Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment

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Abstract Starting from the sequences of magainin 2 analogs, peptides with slightly increased hydrophobic moment (μ) but retained other structural parameters were designed. Circular dichroism investigations revealed that all peptides adopt an α-helical conformation when bound to phospholipid vesicles. Analogs with increased μ were considerably more active in permeabilizing vesicles mainly composed of zwitterionic lipid. In addition, the antibacterial and hemolytic activities of these analogs were enhanced. Correlation of permeabilization and binding indicated that the activity increase is predominantly caused by an increased membrane affinity of the peptides due to strengthened hydrophobic interactions.

Key words: Antibacterial peptide; Antimicrobial peptide; Amphipathic helix; Hydrophobic moment; Magainin

1. Introduction

The potential to adopt an amphipathic α-helical conformation is a common structural feature of a multitude of membrane-lytic cytotoxic and antimicrobial peptides. An amphipathic conformation allows the peptides to incorporate into the membrane with the charged and polar amino acid side chains exposed to the lipid headgroups and the hydrophobic area embedded into the acyl chain region. After incorporation, the peptides enhance the permeability of membranes by different mechanisms such as pore formation or disruption of the integrity of the membrane (for review see [1,2]). Besides amphipathicity, other structural parameters such as overall hydrophobicity, peptide charge, position of charged residues and relative size of hydrophobic and hydrophilic cores are believed to influence peptide-membrane interaction [3–10].

Several structure function studies of natural peptide sequences and the de novo design of antimicrobial peptides revealed the general importance of amphipathicity, quantitatively expressed by the hydrophobic moment (μ) [11], for membrane activity of helical peptides [4,7,12–16]. However, these investigations did not sufficiently consider that modifications of peptide sequence generally alter more than one structural parameter capable of modulating membrane activity. For instance, incorporation of bulky hydrophobic residues into the hydrophobic region of an amphipathic helix increases not only the hydrophobic moment (μ) but also the overall hydrophobicity. Likewise, incorporation of hydrophilic, positively charged amino acids in the polar region enhances the hydrophilicity and the overall peptide charge in addition to μ. In such cases, it is impossible to assign the observed effect exclusively to the alteration of μ. Consequently, there is a considerable need of quantitative experimental data especially on the influence of small modifications of μ on membrane activity.

To overcome the problem of complex changes of structural parameters due to peptide sequence modifications, we employ an approach of minimal sequence modification which allows the modification of one structural parameter while keeping the others largely constant [6,9]. Our recent investigation showed that slight modifications of structural parameters may substantially modify the antibacterial and hemolytic activities of amphipathic peptides [10]. In this work we investigated in detail the influence of μ on membrane activity and elucidated the biophysical principles providing the basis for the modulation of membrane activity by μ. Starting from two analogs of the antibacterial frog peptide magainin 2, peptides with enhanced μ but retained hydrophobicity and number and positions of charged residues were synthesized. We studied the influence of μ on the helicity of the peptides and investigated the role of the interplay of μ and membrane charge for the membrane-permeabilizing activity. Binding studies and their correlation with membrane permeabilization revealed the influence of μ on both steps of the membrane-permeabilizing process.

2. Materials and methods

2.1. Materials

The lipids were purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA. The Fmoc amino acids were obtained from Novabiochem, Bad Soden, Germany. All other chemicals were of analytical or reagent grade. Peptides were synthesized by automated solid phase methods using Fmoc chemistry on a MilliGen 9050 peptide synthesizer (Millipore, MA, USA) [17]. Tenta Gel S RAM resin (0.21 mmol/g; RAPP Polymere, Tübingen, Germany) was used as the solid support. Purification was carried out by preparative HPLC to give final products >95% pure by HPLC analysis. All peptides were characterized by mass spectrometry (MALDI II; Kratos, Manchester, UK) and quantitative amino acid analysis (LC 3000, Biotronik-Eppendorf, Germany).

2.2. Vesicle preparation

After vortexing the dried lipid in buffer (for CD: 10 mM Tris, 154

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Abbreviations: μ, hydrophobic moment; Tris, tris(hydroxymethyl)-aminomethane; CD, circular dichroism; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; MIC, minimum inhibitory concentration; M2a, magainin 2 amide; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol; PC, phosphatidylcholine

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mM NaF, 0.1 mM EDTA, (pH 7.4), for calcein release: 10 mM Tris, 70 mM calcein, 0.1 mM EDTA (pH 7.4)) small unilamellar vesicles (SUVs) were prepared by sonication (under nitrogen, in ice water) for 25 min using a titanium tip ultrasonicator. Titanium debris was removed by centrifugation.

Calcein containing large unilamellar vesicles (LUVs) were prepared by the extrusion technique [18]: A lipid suspension (buffer: 70 mM calcein, 154 mM NaCl, 10 mM Tris, 0.1 mM EDTA, (pH 7.4)) was freeze-thawed in liquid nitrogen for seven cycles and extruded through polycarbonate filters (six times through two stacked 0.4 μm pore size filters followed by ten times through two stacked 0.1 μm pore size filters). Untrapped calcein was removed by gel filtration on a Sephadex G75 column (eluent buffer: 10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, (pH 7.4)). Lipid concentration was determined by phosphorus analysis [19].

2.3. Circular dichroism measurements

CD measurements were carried out on a Jasco 720 spectrometer between 200 and 260 nm at 23°C. Minor contributions of circular dichroism and circular differential scattering by the SUVs were eliminated by subtracting the lipid spectra for the peptide-free suspension. The helicity of the peptides was calculated from the mean residue ellipticity at 222 nm [20].

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. The theoretical principles of the method were described elsewhere [21].

2.4. 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).

2.5. Immobilized artificial membrane chromatography

The retention behavior of the peptides was studied on an IAM.PC.DD column (100×4.6 mm i.d., particle size 5 μm, 300 Å, Regis Technologies, Inc., Morton Grove, IL, USA). Chromatographic measurements were performed on a Jasco gradient HPLC system (Jasco GmbH, Germany) consisting of two PU-980 pumps, an AS-950 autoinjector, and a UV-975 detector operating at 220 nm. The sample concentration was 0.5 mg/ml of peptide in eluent A, and the injection volume was 20 μl. Runs were performed at 23°C (thermostated system) and at an eluent flow rate of 0.8 ml/min. Mobile phases A and B consisted of 10 mM KH_2PO_4/K_2HPO_4, 154 mM NaCl in water, pH 7.4, and 10 mM KH_2PO_4/K_2HPO_4, 154 mM NaCl in 50% acetonitrile/50% water (v/v), pH 7.4, respectively.

The isocratic retention times (t_R) are used to calculate the capacity factors k_{IAM}' using the following equation: $k_{IAM}' = (t_R - t_0)/t_0$, where t_R is the retention time of the peptide and t_0 is the column void volume. A linear relationship exists between $\ln k_{IAM}'$ and the organic mole fraction ϕ, according to $\ln k_{IAM}' = \ln k_0' - S\phi$, where S is a constant and k_0' is the capacity factor in absence of the organic solvent [22]. Hence, $\ln k_0'$ can be determined by linear regression analysis. The capacity factor k_0' is linearly related to the equilibrium partition coefficient between lipid and aqueous phase according to $k_0' = \psi K$, where ψ is the phase ratio of the column.

2.6. Hemolytic and antibacterial activity

The hemolytic activity of the peptides was determined using human red blood cells as described previously [6]. In brief, the peptide containing suspensions were incubated for 30 min at 37°C. After cooling in ice water and centrifugation an aliquot of the supernatant was mixed 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.

Antimicrobial susceptibility testing was performed using a modification of the National Committee for Clinical Laboratory Standards (NCCLS) microdilution broth assay (1997). 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 $1–5\times10^5$ 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 read at 630 nm. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of peptide that completely inhibits growth.

3. Results

3.1. Peptide design

Peptides with enhanced hydrophobic moment (μ) but hydrophobicities comparable to those of magainin 2 amide (M2a) or the more hydrophilic analog $L^2R^{11}A^{20}$ M2a were designed employing essentially the same principles as recently described for peptides with variations in the hydrophobicity [9]. Starting from the sequence of M2a up to 5 amino acids were substituted according to the following rules: (i) The number and positions of charged amino acids were unchanged to avoid modifications of interactions with charged membrane components. (ii) Amino acids in the hydrophilic region of the amphipathic helix were replaced by hydrophilic ones ($H < 0$, [11]), and residues in the hydrophobic region were substituted by hydrophobic amino acids ($H > 0$). Hence, the angles circumscribed by the hydrophobic and hydrophilic helix regions remained unchanged. (iii) The hydrophobicity of the analogs was adjusted to that of the parent peptides M2a or $L^2R^{11}A^{20}$ M2a by appropriate, simultaneous substitutions in the hydrophobic and hydrophilic cores of the helix. (iv) The Gly residues were not substituted to avoid drastic changes in the helical propensity.

The helical wheel presentations of the designed peptides as well as the biophysical data, based on the Eisenberg consensus scale of hydrophobicity [11], are given in Fig. 1. The first pair of peptides consists of $L^2R^{11}A^{20}$ M2a and $I^6R^{11}R^{14}W^{16}$ M2a with hydrophobic moments of 0.287 and 0.332, respectively, and a constant hydrophobicity of -0.097 ± 0.001. The second pair of peptides (M2a and $I^6V^9W^{12}T^{15}I^{17}$ M2a) is characterized by hydrophobic moments of 0.286 and 0.317, respectively, and a higher hydrophobicity of -0.0355 ± 0.0005.

Table 1
α-helicity of the peptides in buffer, SDS and SUV suspensions

Peptide	Lipid/solvent				
	Buffer	SDS	POPC SUVs	POPC/POPG (3:1) SUVs	POPG SUVs
$L^2R^{11}A^{20}$ M2a	0	55	18	56	58
$I^6R^{11}R^{14}W^{16}$ M2a	0	70	58	83	83
M2a	0	68	51	76	77
$I^6V^9W^{12}T^{15}I^{17}$ M2a	0	76	78	87	87

Helical content was evaluated from $[\Theta]_{222}$ [20]. The peptide concentration was 50 μM in buffer (10 mM Tris, 154 mM NaF, 0.1 mM EDTA, (pH 7.4)). The SDS concentration was 25 mM and the lipid concentration was 8.3 mM of POPG and POPC and 13 mM of POPC/POPG (3:1).

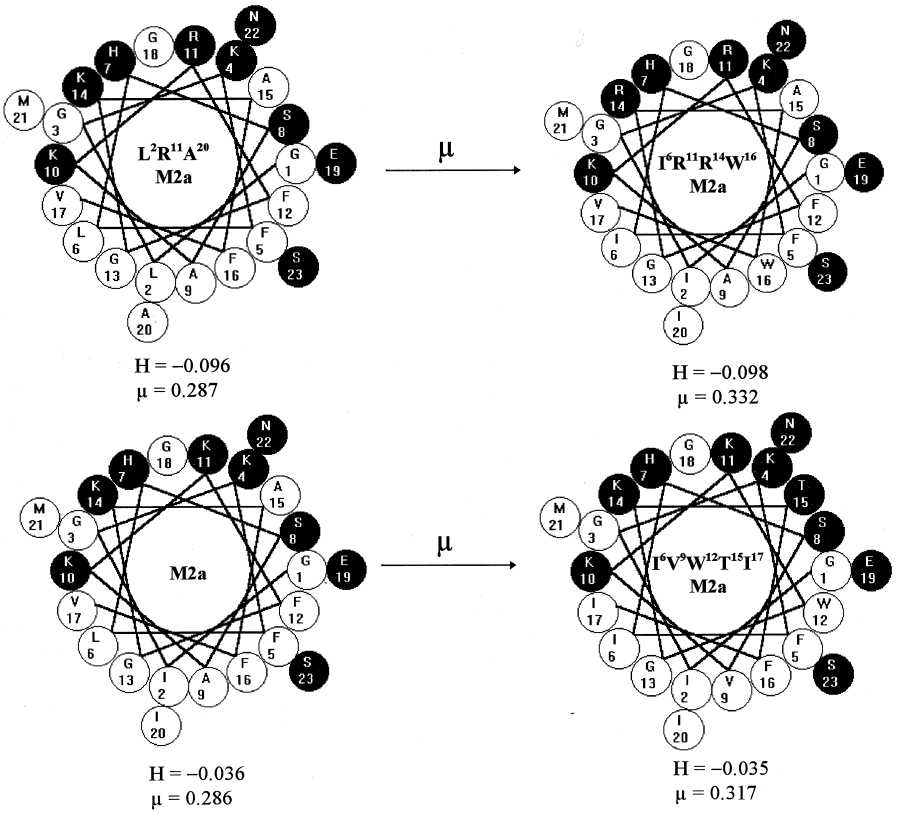

Fig. 1. Helical wheel projection of the parent peptides and the analogs with increased hydrophobic moment μ. Black circles refer to charged or hydrophilic amino acids and white circles to hydrophobic residues. Hydrophobicity (H) and μ were calculated using the consensus scale of hydrophobicity [11]. Both parameters are expressed as mean residue values throughout the paper.

3.2. Conformational investigations

The conformation of the peptides was investigated by CD spectroscopy in Tris-buffered saline, SDS solution and different vesicle suspensions. All peptides adopted an unordered conformation in Tris-buffered saline (Table 1). In SDS solution as well as in the presence of negatively charged POPG and POPC/POPG (3:1) SUVs the peptides folded into mainly α-helical conformations. Interestingly, analogs with enhanced μ were more helical than the corresponding peptides with lower μ but the same hydrophobicity. Titration experiments with negatively charged vesicles revealed that, under the conditions used, all peptides were completely membrane-bound (not shown). Thus, the differences in helicity in the presence of these vesicles reflect differences in the helicity of the lipid-bound peptides. More pronounced differences in helicity were observed in the presence of zwitterionic POPC SUVs. However, not all the peptide was bound under these conditions and the large differences arose from both differences in the membrane affinity and differences in the helicity of the bound peptide. The strongly enhanced helicity of analogs with increased μ is an indication of their increased affinity to zwitterionic lipids.

3.3. Permeabilization of vesicles and biological activity

The ability of the peptides to permeabilize LUVs of different surface charge (POPG, POPC/POPG (3:1), POPC) was tested by a dye release assay. The EC_{50} values of the initial rate of calcein release were used as a measure of the membrane-permeabilizing activity (Table 2). All analogs were very effective and showed only slight differences in permeabilizing

Table 2
EC_{50} values of calcein release from LUVs, MIC values of antibacterial activity, EC_{50} values of hemolytic activity and capacity factors of peptide retention on a IAM.PC.DD column

Peptide	Dye release EC_{50} (μM)			Antibacterial activity MIC (μg/ml)		Hemolysis EC_{50} (μM)	Capacity factor k_0'
	POPG LUVs	POPC/POPG (3:1) LUVs	POPC LUVs	*E. coli*	*P. aeruginosa*	Human erythrocytes	IAM.PC.DD column
$L^2R^{11}A^{20}$ M2a	0.16 ± 0.01	7.45 ± 0.05	65 ± 1	256	> 256	> 1000	240
$I^6R^{11}R^{14}W^{16}$ M2a	0.13 ± 0.00	0.85 ± 0.05	3.4 ± 0.2	128	256	503	1980
M2a	0.18 ± 0.01	1.2 ± 0.1	5.8 ± 0.2	128	256	428	700
$I^6V^9W^{12}T^{15}I^{17}$ M2a	0.34 ± 0.01	0.38 ± 0.03	0.25 ± 0.05	8	64	56	3530

EC_{50} values of dye release are the peptide concentrations inducing 50% dye release after 1 min. Values are the mean of two independent measurements ± error of the mean. The lipid concentration was 25 μM in buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, (pH 7.4)). MIC is the minimum inhibitory concentration of bacterial growth and EC_{50} of hemolysis is the concentration of half-maximal lysis of human erythrocytes.

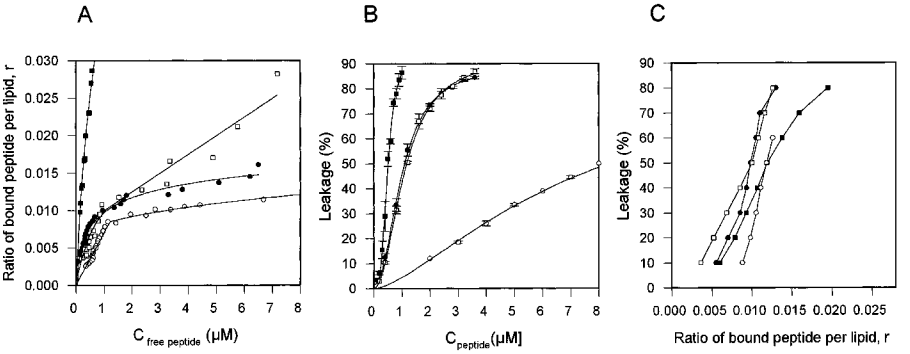

Fig. 2. A: Binding isotherms of peptides on POPC/POPG (3:1) SUVs at 23°C. B: Dependence of calcein leakage from POPC/POPG (3:1) SUVs on total peptide concentration ($c_{peptide}$). Calcein leakage is defined as the percent leakage after 30 min at a lipid concentration of 25 μM. The values are the mean of two independent measures ± error. C: Relationship between the percent calcein leakage and the molar ratio of bound peptide per lipid (r). Symbols: $L^2R^{11}A^{20}$ M2a (○), $I^6R^{11}R^{14}W^{16}$ M2a (□) M2a (●), $I^6V^9W^{12}T^{15}I^{17}$ M2a (■). The r values corresponding to 50% release ($r_{50\%}$) were found to be 0.0118 ± 0.0008 for $L^2R^{11}A^{20}$ M2a, 0.0100 ± 0.0013 for $I^6R^{11}R^{14}W^{16}$ M2a, 0.0098 ± 0.0010 for M2a and 0.0122 ± 0.0016 for $I^6V^9W^{12}T^{15}I^{17}$ M2a. The error of $r_{50\%}$ was estimated from the error of the calcein leakage and the uncertainty of the binding isotherm.

highly negatively charged POPG LUVs. In contrast, the ability of the analogs to permeabilize LUVs composed of zwitterionic POPC was highly differentiated. An increase of μ of $L^2R^{11}A^{20}$ M2a from 0.287 to 0.332 ($I^6R^{11}R^{14}W^{16}$ M2a) resulted in a 19-fold higher activity. Likewise, enhancement of μ of M2a from 0.286 to 0.317 ($I^6V^9W^{12}T^{15}I^{17}$ M2a) increased the permeabilizing activity by a factor of about 23. The same relation between μ and permeabilizing activity was found at mixed POPC/POPG (3:1) LUVs. However, the differences between analogs of different μ were less pronounced for mixed vesicles.

$L^2R^{11}A^{20}$ M2a and M2a were highly selective in permeabilizing negatively charged (POPG) over zwitterionic membranes (POPC). Enhancement of μ significantly increased activity on POPC vesicles but scarcely influenced activity on POPG vesicles. In consequence, the selectivity of analogs with increased μ for negatively charged model membranes was reduced.

The activity of the peptides on PC-rich model membranes correlated well with the antibacterial and hemolytic activity (Table 2). Increase of μ for $L^2R^{11}A^{20}$ M2a and M2a resulted in analogs with enhanced antibacterial and hemolytic activity ($I^6R^{11}R^{14}W^{16}$ M2a, $I^6V^9W^{12}T^{15}I^{17}$ M2a). Increase of μ for the native sequence (M2a → $I^6V^9W^{12}T^{15}I^{17}$ M2a) did not reduce selectivity for *E. coli* over erythrocytes. However, the activity against *P. aeruginosa* was enhanced by a factor of four whereas the hemolytic activity was increased 8-fold, thus reflecting a slightly reduced antibacterial specificity.

3.4. Binding investigations

CD-derived binding isotherms of the peptides on POPC/POPG (3:1) SUVs revealed a strongly enhanced lipid affinity for analogs with increased μ (Fig. 2A). In addition, the dye-releasing activities of these analogs on POPC/POPG (3:1) SUVs were clearly higher than those of the corresponding peptides with a lower μ (Fig. 2B). Binding of the peptides was correlated with the dose-response curves of dye release in order to calculate the ratios of bound peptide per lipid corresponding to a given release effect (Fig. 2C). The efficiency of the bound peptide fraction to induce 50% dye release was only between 10 and 12 bound molecules per thousand lipid molecules showing little variability among all the analogs.

The binding of peptides to lipid was also investigated by immobilized artificial membrane chromatography on an IAM.PC.DD column. This column was recently developed to mimic the environment of phosphatidylcholine [23]. The capacity factor k_0' of a peptide is linearly related to the partition constant between aqueous and lipid phase, and can therefore be used as a measure for the lipid affinity of the peptide (Table 2). $I^6R^{11}R^{14}W^{16}$ M2a showed about an 8-fold higher k_0' value than the corresponding analog with a lower μ. Likewise, the lipid affinity of $I^6V^9W^{12}T^{15}I^{17}$ M2a was about 5-fold higher than that of M2a.

4. Discussion

The general importance of amphipathicity for antibacterial and hemolytic activity of helical peptides has long been recognized. Sixteen years ago, DeGrado et al. substituted residues 1 to 20 of melittin with a sequence capable of forming an idealized amphipathic α-helix [12]. This led to a more hemolytic peptide and impressively proved the effect of the amphipathic helix on activity. More recently, there have been extensive, successful studies directed to design basic amphipathic model peptides with lytic activity [3,13–15]. The role of amphipathicity has also been studied by strengthening or weakening of helicity due to incorporation of helix-promoting or helix-destabilizing residues [6,24–27]. However, until now no studies have been devoted to quantitatively assess the influence of changes in the hydrophobic moment (μ) on membrane activity and to exclude simultaneously the effect of variations of other structural parameters.

In this work, we studied the impact of μ on membrane activity of magainin 2 analog peptides keeping overall charge and position of charged amino acids constant and hydrophobicities comparable to those of the parent peptides.

CD investigations revealed that all peptides adopt an α-helical conformation in SDS solution as well as in the presence of phospholipid vesicles. Under the conditions used, the peptides were completely membrane-bound in POPG and POPC/POPG (3:1) SUV suspension. Lipid-bound M2a has

recently been reported to be helical over the entire peptide chain [28,29]. Because all analogs were derived from M2a by a few, conservative amino acid substitutions distributed over the whole peptide chain, we assume that differences in the helicities result from an overall stabilization or destabilization of the helix rather than from an extension or shortening of the helical region.

Lytic natural and model peptides comprise a large range of hydrophobic moment values, e.g. mastoparan: $\mu = 0.221$; PGLa: $\mu = 0.260$; bombolitin I: $\mu = 0.284$; δ-hemolysin: $\mu = 0.380$; model peptide $L_{15}K_7$ [15]: $\mu = 0.443$; model peptide M2 [7]: $\mu = 0.471$; model peptide Hel9-9 [8]: $\mu = 0.513$. However, the peptides investigated in this study were characterized by comparatively small differences in μ (between 0.286 and 0.332). Nevertheless, small increases of μ, caused by few, conservative amino acid substitutions, were found to strongly enhance the permeabilizing activity on PC-rich LUVs as well as antibacterial and hemolytic activity (Table 2). Furthermore, the interplay of hydrophobicity and μ is interesting to note: While a reduction of the hydrophobicity of M2a from −0.036 to −0.096 ($L^2R^{11}A^{20}$ M2a) substantially decreased activity at PC-rich membranes, the activity could be completely restored, even at this low hydrophobicity, by enhancing μ from 0.287 to 0.332 ($I^6R^{11}R^{14}W^{16}$ M2a).

The finding that a slight increase of μ may drastically enhance membrane activity, if other structural parameters are kept constant, may be useful for the design of more active antibacterial substances, but should also be considered when assessing the consequences of sequence modifications. Kiyota et al. [8] recently compared the membrane activity of amphipathic model peptides with varying ratios of Leu to Lys. They discussed their results with regard to the hydrophobic-hydrophilic balance assuming that all analogs had negligible differences in μ (μ was between 0.39 and 0.51). However, the results presented here clearly show that such differences in μ must not be neglected.

Also, the influence of μ is often overcompensated by other effects and hence difficult to evaluate. Matsuzaki et al. did not find a correlation between μ and lytic activity comparing magainin 2, alamethicin and melittin [30]. This is not surprising considering the large differences of other structural parameters such as hydrophobicity and overall charge as well as the different modes of action of these peptides. However, the absence of a correlation between distantly related peptides does not exclude the possibility that the lytic activity of each of the peptides can be enhanced by an increase of its μ.

In contrast to their action on PC-rich vesicles, all peptides were almost equally potent in permeabilizing highly negatively charged POPG LUVs. Clearly, a correlation between biological activity and permeabilization of POPG vesicles does not exist. In spite of this fact, comparison of peptide activity on membranes of different surface charge gives information about the role of electrostatic and hydrophobic interactions in the membrane permeabilization process. The differences in the permeabilizing activity between peptides of different μ becomes more pronounced with decreasing content of negatively charged lipid within the membrane (Table 2). Consequently, the capability of μ to modulate membrane activity increased with decreasing electrostatic peptide-lipid interactions and an increasing role of hydrophobic interactions.

The enhanced membrane-permeabilizing activity of analogs with increased μ at PC-rich membranes can be caused by an increased membrane affinity or by an enhanced permeabilizing efficiency of the membrane-bound peptide fraction. The binding of the peptides to PC-rich membranes was investigated by CD spectroscopy on POPC/POPG (3:1) SUVs and by immobilized artificial membrane chromatography (IAM.PC.DD, model for PC membranes). Both methods revealed an increased lipid affinity for analogs with an enhanced μ (Fig. 2A, Table 2) and therefore a direct correlation between membrane-permeabilizing activity and membrane affinity. In contrast, the permeabilizing efficiency of the membrane-bound peptide fraction showed little variability (Fig. 2C) and did not correlate with the overall membrane-permeabilizing effect (Fig. 2B). Consequently, the higher permeabilization activity of analogs with increased μ is mainly caused by an increased lipid affinity of these analogs.

In summary, our results indicate that slight enhancement of μ by a few, conservative amino acid substitutions drastically increased membrane activity of magainin analog peptides. The activity increase was mainly caused by an increased binding of the peptides to the membrane due to increased hydrophobic interactions between lipid acyl chains and the hydrophobic helix core.

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