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Review

Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells

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Abstract

Antibacterial, membrane-lytic peptides belong to the innate immune system and host defense mechanism of a multitude of animals and plants. The largest group of peptide antibiotics comprises peptides which fold into an amphipathic α-helical conformation when interacting with the target. The activity of these peptides is thought to be determined by global structural parameters rather than by the specific amino acid sequence. This review is concerned with the influence of structural parameters, such as peptide helicity, hydrophobicity, hydrophobic moment, peptide charge and the size of the hydrophobic/hydrophilic domain, on membrane activity and selectivity. The potential of these parameters to increase the antibacterial activity and to improve the prokaryotic selectivity of natural and model peptides is assessed. Furthermore, biophysical studies are summarized which elucidated the molecular basis for activity and selectivity modulations on the level of model membranes. Finally, the knowledge about the role of peptide structural parameters is applied to understand the different activity spectra of natural membrane-lytic peptides.

Keywords: Antimicrobial peptides; Membrane activity; Amphipathicity; Helicity; Hydrophobicity; Hydrophobic moment; Peptide charge; Polar/hydrophobic angle

Contents

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1. Introduction

Peptides with cell-lytic activity are ubiquitous in nature, constituting a defense system against invading microorganisms in many organisms (for reviews, see [1–3]). The peptides can be classified into several groups on the basis of their origin, activity spectrum or structure (for a review, see [4]). Helical peptides, typified by melittin from bees, magainins from frogs and cecropin P1 from pig, are the most widely distributed and have the broadest spectrum of activity (Table 1, Fig. 1). In this review we will focus on linear peptides which assume an α-helical conformation when interacting with the target.

The challenge of bacterial resistance to conventional antibiotics [5], and the remarkable antibacterial selectivity of many peptides and their unique mode of action, have made such peptides promising candidates for the development of a new class of antibiotics [6,7]. Considerable effort has been made to elucidate the mode of action and to understand the structural basis of membrane selectivity with the goal of optimizing peptide antimicrobial activity (for reviews, see [8–10]).

The available evidence suggests that the peptides exert their cell-lytic effect by a two-step mechanism consisting of (i) binding to the cell surface and (ii) membrane permeabilization. Largely unstructured in solution, linear peptides fold into an amphipathic helix upon binding to the target membrane, insert into the membrane and disturb the barrier function in different ways. Membrane permeabilization leads to a breakdown of the transmembranal potential and of ion gradients and causes leakage of cell contents, resulting finally in cell death. In addition to a disturbance of the membrane function by formation of ion channels, two other mechanisms of membrane permeabilization are at present under discussion. A global disturbance of the lipid arrangement resulting from a very dense peptide accumulation has been proposed for a variety of peptides and was designated the 'carpet mechanism' (for a review, see [11]). This mode of action is supported by the facts that peptides mainly adopt a parallel orientation to the lipid bilayer and permeabilize it even in the absence of a transmembrane potential. The second mechanism is pore formation. Local thinning of the membrane at the site of peptide binding [12] may favor the formation of transitory pores connecting the inner and outer leaflets by a complex peptide lipid arrangement as recently suggested for magainin and melittin (for a review, see [9]).

Distinct differences in the membrane properties of prokaryotic and eukaryotic cells appear to regulate activity and specificity. Antimicrobial peptides have been postulated to overcome the high negative charge of the outer wall of Gram-negative bacteria by inducing transient lesions large enough to permit the passage of peptides (self-promoted uptake) [6]. Negatively charged lipids favor peptide binding and an inside negative transmembrane potential supports penetration of the inner membrane. The cytoplasmic membrane of Gram-positive bacteria is directly exposed to the lytic peptides and its negative charge facilitates interaction with cationic compounds.

For a peptide to act as part of the host defence system or to be of pharmaceutical interest, high antimicrobial activity must be combined by a low activ-

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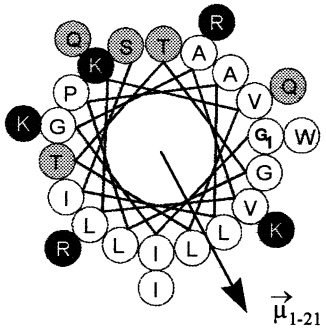
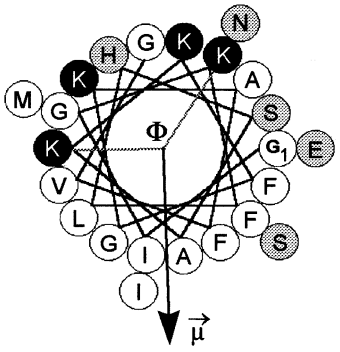
Fig. 1. Helical wheel projections and schematic drawings of the amphipathic helices of melittin, magainin 2 amide, cecropin P1 and a KLAL model peptide illustrating the different structural features. The one letter code for amino acids is used. Hydrophobic residues are shown in white, polar residues in gray and cationic residues in black circles. N is the number of residues. The total charge Q was calculated under the assumption that K, R and the N-terminal NH_2 are positively charged and E and the C-terminal COOH bear a negative charge. H is the mean residue hydrophobicity, calculated on the basis of the Eisenberg [45] consensus scale of hydrophobicity. The angle subtended by the charged residues on the helix surface is denoted by Φ. H_h is the hydrophobicity of the non-charged domain (360°−Φ). μ is the hydrophobic moment calculated as the vector sum of the hydrophobicities of all residues, assuming an ideal α-helix. H and μ of melittin were calculated for the α-helical region only (residues 1–21 [92]).



Melittin

GIGAV LKVLT TGLPA LISWI KRKRQ Q-NH_2

N= 26; Q= +6; $H_{1\text{-}21}$= 0.183; $|\mu|_{1\text{-}21}$= 0.266

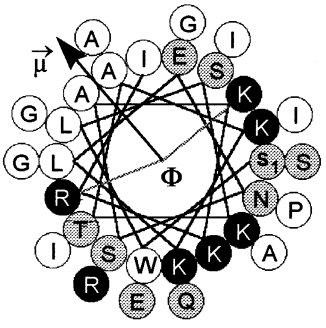


Magainin

GIGKF LHSAK KFGKA FVGEI MNS-NH_2

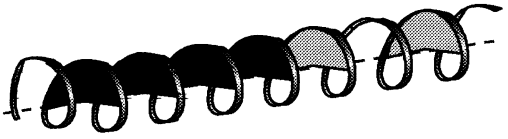
N= 23; Q= +4; H= -0.036; $|\mu|$= 0.286; Φ= 120°

H_h= 0.331



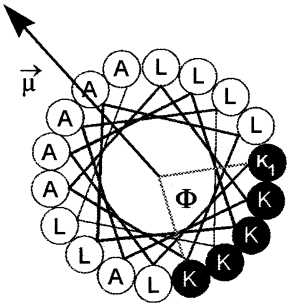
Cecropin P1

SWLSK TAKKL ENSAK KRISE GIAIA IQGGP R



N= 31; Q= +5; H= -0.230; $|\mu|$ = 0.234; Φ= 200°

H_h= 0.239



KLAL-Model

KLALK LALKA LKAAL KLA-NH_2

N= 18; Q= +5; H= -0.016; $|\mu|$ = 0.334; Φ= 80°

H_h= 0.401

Table 1
Origin and properties of representative cytolytic peptides

Origin	Peptide	Number of residues, total charge	Secondary structure	Cytolytic activity			Reference
				RBC	Bacteria		
					Gram$^+$	Gram$^-$	
Microorganisms							
Bacterium *Staphylococcus aureus*	δ-Hemolysin	27, linear 0	α-Helical	++	−	−	[88]
Insects							
Bee venom	Melittin	26, linear +6	α-Helical	++	++	++	[89]
Amphibians							
Frog skin *Xenopus laevis*	Magainin 2	23, linear +4	α-Helical	−	++	++	[90]
Mammals							
Porcine small intestine	Cecropin P1	31, linear +5	α-Helical	−	+	++	[91]

++, + and − denote high, low and practically no biological activity, respectively; RBC, red blood cells.

Table 2
Effect of parameter modification of magainin 2 amide and of the amphipathic KLAL model peptide on affinity, permeabilizing efficiency and activity on neutral (erythrocyte membrane) phosphatidylcholine (POPC) and negatively charged (bacterial membrane) POPC/POPG bilayers and the hemolytic and antimicrobial activity

1/MIC	activity	efficiency	affinity	parameter	affinity	efficiency	activity	1/EC_{50}
E. coli	POPC/POPG				POPC			RBC
⇓	⇓	n.d.	⇓	α ⇓	⇓⇓	⇓⇓	⇓⇓	⇓⇓
⇓	⇓	≈	⇓	**H** ⇓	⇓⇓	⇓	⇓⇓	⇓⇓
⇓	⇓	≈	⇓	**μ** ⇓	⇓⇓	⇓	⇓⇓	⇓⇓
⇑	⇑	⇓	⇑	Φ⇑/Ψ⇓	⇑[(1)]	≈[(1)]	⇑[(1)]	⇑

Small and large up and down arrows and ≈ symbolize small/large increases, reductions and almost no change of the corresponding feature, respectively. MIC, minimal inhibitory concentration of bacterial growth; RBC, red blood cells; EC_{50}, concentration of half-maximal hemolysis; n.d., not determined; [(1)]determined only for KLAL; α, helicity; H, hydrophobicity; μ, hydrophobic moment; Φ, angle subtended by cationic residues; $\Psi = (360° - \Phi)$, angle of the hydrophobic domain. For interpretation of the table it is to consider that the peptide affinity is by orders of magnitude higher for negatively charged lipid bilayers compared to neutral ones while the permeabilizing efficiency is much higher on neutral than on negatively charged membranes.

ity against red blood cells. The membrane of red blood cells, typically for eukaryotic cells, is composed exclusively of neutral phospholipids and has a high cholesterol content [13].

Different strategies have been developed to generate highly active antimicrobial peptides which possess a low hemolytic activity. One approach has involved the analysis of sequences of naturally occurring antimicrobial and hemolytic peptides in order to extract sequence regions that may contribute to activity, and thereafter to synthesize peptide analogues according to these patterns [14–16]. The second method uses combinational libraries and has been successfully applied to the development of helical antimicrobial peptides [17]. The third method is based on systematic studies of the role of structural properties of helical peptides in membrane interaction (for a review, see [10]). A diversity of studies have described the influence of peptide charge, helicity, intrinsic hydrophobicity, hydrophobic moment and the size of the polar/hydrophobic domain (cf. Table 2) on the permeabilizing effect on lipid bilayers and on antimicrobial and hemolytic activity. Most studies, however, have not considered that sequence modifications usually result in complex changes of more than one structural parameter, making it difficult to trace activity differences back to a specific structural motif.

This review addresses the question of the molecular basis of membrane activity and selectivity.

A variety of membrane model systems and a rich arsenal of methods for physico-chemical studies of peptide-membrane interaction (for reviews, see [8,9]) provide the basis for the interpretation of biological results. Based on our own systematic studies, we focus on advances in understanding the role of helicity, hydrophobicity, hydrophobic moment, the size of the polar/apolar helix domain and peptide charge for both binding and permeabilizing efficiency. The correlation of the effects on model membranes with activity against bacteria and red blood cells shows that these parameters may provide a powerful basis for the optimization of peptide structure with respect to antimicrobial activity. Furthermore, the data suggest that ion channels, pores and global membrane rupture do not represent three completely different modes of action, but rather that there is a continuous graduation between them. The predominance of a specific membrane-permeabilizing mechanism is determined by the interplay of structural motifs of the peptides and by their target-dependent influence on the binding and permeabilization steps.

2. Structural parameters capable of modulating activity and selectivity

2.1. Helicity

The amphipathic helix is a membrane-binding motif in many proteins and peptides formed by linear amino acid chains with a periodicity of polar and apolar residues of about three to four (Fig. 1) [18]. This structure, with the polar side chains aligned along one side and the hydrophobic residues along the opposite side of the helical coat, allows an optimal interaction of the peptides with the amphiphilic structure of the biological membrane.

It is well known that certain amino acids favor the adoption of the helical structure while others destabilize it [19]. Thus, early studies directed towards enhancing membrane activity and improving the antimicrobial effect were based on appropriate amino acid substitutions to increase peptide helicity. Such an increased helicity, attained by either deleting glycine or substituting it with leucine in the N-terminal helix of melittin, correlated well with an enhanced hemolytic and antibacterial activity of the peptide [20,21]. However, substitution of lysine at position 7 by glycine, reducing the helix propensity, had little influence on the antimicrobial and hemolytic activity [22]. Substitution of glycine residues by helix-promoting alanine drastically improved the antibacterial and hemolytic activity of magainin peptides [23,24]. Other magainin 2-derived peptides, which were designed to have more amphipathic α-helix than magainin 2, showed especially improved antibacterial activity [25]. Comparable results were reported for substitution of helix-breaking proline residues. The 1–18 stretch of the 33-residue shark repellent neurotoxin pardaxin is highly active against *Escherichia coli*, but does not show cytolytic activity. Substitution of proline 7 by alanine distinctly enhanced helicity and induced hemolytic activity [26].

Inversely, substitutions which prevent folding of

melittin into a helical conformation resulted in a loss of both hemolytic and antimicrobial activity [27]. Furthermore, incorporation of proline in the N-terminal helix of insect cecropin A [28] and cecropin P1 [29] reduced activity against different bacterial strains, which correlated well with decreased helicity.

Although these findings unquestionably demonstrate the general importance of the amphipathic helix for the biological effect, a quantitative correlation of helicity with membrane activity was seldom demonstrated. One reason is that in addition to modification of peptide helicity, amino acid substitutions involving changes in the character of the side chain (charge, hydrophobicity, surface area) affect other peptide properties such as the hydrophobic moment, the hydrophobicity, and the size of the polar/hydrophobic domain, as well as charge and charge distribution.

More detailed information concerning the role of helicity in the antimicrobial and hemolytic effects came from studies of peptides modified by replacement of L-amino acids by their corresponding enantiomers. D-Amino acid substitutions locally disturb helix formation of linear peptides while retaining the sequence, intrinsic hydrophobicity and charge. Pouny and Shai [30] reported that helix disturbance by D-amino acid substitution in pardaxin distinctly reduced the hemolytic activity of the peptide. Although no correlation between helicity and the efficiency to induce dye release from lipid vesicles was found, these early studies showed that peptide helicity might be more important for activity on neutral than on negatively charged lipid bilayer membranes.

Replacement of two adjacent amino acid residues by their D-isomers has been found to induce a more pronounced local disturbance of helix conformation than does single amino acid substitution [31]. A systematic exchange of two neighboring residues in magainin by their D-enantiomers (double D-amino acid replacement set) resulted in decreased helicity in the membrane-bound state and distinctly reduced its permeabilizing activity against neutral and moderately negatively charged lipid vesicles. In contrast, almost no differences were found in the peptide concentrations inducing permeability in highly negatively charged phosphatidylglycerol membranes [32]. Investigations with a cationic amphipathic KLAL model peptide (Fig. 1) confirmed that the amphipathic helix is essential for the action on neutral lipid membranes but appears less important for the permeabilization of negatively charged bilayers [33].

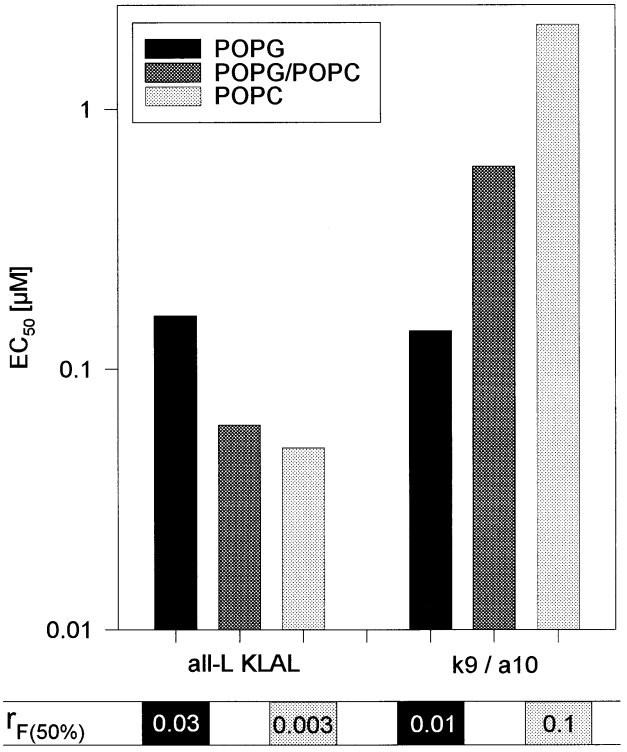


Fig. 2. Permeabilizing activity of the amphipathic helical model peptide KLAL and of a non-helical analogue produced by substitution of residues 9 and 10 by the corresponding D-amino acids on negative charged phosphatidylglycerol (POPG), phosphatidylcholine (POPC) and mixed POPC/POPG (3/1) vesicles (lipid concentration, 12 μM). EC_{50} is the concentration required to induce $F = 50\%$ dye release. The permeabilizing efficiency of bilayer bound peptides is represented by $r_{F(50\%)}$ denoting the molar ratio of bound peptide per lipid inducing 50% dye release. There is no difference in the activity of the two structurally different peptides on highly negatively charged vesicles. With reduction of membrane charge the permeabilizing efficiency of the helical peptide increases by a factor of 10 resulting in enhanced activity despite low binding. The ability of the non-helical analogue to induce dye release from neutral vesicles is much reduced. This is caused by a distinctly reduced permeabilizing efficiency in addition to a decreased affinity [33].

Studies with double D-amino acid substitution sets provided a deeper insight into the relevance of helicity to peptide binding and their ability to permeabilize lipid membranes. The high affinity of the cationic peptides to negatively charged membranes suggests that the accumulation is driven by electrostatic forces. However, the high affinity is offset by a

comparably low permeabilizing efficiency as documented for the KLAL model, in which a high number of bound molecules per lipid appears necessary to induce dye release from lipid vesicles (Fig. 2). An identical membrane affinity and permeabilizing efficiency, despite distinct structural differences, suggest that high binding is sufficient to destabilize highly negatively charged lipid membranes [33].

With reduction of electrostatic interactions in the case of a reduced lipid bilayer charge, the peptide affinity decreases [32,33]. Concomitantly, hydrophobic interactions become more relevant. This is confirmed by the observed reduction of the bilayer affinity of analogues with a disturbed amphipathic helix. On the progressive reduction of membrane lipid negative charge, the ability of KLAL to disturb the neutral lipid bilayer distinctly increases (Fig. 2). The high permeabilizing efficiency decreases, however, as soon as the amphipathic helix is disturbed. In conclusion, the high activity of peptides against neutral membranes is related to a well established hydrophobic peptide domain which provides the high membrane permeabilizing efficiency.

A relationship between helicity and activity is also found on the cell membrane level. KLAL analogues with low helicity, low affinity, and low permeabilizing efficiency on neutral membranes (charge corresponds to erythrocyte membrane) are unable to lyse red blood cells. On bacterial membranes, however, the relation between helicity and activity is more complex. Effects determined by charge and hydrophobic peptide-membrane interactions superimpose. While the model peptides displayed only moderate changes in antimicrobial activity with reduced helicity, the antimicrobial activity of magainin double D-isomers practically disappeared [33].

Comparable results have been reported for diastereomers of melittin [34] and pardaxin [35]. The D-amino acid-containing peptides lost their helix structure, which abrogated their hemolytic activity but did not diminish their antibacterial activity. The antimicrobial activity correlated with peptide binding and the membrane-destabilizing effect on liposomes. Only hemolytic peptides bind to and destabilize zwitterionic phospholipid membranes, while the diastereomers interact only with negatively charged phospholipids. Studies of D-amino acid-containing KL model peptides [36] complete the picture. The non-helical peptides are very weakly or not active against neutral lipids, but the permeability enhancing effect of the stereoisomers on negatively charged phosphatidylethanolamine/phosphatidylglycerol liposomes is high and little differentiated and correlates with the antibacterial activity against *E. coli*.

Helix-inducing organic solvents such as TFE are frequently referred to as 'membrane mimicking'. However, structure-inducing driving forces in isotropic solvents and on the non-isotropic membrane interface are generally different. Alcohols weaken non-local hydrophobic interactions and favor local polar interactions, such as the formation of intramolecular hydrogen bonds [37]. On lipid model membranes the hydrophobic interactions between peptide side chains and lipid acyl chains appear to be the primary forces driving secondary structure formation [38]. Wimley and White [39] have shown that the very unfavorable free energy contribution of partitioning of the polar peptide bonds into the lipid bilayer hampers peptide insertion. They proposed that intramolecular NH-CO hydrogen bonding reduces the energy costs of partitioning and thereby promotes helix formation (for a review, see [40]). Studies with magainin peptides show that helicity is independent of the membrane charge, indicating that electrostatic interactions driving the accumulation of the cationic peptide at negatively charged membranes have little influence on the peptide conformation [32].

As a result of different driving forces, the secondary structure of lytic peptides may be quite different in structure-inducing organic solution or when bound to lipid membranes. This is convincingly demonstrated by conformational studies with pardaxin peptides. These peptides were shown to adopt a helical or β-sheet structure depending on whether lipid vesicles or different concentrations of TFE and sodium dodecyl sulfate (SDS) were used in the experiments [26,41]. An amphipathic model peptide was found to be helical in the presence of organic solvents but β-structured in the presence of SDS or upon binding to the interface of a C18-acyl chain-coated plate [42]. Considerable differences in structure have also been reported for cecropin-magainin hybrids, displaying a helical content of 56% in TFE but only 17% in the presence of negatively charged SDS micelles [43]. The helicity of magainin 2 amide

was found to be about 55% in TFE/buffer and 76% in the lipid membrane-bound state [32].

The problem of determining the structure of lipid-bound peptides arises mainly from limitation of the available methods. The method of choice is two-dimensional ^{1}H-NMR spectroscopy; the application of this technique to the study of lipid vesicle-associated peptides has, however, proved problematical because of slow molecular reorientation rates involved. Other spectroscopic methods such as circular dichroism (CD), Raman and Fourier transformed infrared spectroscopy (FTIR) can be used to obtain information about peptide conformation in the model membrane-bound state. The disadvantage of these techniques is that they provide an overall picture of the conformation but do not generally allow localization of structural motifs within the peptide sequence. An approach to obtain such information using CD spectroscopy has recently been developed by combining this method with systematic double D-amino acid substitution [32,33]. Studies with a magainin double D-replacement set [32] confirmed the results obtained by solid state NMR measurements of selectively ^{15}N labeled magainin analogues [44], indicating that the peptide is completely helical in the lipid-bound state. The high sensitivity of CD spectroscopy additionally revealed that the magainin helix is rather flexible at the N-terminus and more stable in the region ranging from residue 9 to 20. The same approach led to the result that a potentially amphipathic KLAL model peptide in a membrane environment is characterized by a central helix and flexible N- and C-termini [33].

Finally, it should be emphasized again that valid structure-activity correlation studies require extensive knowledge concerning the conformation of the lipid membrane-bound peptide.

2.2. Hydrophobic moment

The hydrophobic moment is a quantitative measure of peptide amphipathicity. It is defined as the vector sum of the hydrophobicities of the individual amino acids (Fig. 1) [45]. Originally, this concept was developed to study protein folding [46], but the more general definitions were subsequently found to be applicable to membrane-related protein and peptide structures. The hydrophobic moment plot, i.e. the dependence of the hydrophobic moment per residue on the mean residue hydrophobicity, may be used to predict the membrane activity of peptide helices. According to this plot, membrane lytic peptides tend to cluster in a region of high hydrophobic moment and moderate hydrophobicity. However, a *complete* correlation of the hydrophobic moment and activity is rendered more difficult because of two factors.

(i) In many peptides, such as melittin (Fig. 1), the hydrophobic and hydrophilic residues are not regularly distributed within the chain. As a consequence, there are regions characterized by higher and lower hydrophobic moments. An extension of the hydrophobic moment concept is the hydrophobicity potential, depicting both the hydrophobic gradient along a peptide sequence and the hydrophobic contours around a helix [47]. However, most authors prefer to use the traditional hydrophobic moment to assess amphipathicity.

(ii) The hydrophobic moment is calculated on the basis of an ideal helix but the helicity of membrane-bound peptides is often considerably lower than 100%. As shown for magainin [32], chain regions with highest amphipathicity and the most stable helix region provide the greatest contribution to peptide activity. However, the problem of simultaneous consideration of changes in amphipathicity as result of modification of both the secondary structure and the peptide sequence, has not been satisfactory solved. Matsuzaki et al. [48] did not find a correlation between the hydrophobic moment and the lytic activity of different peptides. This was not surprising considering the large differences in hydrophobicity, overall charge, and secondary structure of peptides such as magainin, alamethicin, tachyplesin and gramicidin S. In contrast, the gradual reduction of the hydrophobic moment of melittin by substitution of lysine in the hydrophobic N-terminal helix led to a loss of antimicrobial and hemolytic activity [27]. Recent studies with a highly polar C-terminal melittin fragment showed that minor rearrangement in the sequence, correlated with an increase of μ, resulted in a considerably increased antimicrobial activity [49]. An attempt to quantify the dependence of the antimicrobial activity of magainin 2 and 12 derived analogues led to the conclusion that the mean hydrophobic moment is a more important factor governing antimicrobial activity than are hydrophobicity or helix content [50].

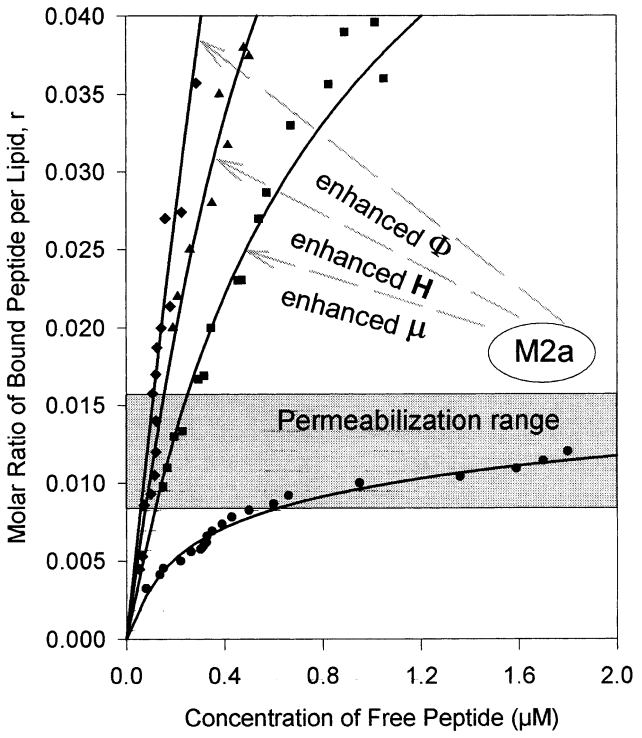


Fig. 3. Influence of structural parameters (μ, hydrophobic moment; H, hydrophobicity; Φ, angle of the polar helix domain) of magainin peptides on the lipid affinity (POPC/POPG 3:1 vesicles). The peptides shown are: ●, M2a (magainin 2 amide) [60]; ♦, 140° M2a (enhanced Φ) [71]; ▲, $I^6A^8L^{15}I^{17}$ M2a (enhanced H) [60]; ■, $I^6V^9W^{12}T^{15}I^{17}$ M2a (enhanced μ) [52]. The permeabilization range indicates the molar ratio of bound peptide per lipid, where the POPC/POPG (3:1) membrane becomes permeable.

The relation between hydrophobic moment and activity has also been studied by the use of cationic model peptides composed of only a few different amino acids. Blondelle et al. [51] reported that introduction of lysine into the hydrophobic region of a model peptide enhanced the antimicrobial activity, although the substitution also disturbed the hydrophobic domain and decreased the hydrophobic moment, as reflected by a lower retention time in reversed phase HPLC. Introduction of hydrophobic residues in the hydrophilic helix face resulted in compounds which displayed also higher antibacterial activity than the parent sequence and were additionally considerably hemolytic. Since the bioactivity of each of the two series of peptides was almost independent of the site of substitutions, it is obvious that parameters other than the hydrophobic moment predominate in this instance.

In addition to our efforts to understand the general role of the amphipathic helix for the membrane lytic effect, we investigated in detail the influence of the hydrophobic moment on antimicrobial and hemolytic activity and analyzed the biophysical principles involved in the modulation of membrane activity [52–54]. To overcome the problem of simultaneous changes of other parameters, we employed an approach of minimal sequence modification. According to this approach, the hydrophobic moment was modulated by a few conservative amino acid substitutions, while the hydrophobicity, the peptide charge, the chain length and the angles subtended by the hydrophilic and hydrophobic helix faces were largely kept constant. Studies with analogues of the antibacterial magainin 2 amide indicated that slight enhancement of the hydrophobic moment by a few conservative amino acid substitutions substantially increased the antimicrobial activity but also induced hemolytic activity. The activity increase on moderately negatively charged lipid bilayers paralleling bioactivity was mainly caused by enhanced binding due to strengthened hydrophobic interactions (Fig. 3). The permeabilizing efficiency of the membrane-bound peptides was barely affected [52]. Additional studies with KLAL model peptides revealed that the hydrophobic moment plays a minor role in the permeabilization of highly charged lipid membranes but substantially influences their effect on neutral lipid membranes. This observation confirms that changes of the hydrophobic moment predominately influence hydrophobic peptide-lipid bilayer interactions [54]. Interestingly, the activity modulating effect of the hydrophobic moment decreases for peptides of very low as well as high hydrophobicity [53]. Obviously, there is a limited hydrophobicity range within which the hydrophobic moment is a strong modulator of membrane disrupting activity.

In conclusion, modifications of the hydrophobic moment are an applicable tool to regulate the bioactivity of peptides. However, the opportunity to design peptides with substantially increased antimicrobial activity, while retaining selectivity, remains limited.

2.3. Hydrophobicity

The peptide hydrophobicity reflects the intrinsic

capability of a peptide to move from an aqueous into a hydrophobic phase. Hydrophobic interactions, in addition to other forces such as electrostatic interactions, conformational transitions and van der Waals interactions, play a major role in the partitioning of antibacterial peptides into the lipid bilayer of biological membranes. Peptide hydrophobicity is hence expected to be a strong modulator of membrane activity. With respect to hydrophobicity, two general requirements have to be fulfilled for a membranelytic peptide to be part of the host defense system: (i) the peptide has to be sufficiently soluble in water to allow storage in high concentration and to enable rapid transport to the microbial target (low hydrophobicity required) and (ii) it must be able, at the same time, to interact with the hydrophobic region of the bilayer in order to disturb the bilayer structure and to enhance the membrane permeability (high hydrophobicity required). However, too high a hydrophobicity would result in self-association or even precipitation of the peptide in water and would thus prevent its transport to the microbial target. On the other hand, a peptide with a very low hydrophobicity has an insufficient lipid affinity.

Several studies have addressed the question of the relation between hydrophobicity and antibacterial activity. Juvvadi [55] reported that the antibacterial activity of a cecropin A-melittin hybrid peptide could well be modulated by the hydrophobicity of the residue in position 8. Likewise, hydrophobicity was, beside amphipathicity, the most important factor determining the antibacterial effect of a set of analogues of the antimicrobial peptide CAMEL0 [56].

However, hydrophobicity was much less important than the hydrophobic moment for the antibacterial activity of peptides derived from the antisense sequence of magainin 2 [50]. Several studies using model peptides even indicated that no strong correlation exists between antibacterial effect and hydrophobicity [51,57,58].

A more direct relation seems to exist between hemolytic activity and hydrophobicity. For model peptides of different chain lengths and peptide hydrophobicity, a clear correlation between hydrophobicity and the ability to induce hemolysis has been observed [58]. Blondelle and Houghten [51] reported that the decrease of hydrophobicity resulting from substitution of lysine for leucine in the hydrophobic region of an amphipathic model peptide considerably reduced its hemolytic activity. On the other hand, replacement of lysine by leucine in the hydrophilic helix region, increasing the hydrophobicity, enhanced the hemolytic effect. A strong correlation between hemolysis and the RP-HPLC retention times, and hence hydrophobicity, was also found for analogues of the frog peptide brevinin 1E [59].

It should be emphasized that the results summarized above are generally derived from a comparison of peptides which differ in more than one structural parameter. For example, replacement of a lysine residue for leucine in the hydrophobic helix face [51] not only reduces the hydrophobicity, but also modifies amphipathicity and overall charge (see also Sections 2.1, 2.2 and 2.5). Deletion of N-terminal residues from brevinin 1E affects not only its hydrophobicity but also its helix propensity and the hydrophobic moment [59]. Such simultaneous alteration of more than one structural parameter complicates assessment of the role of hydrophobicity and may be one reason for the contradictory results obtained for the relation between hydrophobicity and antibacterial effect.

In order to address this problem, we have designed peptide analogues with varied hydrophobicity but with other structural parameters largely unchanged. Hydrophobicity-modified analogues were derived from the amino acid sequence of the bacteria-selective magainin 2 amide and the amphipathic helical model peptide KLAL, which exhibits high antibacterial as well as hemolytic activity [33,53,60]. In agreement with the work discussed above, a strong correlation between hydrophobicity and the hemolytic effect was evident in both peptide sets. Likewise, the antibacterial activity against *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was found to increase with increasing peptide hydrophobicity. However, the relative influence of hydrophobicity was often larger for the hemolytic than for the antibacterial effect. Thus, the more pronounced reduction of hemolytic activity compared to antimicrobial activity with decreasing hydrophobicity led to a selectively antimicrobial KLAL analogue. An enhanced antibacterial specificity can thus be achieved by reducing the peptide hydrophobicity. An additional consequence of this finding is that a hydrophobicity-based gain in antibacterial activity is likely

accompanied by a reduced antibacterial specificity. Such a reduced antibacterial specificity with increasing peptide hydrophobicity was also observed for PGLa analogues [61].

The molecular basis for the larger influence of hydrophobicity on the hemolytic than on the antibacterial effect was investigated using model membranes [33,60]. Hydrophobicity modifications led to large differences in the potential of peptides to permeabilize electrically neutral phosphatidylcholine membranes. However, the differences between analogues of varied hydrophobicity considerably decreased with increasing content of negatively charged lipids in the model membrane. Obviously, the electrostatic accumulation of basic peptides in the vicinity of the negatively charged membrane reduces the relative contribution of hydrophobicity to the overall effect.

Another important question is to clarify whether hydrophobicity-induced activity modifications arise exclusively from changes in the lipid affinity or also from differences in the membrane-disturbing activity of the bound peptide. For the magainin hydrophobicity set, the analysis of binding isotherms and activity profiles determined for moderately negatively charged lipid bilayers revealed that hydrophobicity mainly modulated the lipid affinity (Fig. 3) [60]. An influence on the membrane-disturbing activity of the bound peptide was found for the interaction of hydrophobicity modified KLAL peptides with neutral phosphatidylcholine bilayers (Dathe, unpublished results). On highly negatively charged membranes, modification of hydrophobicity of the cationic peptides is without any relevance.

2.4. *Angle subtended by the hydrophilic/hydrophobic helix surfaces*

Membrane binding involves penetration of the peptide's hydrophobic helix face into the lipid acyl chain region, whereas the hydrophilic peptide groups remain in contact with the polar lipid head groups and the aqueous surroundings. Membrane binding and perturbation are hence expected to depend on the relative size of the hydrophilic and hydrophobic helix cores. A simple parameter used to express the hydrophilic/hydrophobic residue distribution within an amphipathic helical peptide is the angle subtended by the hydrophobic or hydrophilic helix face. Independent of the hydrophobicity and the hydrophobic moment, the hydrophobic/hydrophilic angle is expected to influence the location of the peptide within the membrane and the structure of the transmembranal pores. Furthermore, the distribution of polar and non-polar residues may induce a positive or negative membrane curvature strain, thereby destabilizing lipid bilayer membranes.

A simple classification of the membrane location of membrane-binding peptides with regard to their hydrophobic/hydrophilic domain size was suggested by Brasseur et al. [62]. According to their classification, peptides with a small hydrophilic angle and a high overall hydrophobicity associate to form transmembranal pores, whereas peptides with approximately equivalent hydrophilic and hydrophobic cores orient parallel to the membrane surface. The currently available experimental data for native antibacterial peptides suggest that these are predominantly located parallel to the membrane surface, independent of their hydrophilic/hydrophobic angle [44,63–65]. However, these findings do not exclude the possibility that a minor fraction of the bound peptide is involved in the formation of transmembranal pores.

Kiyota et al. [66] investigated 18-residue amphipathic model peptides with rather drastically varied hydrophobic/hydrophilic domain sizes. The peptides consisted solely of lysine and leucine in a ratio varying between 5:13 and 13:5. The ability of the analogues to induce lipid vesicle leakage and hemolysis completely paralleled the magnitude of the hydrophobic area. A deep penetration into the hydrophobic bilayer core was observed for peptides containing at least nine leucine residues. Peptides with less leucine residues were only able to interact with negatively charged membranes. Obviously, a broad hydrophobic core is a prerequisite for effective hydrophobic membrane perturbation. It should be mentioned that these results can also be interpreted on the basis of a modified peptide hydrophobicity and charge. Specific conclusions concerning the role of the polar/non-polar angle cannot be drawn.

An attempt to understand the influence of the angle subtended by the polar/non-polar helix face was made by Tytler et al. [67]. According to the classification of Segrest et al. [68], many antibacterial α-helical peptides belong to the class L (lytic) amphipathic peptides, characterized by a high mean residue

hydrophobic moment and a narrow hydrophilic helix face containing predominantly lysine residues, whose long side chains can allow increased penetration of the peptide into the membrane. The inverted wedge shape of class L peptides leads to the induction of a negative curvature strain and the resulting bilayer-destabilizing effect has been suggested to be the molecular basis of membrane permeabilization [67,69]. In addition, the potential of a peptide to induce a negative curvature strain facilitates membrane fusion, thereby providing an alternative mechanism of membrane permeabilization [70]. The apex angle of the inverted wedge is mainly determined by the angles circumscribed by the hydrophilic and hydrophobic helix regions and the hydrophilic/hydrophobic domain sizes should hence have a marked influence on the peptide potential to induce curvature strain. In order to test this hypothesis, Tytler et al. [67] investigated three peptides derived from the model peptide 18L with polar face angle of 100°, 120°, and 140°, respectively, and reported a decrease in the hemolytic activity with increasing angle. Differential scanning calorimetry experiments revealed that 18L promotes the formation of the inverted hexagonal phase (H_{II}) of phosphatidylethanolamine and induces a negative curvature strain. The decreased hemolytic effect with increasing polar angle (comprised mainly by positively charged lysine residues) was hence explained by the reduced effectiveness in inducing negative curvature strain. It should be mentioned that the analogues with increased polar angle also possessed a reduced hydrophobic moment. Therefore, the decreased hemolytic effect may at least partially arise from the reduced amphipathicity.

Membrane destabilization can generally also be induced by a positive curvature strain. In fact, in contradiction to the expectation for class L peptides, recent investigations of the antibacterial peptides magainin 2 amide (120° polar angle) [71], PGLa (80° polar angle) and the model peptide KLAL (80° polar angle) (Wieprecht and Dathe, unpublished results) have shown that these peptides *raise* the bilayer-to-hexagonal phase transition temperature of a phosphatidylethanolamine membrane and are hence able to induce a positive curvature strain in a strain-free membrane. In order to investigate systematically the influence of the polar/non-polar helical angles on the membrane perturbation activity, we have designed peptide sets in which the polar/positively charged angle of magainin and KLAL was varied between 80° and 180°. In accordance with our design principles, the hydrophobicity, the hydrophobic moment and the number of charged residues were maintained constant within each peptide set. Differential scanning calorimetry revealed that all analogues, independent of their polar angle, raised the bilayer-to-hexagonal phase transition temperature of phosphatidylethanolamine and hence induced positive curvature strain [71] (Wieprecht and Dathe, unpublished results). For the magainin set, the activity to permeabilize model membranes, the antibacterial activity as well as the hemolytic activity were enhanced in those analogues with a large positively charged domain. The enhanced activity of these analogues was traced back to an increased lipid affinity (Fig. 3). However, the membrane-disturbing efficiency of the membrane-bound peptide decreased with increasing angle subtended by the positively charged helix face. This finding is in accordance with a pore formation model recently suggested for magainin [72]. The membrane-permeabilizing efficiency of analogues with enhanced angle of positively charged residues is reduced due to electrostatic repulsion between adjacent helices within the pore, thus resulting in a decreased pore-forming probability and/or pore destabilization. Recently, Matsuzaki et al. [73] suggested that the potential of magainin to induce positive curvature strain might even facilitate the formation of a torus-type pore.

In contrast to the results obtained for the magainins, modulation of the size of the hydrophobic/hydrophilic domains of the more hydrophobic KLAL model peptides had only a slight influence on their antimicrobial and hemolytic activities [53]. The moderate activity changes on red blood cells correlated with a low influence of the size of the charged/hydrophobic domain on the permeabilizing activity on neutral lipid bilayers. This highlights the dominance of the intrinsic hydrophobicity of the KLAL peptides and shows that the modulating potential of the size of the polar/hydrophobic domains is generally connected with the magnitude of other structural parameters. The ability of the KLAL and magainin peptides to induce dye release from highly negatively charged phosphatidylglycerol vesicles decreased with increasing polar angle [54]. Since differences in

the membrane affinity of the analogues are negligible at highly negatively charged membranes, it is evident that changes of the balance between the polar and hydrophobic domain of this type of peptides mainly affect the permeabilizing efficiency.

2.5. Charge

Most natural antibacterial and hemolytic peptide are positively charged. The specific role of a positive peptide charge for the interaction with the negatively charged membranes of Gram-positive and Gram-negative bacteria has long been recognized and is documented by a variety of attempts to induce or to improve antimicrobial activity by charge modification. The electrically neutral δ-hemolysin (Table 1) is highly hemolytic but devoid of antimicrobial activity. Introduction of a cationic net charge by substitution of negatively charged residues by lysine conferred upon the peptide potent antibacterial activity [74]. The addition of ten lysine residues to the N-terminus of the antibacterial magainin 2 drastically increased antibacterial activity without enhancing hemolytic activity [75]. Also, glutamic acid exchange by alanine [76] or glutamine [77] enhanced the positive charge of magainin and increased antimicrobial potency. Conversely, a gradual reduction of the positive peptide charge resulted in a loss of antimicrobial activity, while the hemolytic activity was less influenced or even increased as shown for magainin [77] and the N-terminal stretch of pardaxin [26].

There is, however, no simple correlation between peptide charge and antimicrobial activity. In model peptides composed solely of leucine and lysine residues, enhancement as well as reduction of charge led to analogues with increased antibacterial activity [51]. We again emphasize that these substitutions also modify other peptide structural parameters. Other studies of synthetic model peptides [66] and magainin analogues (Dathe et al., to be published) showed that highly cationic peptides might even be devoid of antibacterial activity though they are highly hemolytic.

Our studies of the role of amphipathicity and hydrophobicity in the antimicrobial and hemolytic action of helical peptides led to the suggestion that peptide-membrane interactions are determined by a sensitive balance of electrostatic and hydrophobic interactions [53]. Reinforcement of electrostatic interactions by modification of the positive peptide charge, together with a conservation of a high permeabilizing efficiency based on a well developed hydrophobic helix domain, is expected to enhance the antibacterial activity and to improve prokaryotic selectivity. Following the strategy of systematic variation of one parameter while conserving all others, we studied magainin analogues of modified charge (Dathe, to be published). As expected, the increase of peptide charge enhanced the activity against Gram-positive as well as Gram-negative bacteria. Simultaneously, the hemolytic activity practically disappeared. Compared to the native magainin 2 amide, the antimicrobial activity of the most potent analogue was increased by a factor of about 50, and the selectivity for *E. coli* could be substantially improved. However, extension of the cationic charge beyond a threshold reduced the bactericidal activity and very high hemolytic activity resulted in a loss of prokaryotic specificity.

To elucidate the basic principles underlying the membrane permeabilizing process, it is important to know how peptide charge modulates membrane affinity and the permeabilizing efficiency of peptides. The role of electrostatic interactions in the binding step is well established. Titration calorimetry studies showed that binding can be treated as a simple partition of peptide molecules between the aqueous phase and the membrane surface, provided that the effect of electrostatic attraction to or repulsion from the membrane surface is properly corrected (for a review, see [78]). Using the Gouy-Chapman theory to correct for electrostatic effects, it was recently shown that the enhanced lipid affinity of magainin for negatively charged membranes is a consequence of the increased peptide concentration in the vicinity of the membrane due to the negative surface potential. A specific interaction of the positively charged peptide with the negatively charged membrane could be excluded [79,80]. Increasing affinity in connection with enhanced negative charge density of lipid bilayers was also reported for melittin [81], cecropin B [82], cecropin P1 [29] or magainin [32,83]. Further insight into the role of electrostatic interactions comes from studies with de novo designed compounds [84]. Cationic peptides containing a sequence region composed exclusively of hydrophobic residues

spontaneously inserted into negatively charged as well as neutral phospholipid bilayers. However, the introduction of less hydrophobic residues into the hydrophobic block resulted in analogues possessing selectivity for negatively charge membranes. This underlines the importance of electrostatic interactions between the lipid head groups and basic residues of the peptide for the membrane binding of peptides lacking a purely hydrophobic chain region. This principle is also well documented in native peptides, as a comparison of melittin and magainin reveals (Fig. 1, Table 1). Melittin, characterized by a very hydrophobic N-terminus and an extremely polar, charged C-terminus, interacts with neutral as well as negatively charged lipid bilayers and lyses red blood cells and bacterial membranes. In contrast, magainin, which lacks a hydrophobic chain segment, preferentially interacts with negatively charged lipid bilayers and possesses antibacterial specificity.

Electrostatic peptide-membrane lipid interactions are also an important determinant of the permeabilizing efficiency of peptides. Inhibition of the lytic potential with increasing negative membrane charge has been reported for a KLAL model peptide and magainin 2 amide [33,60]. The reduction of the permeabilizing efficiency (Fig. 2) has been proposed to result from strong electrostatic peptide-lipid interactions which anchor the cationic peptide in the lipid head group region. Inhibited membrane immersion has also been suggested to be responsible for the reduced lytic activity of melittin against highly negatively charged lipid bilayers [85]. Competing effects are also operative when the cationic charge of magainin is enhanced [77]. An increased charge improved peptide binding to the negatively charged lipid membrane, but intermolecular repulsion between highly cationic peptide helices within the pore structure disturbed the permeabilizing mechanism and thus reduced lytic activity.

The studies above emphasize the importance of cationic peptide charge for the recognition of bacterial membranes. However, because of the sensitive balance of electrostatic and hydrophobic peptide-membrane interactions, a simple correlation between the number and position of cationic residues on the one hand and activity and antimicrobial specificity on the other hand does not exist.

3. Summary: the potential of global structural parameters to modulate activity

In recent years, our knowledge concerning the role of structural motifs in antimicrobial peptides for membrane activity and selectivity has been significantly increased.

Studies of a variety of natural peptides and their chemically modified analogues, as well as model compounds, convincingly demonstrated that a cationic charge is essential for the antimicrobial effect. Since electrostatic interactions are advantageous for binding but tend to decrease the ability of peptides to permeabilize negatively charged membranes, the importance of charge for the antimicrobial effect consists in (i) recognition of the bacterial membrane and (ii) high peptide accumulation in the vicinity of the target membrane. To possess antimicrobial activity, less hydrophobic peptides should have a well developed hydrophobic domain providing the peptide with a high hydrophobic moment, while peptides with low amphipathicity may display antimicrobial activity if they possess an appropriate high intrinsic hydrophobicity.

The concentration of natural antimicrobial peptides necessary to inhibit bacterial growth is usually in the micromolar range. All studies published so far indicate that the opportunities to distinctly increase activity by modification of structural motifs are limited. The growth-inhibiting concentration of most effective peptide analogues has been found to be only slightly below 1 μM.

The importance of the peptide structural parameters charge, helicity, hydrophobic moment, hydrophobicity and domain size (Table 2) consists in their different roles in the interaction with the membranes of prokaryotic and eukaryotic cells.

With reduction of electrostatic interactions due to a decreased membrane charge, the activity-modulating potential of hydrophobicity, the hydrophobic moment and the size of the hydrophobic domain becomes more pronounced (Table 2). The effective disturbance of the electrically neutral membrane is more dependent on a high permeabilizing efficiency than on a high lipid affinity. Since the hemolytic activity can be effectively reduced through reduction of amphipathicity (helix disturbance, reduction of the hydrophobic moment), lowering the size of the hy-

drophobic domain and decreasing the intrinsic hydrophobicity, these variables provide a powerful basis for the improvement of the antimicrobial selectivity.

The results summarized in this review are in accordance with a model of peptide-lipid interactions which explains the different activities by changes in the membrane affinity and in the location of bound peptides. On negatively charged membranes electrostatic interactions cause a high peptide accumulation and a tight binding of the peptide molecules to the lipid head groups. This surface state is less effective for permeabilization. On neutral membranes, hydrophobic interactions become significant, and despite low binding affinities, the peptides may penetrate deeply into the hydrophobic membrane region and thereby effectively disturb the barrier function of the lipid bilayer.

This mechanistic picture of differences in the dominance of electrostatic and hydrophobic peptide-membrane lipid interactions provides an explanation for the different activity spectra (Table 1) of peptides such as the hemolytic δ-hemolysin, the hemolytic and antibacterial melittin and the antibacterial magainin and cecropin P1, which is selective for Gram-negative bacteria.

δ-Hemolysin is inactive against bacteria, since a net charge of zero prevents peptide accumulation at negatively charged bacterial membranes. However, the high hydrophobic moment of the well developed amphipathic helix allows binding to and penetration into the neutral membranes of erythrocytes.

The activity of melittin on eukaryotic cell membranes is mediated by strong hydrophobic interactions between the extensive hydrophobic N-terminal peptide domain and the neutral membrane (Fig. 1). The large angle of the N-terminal hydrophobic helix domain and the low charge of its small polar coat support a deep penetration into the hydrophobic membrane interior and might favor the association of membrane-bound peptides to form transmembrane ion channels. The effect on prokaryotic membranes is additionally favored by ionic interactions of the charged C-terminus, causing peptide accumulation in the outer leaflet of negatively charged membranes.

In magainin, structural motifs favoring hydrophobic interactions are poorly developed: the cationic charge is spread over the whole peptide chain and the hydrophobicity and hydrophobic moment are only moderate (Fig. 1). Hence, magainins are not hemolytic. However, electrostatic contributions to membrane interactions result in antimicrobial activity against Gram-positive bacteria. The activity against Gram-negative bacteria is even higher because of additional pronounced accumulation at the outer wall. The large, mildly hydrophobic domain allows shallow insertion of the helical rod parallel to the membrane surface. Reorientation of magainin helices making the outer and inner leaflets a continuum while keeping the charged peptide face always in contact with the lipid head groups may lead to complex transbilayer pores.

A high cationic charge, low hydrophobicity, low hydrophobic moment and a small hydrophobic domain prevent interaction of cecropin P1 (Fig. 1) with the neutral membrane of erythrocytes. However, electrostatic forces mediate high activity of cecropin P1 against bacteria. The effect of the poorly developed hydrophobic domain which seems to inhibit peptide penetration into the hydrophobic membrane region of Gram-positive bacteria is partially compensated by a pronounced accumulation on the envelope of Gram-negative bacteria to give high activity. It is reasonable to assume that the peptide accumulation in the outer leaflet of the negatively charged target membrane enhances the interfacial tension disturbing the bilayer packing and finally leading to membrane disruption.

In summary, the structural parameters of membrane-lytic peptides discussed in this review are effective modulators of membrane activity. Provided the properties of the target membrane are known, the balance between hemolytic and antimicrobial activity can be optimized by variation of the individual parameters. However, general optimization of activity against a broad spectrum of pathogens is a multidimensional problem and has proved difficult to solve. Each peptide has its unique spectrum of activity determined by the combination of the structural motifs discussed. Furthermore, the differences in lipid composition and the architecture of the membranes of different target cells complicate activity optimization. Finally, recent reports concerning the translocation of amphipathic peptides through cell membranes [86] and peptide binding to DNA [87]

lead to speculations that the mechanism of action could be more complex than originally anticipated and might involve also events others than the breakdown of the membrane barrier function.

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