

Optimization of the antimicrobial activity of magainin peptides by modification of charge

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Abstract Investigation of magainin II amide analogs with cationic charges ranging between +3 and +7 showed that enhancement of the peptide charge up to a threshold value of +5 and conservation of appropriate hydrophobic properties optimized the antimicrobial activity and selectivity. High selectivity was the result of both enhanced antimicrobial and reduced hemolytic activity. Charge increase beyond +5 with retention of other structural motifs led to a dramatic increase of hemolytic activity and loss of antimicrobial selectivity. Selectivity could be restored by reduction of the hydrophobicity of the hydrophobic helix surface (H_{hd}), a structural parameter not previously considered to modulate activity. Dye release experiments with lipid vesicles revealed that the potential of peptide charge to modulate membrane activity is limited: on highly negatively charged 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol bilayers, reinforcement of electrostatic interactions had an activity-reducing effect. On neutral 1-palmitoyl-2-oleoylphosphatidylcholine bilayers, the high activity was determined by H_{hd} . H_{hd} values above a certain threshold led to effective permeabilization of all lipid systems and even compensated for the activity-reducing effect of charge increase on highly negatively charged membranes. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Antibacterial peptide; Magainin; Peptide charge; Hemolysis

1. Introduction

Magainin II amide (MIIa, GIGKF LHS AK KFGKA FVGEI MNS HN₂) and related peptides are potent antimicrobial compounds with a broad spectrum of activity [1].

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Abbreviations: CD, circular dichroism; EDTA, ethylenediamine-tetraacetic acid; HPLC, high performance liquid chromatography; MIIa, magainin II amide; MIC, minimum inhibitory concentration; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol; SUVs, small unilamellar vesicles; LUVs, large unilamellar vesicles; TFE, 2,2,2-trifluoroethanol; Tris, tris(hydroxymethyl)aminomethane; H , hydrophobicity; H_{hd} , hydrophobicity of the hydrophobic helix surface; Fmoc, 9-fluorenylmethoxy-carbonyl; OD, optical density

Their effect is the result of a direct interaction with the lipid matrix of the target membrane leading to the disturbance of its barrier function. In the first stage of the permeabilization process peptides bind to the cell surface. The second step involves insertion into and destabilization of the membrane. A number of studies have shown that magainins might be good candidates for therapeutic agents [2]. Therefore, considerable effort has been focussed on optimizing peptide-lipid interactions to improve antimicrobial activity and selectivity [3,4]. For both recognition and permeabilization of the bacterial membrane, electrostatic attraction between the cationic peptides and the negatively charged cell surface plays an important role. The enhancement of the total cationic peptide charge often resulted in higher antimicrobial activity [4–7] attributed to increased affinity for the bacterial membrane. However, a higher number of positive groups does not always increase the activity, suggesting a more complex interaction. In model peptides enhancement as well as reduction of charge led to analogs with improved antimicrobial activity [8]. Other studies showed that highly cationic peptides may even be devoid of antimicrobial activity [9]. One reason for our inability to predict reliably the influence of charge modification on antimicrobial and hemolytic activity is the fact that amino acid substitutions result in variation of certain structural parameters which influence peptide activity. Thus, changes in the position of charged residues may influence helicity (α) and the hydrophobic moment (μ) (see e.g. [8]) and an increase in the number of charges (Q) is always connected with reduced peptide hydrophobicity (H) and a modification of μ [9].

Studies of structural parameters characterizing an amphipathic helix led us to suggest that peptide-membrane interaction is determined by a sensitive balance of hydrophobic and electrostatic interactions [4,10]. In this study the influence of peptide charge on membrane activity and selectivity was analyzed. Following our strategy of systematic variation of only one parameter while conserving all other structural motifs [11], peptides of modified charge were synthesized and structurally characterized. Their antimicrobial and hemolytic activities were examined. Studies on model membranes were performed, addressing the question of how peptide charge modulates the two determinants of the permeabilization process: affinity and permeabilizing efficiency. The data indicate that increase of Q , conservation of moderate H and an appropriate μ render magainin more active and selective for bacterial cells. However, there was a threshold value for optimized electrostatic interactions. Additionally, the hydrophobicity of the non-cationic helix surface (H_{hd}) has been recognized as an activity- and selectivity-modifying property.

2. Materials and methods

2.1. Materials

The lipids 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 9-Fluorenylmethoxy-carbonyl (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 standard Fmoc chemistry on a MilliGen 9050 (Millipore, Bedford, MA, USA) peptide synthesizer [12]. Purification by preparative high performance liquid chromatography (HPLC) gave final products >95% pure by HPLC analysis. The 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

Small unilamellar vesicles (SUVs; about 40 mM lipid in 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer containing 154 mM NaF and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) were prepared by sonication and characterized as described [10]. Dye-containing large unilamellar vesicles (LUVs) were prepared by the extrusion of the vortexed lipid suspensions (70 mM calcein, 10 mM Tris, 0.1 mM EDTA, pH 7.4) through polycarbonate filters (six times through two stacked 0.4 μm pore size filters followed by eight times through two stacked 0.1 μm pore size filters) [10]. Untrapped dye was removed from the LUVs by microcolumn centrifugation on Sephadex G50 according to the procedure described by New [13]. Lipid concentration was determined by phosphorus analysis [14].

2.3. Circular dichroism (CD) measurements and dye release

CD measurements were carried out on a Jasco 720 spectrometer as described elsewhere [10]. The α of the peptides was determined from the mean residue ellipticity (Θ) at 222 nm [15]. All data are the means of two independent measurements which did not deviate by more than 5%.

Calcein release from vesicles was determined by measuring the decrease in self-quenching 1 min after injecting an aliquot of dye-containing vesicular suspensions into a cuvette containing stirred peptide solutions (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH 7.4) (excitation at 490 nm, emission at 520 nm) on a Perkin-Elmer LS 50B spectrofluorometer [9]. The fluorescence intensity corresponding to 100% release was determined by addition of Triton X-100 (10% v/v in water). Peptide concentration causing 50% dye efflux (EC_{50}) was estimated from dose-response curves.

Apparent binding constants for peptide-lipid interaction were determined from binding isotherms evaluated from the change of the

peptide CD according to the procedure described by Schwarz and Beschiaschvili [16]. Binding isotherms from calcein-releasing experiments were derived according to Matsuzaki et al. [17].

2.4. Biological activity

Hemolytic activity was determined using human red blood cells (Blutspendedienst des DRK, Berlin, Germany) by measuring the peptide-induced changes of the optical density (OD) at 540 nm (Lambda 9, Perkin-Elmer, Germany) [10]. Peptide concentrations causing 50% hemolysis (EC_{50}) were derived from dose-response curves. Values from repeat experiments differed by less than 5%.

Escherichia coli (DH5 α strain) and *Bacillus subtilis* (PY 22 strain) cultivated in Luria broth (Gibco BRL, Paisley, UK) were used to test the antibacterial activity of the peptides. The inoculum was prepared from mid-logarithmic phase cultures ($\text{OD}_{600} = 0.5$). Aliquots of cell suspension were added to the wells of a microtiter plate containing peptide solutions of different concentrations. The final density of bacteria in the wells was 1.25×10^6 CFU/ml. The peptide concentration ranged from 0.04 to 80 μM in two-fold dilutions. All peptides were tested in duplicate. After incubation overnight at 37°C with gentle shaking, the absorbance was read at 600 nm (Autoreader EL 311, Bio-Tek Instruments, USA). The minimum inhibitory concentration (MIC) is defined as the lowest concentration of peptide at which there was no change in OD.

3. Results

3.1. Peptide design and structural characterization

MIIa peptides of modified cationic charge (Q) were designed following our strategy of keeping all other structural motifs, such as α , H , μ and ϕ , largely constant (Fig. 1, Table 1). The set is based on an analog with high antimicrobial and moderate hemolytic activity which differs from MIIa by an enhanced μ [4]. The peptides ($+3 \leq Q \leq +7$) were prepared by variation of the number of lysine residues. Modification of the negative charge was avoided because of E¹⁹ exchange [6] and a negatively charged C-terminus [18] have been reported to substantially reduce the stability of the magainin helix. Histidine has been reported to be uncharged under physiological pH conditions used in this study [19]. Changes of H and μ connected with the manipulation of charge were compensated by the introduction of more hydrophobic residues and variation of the position of several amino acids (Fig. 1). These modifications resulted in changes of the H_{hd} (Table 1), a

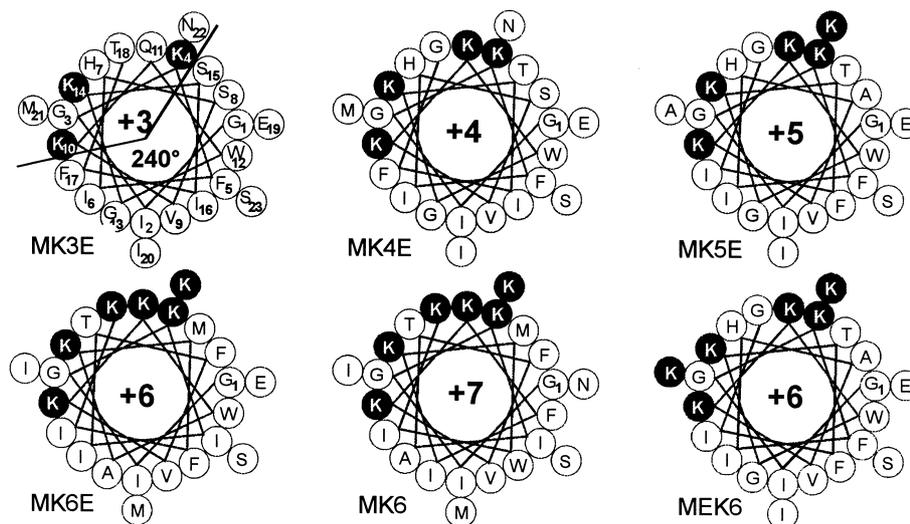


Fig. 1. Helical wheel projection of charge-modified MIIa analogs. Q is given in the center of the wheel. The angle subtended by cationic residues (black circles) is $\phi = 120^\circ$. The 14 amino acid residues covering the angle of 240° were used for calculation of the mean residue H_{hd} .

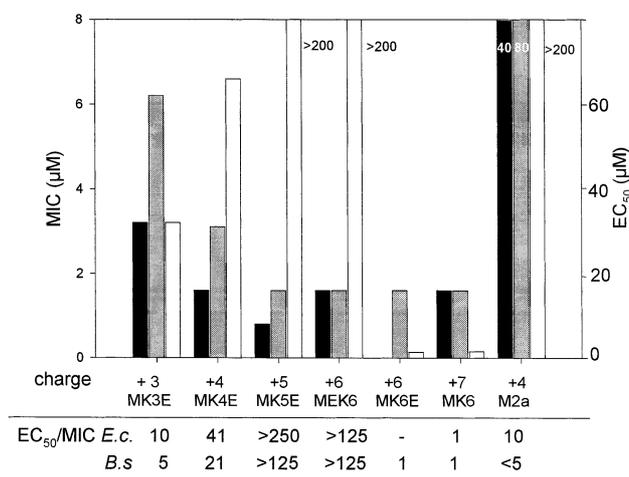


Fig. 2. The biological activity and selectivity of magainin peptides. MIC of bacterial growth against *E. coli* (black bars) and *B. subtilis* (gray bars) (1.25×10^6 CFU/ml) and concentration for half-maximal lysis of red blood cells (white bars) (EC_{50}) (2.3×10^8 cells/ml). The values determined in two experiments differed by less than 5%. The MICs of MIIa were $40 \mu\text{M}$ (*E. coli*) and $> 80 \mu\text{M}$ (*B. subtilis*), the EC_{50} was $200 \mu\text{M}$ [6]. The prokaryotic specificity was determined as the quotient of EC_{50} for human red blood cells and MIC of bacterial growth (EC_{50}/MIC).

structural motif not considered so far as a potential activity-modulating parameter. To assess the role of H_{hd} , an additional analog (MEK6) characterized by six positive charges and the H_{hd} of MK5E was prepared (Table 1). The increase of charge from +5 to +6 and the conservation of H_{hd} , however, resulted in reduced total H . The analog MK6 (N in position 19 and thus bearing seven positive charges) was included in the studies to confirm the surprising results with MK6E characterized by a pronounced H_{hd} .

All peptides showed pronounced α in 2,2,2-trifluoroethanol (TFE)/buffer (1/1 v/v) mixture (Table 1). The high α , exceeding 86% for the two analogs MK6E and MK6 with an H_{hd} of about 0.36, can be associated with the substitution of $G^{13,18}$ by helix-promoting lysine residues [20]. The retention time (t_{R}) in RP-HPLC reflecting affinity to the hydrophobic stationary phase is closely related to the induced α of peptides [21]. Table 1 shows that the t_{R} of peptide with conserved H , μ was linearly related to the product of $H_{\text{hd}} \times \alpha_{\text{TFE}}$. Peptide α in the lipid-bound state was determined using highly negatively charged POPG and electrically neutral POPC vesicles (Table 1). Titration of peptide solutions with lipid suspensions

Table 1
Structural properties of MIIa peptides bearing various charges

Peptide analog	Q	H	μ	H_{hd}	t_{R} (min)	α_{TFE}	$\alpha_{\text{TFE}} \times H_{\text{hd}}$	α POPG ($c_l/c_p = 50$)	α POPC ($c_l/c_p = 520$)
MK3E	+3	-0.0357	0.32	0.284	20.8	0.65	0.185	0.58	0.72
MK4E	+4	-0.0352	0.32	0.298	19.5	0.50	0.149	0.62	0.71
MK5E	+5	-0.0335	0.33	0.326	20.0	0.54	0.176	0.67	0.72
MK6E	+6	-0.0344	0.33	0.364	25.4	0.86	0.313	0.87	0.95
MK6	+7	-0.0352	0.33	0.362	25.8	0.89	0.323	0.96	0.99
MEK6	+6	-0.0922	0.35	0.326	18.4	0.67	0.218	0.77	0.78

Total Q , H , μ , H_{hd} , HPLC t_{R} and the amount of helix in structure inducing TFE/buffer (1/1 v/v) mixture (α_{TFE}) and in the presence of negatively charged POPG and electrically neutral POPC SUVs suspended in 10 mM Tris buffer containing 150 mM NaF, pH 7.4. The mean H was calculated as the sum of the H s of the individual amino acid residues divided by their number, μ is the vector sum of the H s and H_{hd} was determined as the mean residue H of the 14 residues forming the non-cationic helix surface. The Eisenberg consensus scale of H was used [29]. α was determined according to [20]. $\alpha_{\text{TFE}} \times H_{\text{hd}}$ characterizes the hydrophobic helix surface. The peptide concentration was 10^{-5} M. Because of differing affinities, the ratio of lipid to peptide concentration (c_l/c_p) varied from 50 for POPG to 520 for POPC. For chromatographic details see Section 2. MIIa is characterized by $Q +4$, $H -0.0357$, $H_{\text{hd}} 0.289$, $\mu 0.29$, $\phi 120^\circ$.

showed that constant ellipticity reflecting complete binding to POPG vesicles was reached at a peptide to lipid molar ratio (c_l/c_p) as low as 15. Binding to neutral POPC liposomes required $c_l/c_p \geq 350$ (data not shown). Although completely bound (99.8% > binding > 97%), striking differences in the α of the peptides were observed. H_{hd} values higher than 0.326 distinctly favored helix formation. Additionally, a negative surface charge of the vesicles inhibited helix formation in peptides with $Q < +6$. Both observations point to the dominating role of hydrophobic peptide-lipid interactions for the induction of the peptide helix [21].

3.2. Biological activity

The increase of peptide charge led to enhanced activity against both Gram-positive and Gram-negative bacteria which reached a maximum at a peptide charge of +5 (Fig. 2). The high antibacterial activity was retained on a charge increase beyond +5. The MIC of MK6 against *E. coli* could not be determined reliably. Reduction of OD was associated with precipitation during the incubation time. The increase of antimicrobial activity was accompanied by a substantial reduction of hemolytic activity (Fig. 2). MK5E was practically inactive against red blood cells. Further charge increases for peptides with conserved H , μ and ϕ to +6 and +7 (MK6E, MK6) led to a striking increase of the undesired hemolytic effect. The low hemolytic activity of a six charges-bearing peptide, however, could be restored in MEK6 by decreasing H_{hd} . Compared to the unmodified MIIa, the antimicrobial activity of the optimized MK5E was increased by a factor of 50 and the selectivity was 20 times higher (Fig. 2).

3.3. Bilayer-permeabilizing activity

All peptides showed a high permeabilizing activity against POPG, mixed POPG/POPC and POPC liposomes. However, the activity pattern on the different lipid systems differed (Fig. 3). The activity against POPG liposomes distinctly decreased when increasing peptide charge from +3 to +5. MEK6 (+6 charge, $H_{\text{hd}} 0.326$) fits this profile. However, MK6E and MK6 showed substantially improved activity which is related to the high α in the lipid-bound state and the elevated H_{hd} (Table 1, Fig. 3). No relationship between peptide charge and activity against POPC vesicles was found. The EC_{50} was rather related to peptide α and H_{hd} . Peptide activity decreased upon enhancing the amount of negatively charged lipids in the vesicles (Fig. 3). All peptides, except MK3E, were least active against POPG vesicles, a phenomenon also observed for other peptides [10].

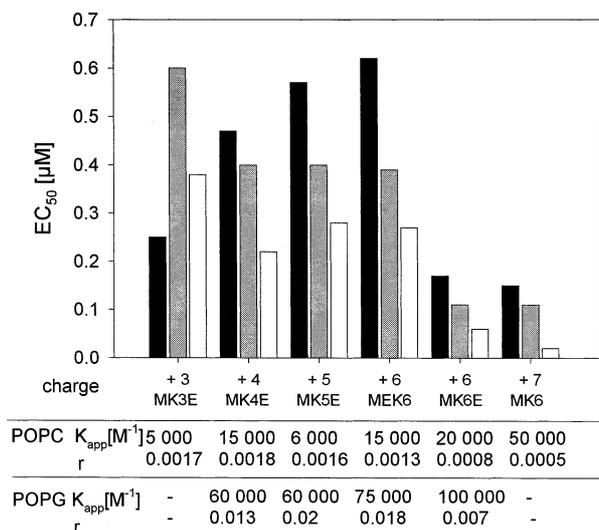


Fig. 3. The lytic activity, affinity and permeabilizing efficiency of magainin peptides. EC_{50} is the peptide concentration inducing 50% dye release from LUVs composed of POPG (black bars), a mixture of POPG/POPC (1/3 mol/mol) (gray bars) and POPC (white bars) at a lipid concentration of 25 μM . K_{app} is the apparent binding constant determined CD-spectroscopically for the peptide interaction with POPC vesicles, and by fluorescence spectroscopy from dye release experiments for POPG liposomes. r is the molar ratio of bound peptide per lipid inducing 50% dye release and is taken as a measure of the bilayer-permeabilizing efficiency of the vesicle-bound peptides.

3.4. Binding and permeabilizing efficiency

Apparent binding constants (K_{app}) to the highly negatively charged POPG LUVs were derived from dye release experiments. This method is suitable if membrane binding correlates directly with membrane leakage [22]: actually, cationic peptide-induced permeabilization of highly negatively charged lipid bilayers is determined by the binding step [10]. K_{app} of the magainin analogs ranging between 50 000 M^{-1} and 100 000 M^{-1} reflect their high affinity to the POPG bilayer (Fig. 3). Binding of MK3E, MK4E, MK5E and MEK6 to neutral POPC SUVs is characterized by K_{app} values in the range of 5000 and 15 000 M^{-1} (Fig. 3). Distinctly increased affinities of MK6E and MK6 ($K_{app} = 20\,000$, 50 000 M^{-1} , respectively), characterized by a pronounced H_{hd} , underline the dominance of hydrophobic interactions. The standard free energy of magainin binding was almost identical for LUVs and SUVs [23].

The discrepancy between the slightly differentiated EC_{50} of the individual peptides for the permeabilization of POPC and POPG vesicles and the pronounced differences in their affinities to highly negatively charged and neutral bilayers (Fig. 3) points to the important role of another regulatory step in the permeabilization process – the ability of bound peptides to disturb the lipid bilayer. The permeabilizing efficiency is quantified by the ratio of bound peptide per lipid (r) required to induce 50% dye efflux. With about one to two peptide molecules bound per 100 POPG molecules to induce dye release, the r values show little variability and the permeabilizing efficiency is very low (Fig. 3). On POPC bilayers, the efficiency is approximately 10 times higher. In both systems MK6E and MK6 were the most effective analogs. The results suggest that besides affinity, the permeabilizing efficiency is also related to peptide α and H_{hd} .

4. Discussion

Earlier studies had shown that α , H , μ and ϕ are effective modulators of antimicrobial activity but preferentially influence the hemolytic effect [4,11]. Thus, modification of these parameters seemed less suitable for the generation of highly active and highly selective antimicrobial peptides. This study shows that one way of making MIIa peptides more antimicrobially active as well as selective is by reinforcement of electrostatic peptide–membrane interaction by enhancement of cationic peptide charge. The optimized activity of MK5E is the result of both an improved antimicrobial effect and a drastically reduced hemolytic activity. However, the possibility of optimizing the antimicrobial effect via modification of peptide charge seems to be limited. Increase of Q beyond +5 may cause the loss of selectivity because reinforcement of the hydrophobicity of the non-polar helix surface favors the hemolytic effect.

Structural studies of vesicle-bound peptides and the comparison of peptide activity on membranes of different surface charges give information regarding the role of electrostatic and hydrophobic interactions in the membrane permeabilization process. All investigated peptides show high helicity upon binding to lipid membranes (Table 1). Lipid-bound MIIa has been found to be helical over the entire peptide chain. We concluded that changes in α should be caused by modification of the overall stability of the helix under the various conditions [24]. Hydrophobic interactions between the peptides and the environment have been reported to be the driving forces for the induction and stabilization of a helical conformation [21]. Thus, differences in the helical content, coupled with modification of peptide and bilayer surface charge, have to be related to changes in the hydrophobic environment of the peptides. In contrast to their conformational preferences in aqueous solvent, the helical proclivity of amino acid residues such as glycine may be distinctly improved in membrane-mimicking environments [21,25]. Additionally, for potentially transmembrane model peptides, the induced α was found to be related to the H of chain segments and the charge properties of lipid micelles [25]. The α of highly hydrophobic sequences was independent of the lipid head group charge. For less hydrophobic sequences anionic lipids played a determining role in helix formation. Comparable roles appear to be valid for the amphipathic magainin peptides. However, inspection of the structural parameters and α (Table 1) of MK5E and MK6 (identical H but different H_{hd}) and MK5E and MEK6 (different H but identical H_{hd}) suggests that it is H_{hd} rather than H which is important for helix induction. While an increase of H_{hd} favors insertion into the acyl chain region of the bilayers and helix formation, a negative bilayer charge inhibits effective hydrophobic peptide–lipid interactions. A value of H_{hd} in the order of 0.36 appears to exist for this series of peptides which, once exceeded, dictates that the peptide adopts an almost complete helical conformation.

The affinity of the investigated peptides to neutral POPC bilayers (Fig. 3) was more than 10 times higher than that of MIIa ($K_{app} = 400\,M^{-1}$) [24]. This enhanced binding is clearly related to the well developed hydrophobic helix surfaces, based on the high α and high H_{hd} . Effective disturbance of the acyl chain region as a result of deep bilayer penetration determines the high permeabilizing efficiency of the peptides. On mixed POPC/POPG bilayers activity-reducing electrostatic

forces become effective but the conserved activity profile points to the dominance of hydrophobic interactions. The activity-reducing effect of the electrostatic peptide–lipid interaction is much more pronounced on POPG bilayers. High, barely differentiated binding constants and comparably low permeabilizing efficiencies determine the effect (Fig. 3). The reduced activity of peptides with enhanced Q has to be associated with the reduced permeabilizing efficiency. Strong charge interactions immobilize the cationic peptides in the lipid head group region and thus inhibit insertion and membrane disturbance [10,26]. A change in the balance of electrostatic versus hydrophobic interactions occurred with MK6E and MK6. For these peptides the high α and H_{hd} values more than compensate for the permeability-reducing effect of increased charge.

The discrepancy between the activity profiles of the peptides studied against biological cells and lipid bilayers (Figs. 2 and 3) reveals the complex nature of peptide interaction with cell membranes and underlines the limitations of model systems to describe phenomena at the biological level. The derived mechanistic picture leads to the following interpretation of the biological data. On the slightly negatively charged membrane of Gram-positive bacteria enhanced peptide accumulation based on increased peptide charge and an appropriate permeabilizing efficiency induced by the hydrophobic helix surface are superimposed. Maximal activity is attained with five cationic charges, indicating that the ionic peptide–membrane interaction is optimized. Conservation of antibacterial activity with extension of peptide charge beyond a threshold level has also been reported for other magainin sequences [3].

With Gram-negative bacteria the outer lipopolysaccharide membrane plays a strong activity-modulating role. Disturbance of the architecture of the outer wall in the rank order of peptide charge [7] supports self-promoted peptide uptake [27] and allows the peptides to reach and permeabilize the inner target. However, reversible outer membrane permeabilization seems to be limited. Peptides of high charge (e.g. MK6) completely destroy the integrity of the outer wall.

The pronounced decrease in hemolytic activity (MK3E > MK4E > MK5E > MEK6) points to electrostatic interactions even for the red blood cell membrane which is composed mainly of zwitterionic lipids. It is possible that sialic acid molecules, located in the outer leaflet of the membrane, may act to distance cationic peptides from the lipid matrix [28] thus inhibiting membrane lysis. Decreasing hemolytic activity with enhanced peptide charge has also been reported for other magainin sequences [7].

In summary, an increase of charge has been found to improve the antimicrobial activity of magainin peptides. The high selectivity of the optimized MK5 analog is based on both reinforcement of activity against bacteria and reduction of the undesired hemolytic effect underlining the therapeutic potential of this analog.

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