moment, but can be expected to drastically modify the interaction with negatively charged cell membranes. Therefore, the overall peptide charge in connection with the hydrophobic properties has to be taken into consideration as an additional factor modulating peptide−lipid interactions.

In summary, slight enhancement of the hydrophobicity of magainin peptides was shown to increase the permeabilizing ability on POPC-rich membranes as well as the hemolytic and antibacterial effect. Furthermore, we could show that enhanced activity is mainly caused by higher binding affinity of the peptides toward the membranes. The selectivity for negatively charged model membranes decreases with increasing hydrophobicity. Also, the antibacterial selectivity for *Pseudomonas aeruginosa* over erythrocytes decreases with growing hydrophobicity. With respect to the preferred interaction of the native magainins with acidic phospholipids, our results suggest that selectivity arises from the positive peptide charge which promotes interaction with negatively charged lipids as well as from a sufficiently low hydrophobicity which prevents a strong interaction with neutral phospholipids.

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