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# General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides

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**Abstract**

Model compounds of modified hydrophobicity ($H$), hydrophobic moment ($\mu$) and angle subtended by charged residues ($\Phi$) were synthesized to define the general roles of structural motifs of cationic helical peptides for membrane activity and selectivity. The peptide sets were based on a highly hydrophobic, non-selective KLA model peptide with high antimicrobial and hemolytic activity. Variation of the investigated parameters was found to be a suitable method for modifying peptide selectivity towards either neutral or highly negatively charged lipid bilayers. $H$ and $\mu$ influenced selectivity preferentially via modification of activity on 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) bilayers, while the size of the polar/hydrophobic angle affected the activity against 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG). The influence of the parameters on the activity determining step was modest in both lipid systems and the activity profiles were the result of the parameters' influence on the second less pronounced permeabilization step. Thus, the activity towards POPC vesicles was determined by the high permeabilizing efficiency, however, changes in the structural parameters preferentially influenced the relatively moderate affinity. In contrast, intensive peptide accumulation via electrostatic interactions was sufficient for the destabilization of highly negatively charged POPG lipid membranes, but changes in the activity profile, as revealed by the modification of $\Phi$, seem to be preferentially caused by variation of the low permeabilizing efficiency. The parameters proved very effective also in modifying antimicrobial and hemolytic activity. However, their influence on cell selectivity was limited. A threshold value of hydrophobicity seems to exist which restricted the activity modifying potential of $\mu$ and $\Phi$ on both lipid bilayers and cell membranes. 

*Keywords:* Antimicrobial peptides; Model peptides; Hydrophobicity; Hydrophobic moment; Liposomes; Permeability; Protoplasts

Abbreviations: BHI, brain heart infusion; CD, circular dichroism; CFU, colony forming units; EDTA, ethylenediaminetetraacetic acid; LB, Luria broth; LUVs, large unilamellar vesicles; MIC, minimal inhibitory concentration; OD, optical density; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol; RP-HPLC, reversed phase-high performance liquid chromatography; SMM, sucrose, maleic acid, MgCl solution; SMMPA, sucrose, maleic acid, MgCl, protein and antibiotica medium; SUVs, small unilamellar vesicles; TFE, 2,2,2-trifluoroethanol; Tris, tris(hydroxymethyl)aminomethane

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## 1. Introduction

The widespread increase of bacterial resistance towards many conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents and new target sites. Initially discovered as a defence system in invertebrates and vertebrates, antimicrobial peptides are attracting increasing interest as potential therapeutics [1,2]. Unlike classical antibiotics which must penetrate the target cell, the principal mode of action of peptides involves perturbation and permeabilization of the cell membrane. This mechanism confers activity towards a broad spectrum of microbial cells, but is also responsible for undesired lytic activity against mammalian cells such as erythrocytes (for reviews, see [3,4]). Much evidence implies that peptide-induced membrane permeabilization is the result of interaction of the peptides with the lipid matrix of the cell envelope. The induction of a specific amphipathic, often helical structure upon interaction with the bilayer surface has been established as a requirement for lytic activity [5]. The lipid matrix of the membrane provides a unique environment for binding of such peptides. Additionally, most of the known antimicrobial peptides bear cationic amino acid residues and interact preferentially with negatively charged membranes. The high amount of anionic lipids in prokaryotic membranes and their absence in the neutral lipid matrix of erythrocytes may account for the antimicrobial activity and selectivity of many cationic peptides. But, unlike magainins [6], dermaseptins [7] from frog skin and the insect cecropins [8] which are selective for bacteria, the bee venom melittin [9] and the neurotoxin pardaxin [10] are lytic to both bacterial and mammalian cells. The specificity is also mimicked in model liposome studies. Magainins [11] and insect cecropins [12] induce leakage preferentially from acidic lipid vesicles. Melittin [13] and pardaxin [14] permeabilize neutral and negatively charged vesicles. Among the helical antimicrobial peptides, magainin analogs [15] and dermaseptin sequences [16] have been suggested as promising candidates for the development of potential antimicrobial therapeutic agents. Since a better understanding of the interplay between membrane properties and the peptides' physico-chemical properties may provide the basis for the design of compounds for directed interaction, many studies have been devoted to the interactions of peptides with model membranes and their biological relevance (for review see [17,18]). The activity modulating role of individual structural parameters, namely peptide helicity ($\alpha$), hydrophobicity ($H$), hydrophobic moment ($\mu$), the angle subtended by charged residues ($\Phi$) as well as the total peptide charge ($Q$) was demonstrated [18–22]. We showed that the strengthening of hydrophobic peptide properties favored the lytic effect on the neutral lipid bilayer of red blood cells and reduced antimicrobial selectivity, since the interaction with negatively charged lipids, characteristic for prokaryotic cell membranes, was only modestly affected. Reduction of the parameters $\alpha$, $H$, $\mu$ and $\Phi$ actually enhanced antimicrobial selectivity but was accompanied by a pronounced decrease of activity.

Besides the characterization of new antibacterial peptides and the design of analogs of natural peptides, model peptides composed of a limited number of different amino acid residues have been studied extensively in order to understand the general aspects of peptide–lipid interaction, [19,23–28]. Many of the earlier studies simultaneously modified several parameters, making it difficult to distinguish the contribution of each individual motif to the overall effect.

We investigated sets of model peptides with individually modified $H$, $\mu$ and $\Phi$, while the other parameters were conserved. The basic KLA compound had pronounced antimicrobial as well as high hemolytic activity. The study was made to determine whether the characteristics described for the activity and selectivity of the antimicrobial magainin 2 amide are generally valid. We examined whether modulation of $H$, $\mu$ and the size of the polar ($\Phi$) and hydrophobic ($\Psi$) helix surfaces would be sufficient to confer membrane selectivity on the non-selective parent peptide. Structural studies of bilayer-associated peptides and the determination of peptide affinity and permeabilizing efficiency on highly negatively charged and electrically neutral lipid bilayers were used to elucidate the driving forces in the bilayer permeabilization process. Pure, rather than mixed lipid systems were used because in the latter case it is difficult to distinguish whether the peptide interacts with the initially provided mixture or with domains locally enriched in one of the components. Addition-

ally, although the outer membrane of Gram-negative bacteria is characterized by an even higher negative charge density the pure 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol (POPG) bilayer was used to mimic its charge properties [29,30].

The results demonstrate that modulation of the parameters was a suitable method for inducing specific interaction with electrically neutral or negatively charged lipid bilayers. The magnitude of a parameter was found to be related to the modifying potential of the motifs. Thus, the high hydrophobicity of the peptide dominated the effect on neutral vesicles and restricted the activity modulating potential of $\mu$ and $\Phi$. The relation of peptide bilayer interactions to the biological effects emphasized the activity modifying role of the parameters and showed that the activities observed on neutral and charged bilayers superimpose on the complex membranes of biological cells. As a consequence, the activity was modified by variation of the parameters but their selectivity influencing potential was limited.

## 2. Materials and methods

### 2.1. Materials

The lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) and POPG were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Calcein was obtained from Fluka Chemie (Neu-Ulm, Germany), and 2,2,2-trifluoroethanol (TFE) from Aldrich-Chemie (Steinheim, Germany). Tris-(hydroxymethyl)aminomethane (Tris), maleic acid, ethylenediaminetetraacetic acid (EDTA), $MgCl_2$ and other chemicals were from Merck (Germany). Luria broth (LB) was from Gibco BRL (UK), sucrose and bovine serum albumin from Sigma (USA), antibiotica medium 3 from Difco (USA), lysozyme from Roanal (Hungary), and brain heart infusion (BHI) from Becton (USA). Sucrose was from Roth (Germany), penicillin G and yeast extract from Serva (Germany).

### 2.2. Peptide synthesis and characterization

Model peptides were synthesized automatically by the solid phase method using standard Fmoc chemistry in the continuous flow mode on a MilliGen 9050 (Millipore, USA) peptide synthesizer [31]. The peptides were purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) to give final products more than 95% pure by HPLC analysis. The peptides were further characterized by matrix assisted laser desorption/ionization mass spectrometry (MALDI-II, Kratos, Manchester, UK) and quantitative amino acid analysis (LC 3000, Biotronik-Eppendorf, Germany). Chromatographic characterization was performed on a Shimadzu LC-10A gradient HPLC system. Runs were carried out on a PolyEncap-7 A 300 (250×4.0 mm i.d.) column (Bischoff Analysentechnik, Germany) using a Shimadzu LC-M10A gradient HPLC system with a diode array detector operating at 220 nm. The sample concentration was 1 mg/ml peptide in eluent A. Mobile phase A was 0.1% trifluoroacetic acid in water and B was 0.1% trifluoroacetic acid in 80% acetonitrile/20% water (v/v). The retention times ($t_R$) of the peptides were determined using a linear gradient of 5–95% B over 40 min at 22°C. The precision of $t_R$ was $\pm 0.1$ min.

Peptide hydrophobicity ($H$), hydrophobic moment ($\mu$) and hydrophobicity of the non-polar helix surface ($H_{hd}$) were calculated using the Eisenberg consensus scale for hydrophobicity [32].

### 2.3. Preparation of small and large unilamellar vesicles

Small unilamellar vesicles (SUVs) were prepared by drying the lipid under high vacuum, suspending the film by vortex mixing in buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH 7.4) to a final lipid concentration of about 30 mM and sonicating the suspension (under nitrogen, in ice water) for 25 min using a titanium tip ultra sonicator. Dynamic light scattering measurements (N4 Plus, Coulter Corporation, USA) confirmed the existence of a main population of POPC and POPG vesicles (more than 95% mass content) with a mean diameter of $45 \pm 3$ nm (polydispersity index 0.3). Calcein containing large unilamellar vesicles (LUVs) were prepared by vortexing the lipid in dye buffer solution (70 mM calcein, 10 mM Tris, 0.1 mM EDTA, pH 7.4). The suspension was freeze-thawed in liquid nitrogen for six cycles and extruded (Lipex Biomembranes, Can-

ada) through polycarbonate filters [33] (six times through two stacked 0.4 μm pore size filters and eight times through two stacked 0.1 μm pore size filters). Untrapped calcein was removed using a mini-column centrifugation method [34]. A plastic syringe (1 ml volume, plugged with a filter pad) mounted in a centrifugation tube was filled with hydrated Sephadex G-50 gel. After spinning at 2000 rpm for 3 min the gel column had dried and parted from the sides of the syringe. 500 μl of the vesicle suspension was dropped onto the gel bed and the liposomes were eluted by centrifugation at 2000 rpm for 3 min. The mean diameter of the vesicles was determined to be $93 \pm 1$ (polydispersity index 0.08). Lipid concentration was determined by phosphorus analysis [35].

### 2.4. Circular dichroism measurements

Circular dichroism (CD) measurements of $10^{-5}$ M peptide solutions in TFE/buffer (10 mM Tris, 154 mM NaF, pH 7.4) (1/1, v/v) and freshly prepared SUV suspensions were carried out on a J-720 spectrometer (Jasco, Japan) at room temperature. Minor contributions of CD and circular differential scattering of the SUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free suspensions. The amount of helix was calculated from the mean residue ellipticity at 222 nm [36]. The error in helicity was $\leq 5\%$.

### 2.5. Dye efflux measurements

LUV suspension (10 μl) was injected into cuvettes containing 2.5 ml of stirred peptide solutions of different concentration. Calcein release from vesicles was monitored fluorimetrically by measuring the decrease in self-quenching (excitation at 490 nm, emission at 520 nm) after 1 min at room temperature on an LS 50B spectrofluorimeter (Perkin Elmer, Germany). The fluorescence intensity corresponding to 100% release was determined after the addition of Triton X-100 (100 μl, 10% v/v in water) [30]. The concentration of half maximal dye release ($EC_{50}$) was determined from dose–response curves. Dye release, $F$ (%), as a function of bound peptide per lipid, $r$, was determined as described [11]. Dose–response curves were determined at three lipid concentrations, usually 12, 36 and 120 μM. Plotting the total peptide concentration $c_p$ as a function of lipid concentration $c_l$ for a given $F$ (%) results in a straight line. According to the mass conservation law $c_p = r \times c_l + c_f$, the slope of the curves gives the degree of binding $r = c_b/c_l$ and the intercept with the $c_p$ axis describes the concentration of free peptide $c_f$.

### 2.6. Peptide binding

Binding isotherms were determined from the change of the CD of peptide solutions (three different concentrations between $5 \times 10^{-5}$ and $2 \times 10^{-6}$ M) after adding different amounts of SUVs. For the determination of the binding isotherms, the relations $F = \Theta_{222}(p) - \Theta_{222}$ and $F = F_\infty(c_b/c_p) = F_\infty(c_l/c_p) \times r$ with $r = c_b/c_l$ were used. $F$ is the relative CD signal, $\Theta_{222}(p)$ the ellipticity at 222 nm in the absence of lipid, $\Theta_{222}$ the measured ellipticity in the presence of lipid, $F_\infty$ is $F$ of the completely lipid-bound peptide, $c_b$ is the concentration of lipid-bound peptide, $c_l$ is the lipid concentration and $c_p$ is the total peptide concentration. Binding isotherms are derived from these equations and the mass conservation law [37]. Binding isotherms for peptide interaction with POPG vesicles were estimated from dye release experiments using the procedure described above [11]. With $r$ and $c_f$ binding isotherms were constructed and the apparent binding constant, $K_{app}$ could be derived from the initial slope of the curves.

### 2.7. Hemolytic assay

The hemolytic activity of the peptides was determined using human red blood cells (Blutspendedienst Deutsches Rotes Kreuz, Berlin, Germany) as described previously [30]. In brief, the suspensions (10 mM Tris, 150 mM NaCl, pH 7.4) containing the peptide and $2.3 \times 10^8$ cells/ml were incubated for 30 min at 37°C. After cooling in ice water and centrifugation, an aliquot of the supernatant was diluted with 0.5% $NH_4OH$ and the optical density was measured at 540 nm (Lambda 9 spectrophotometer, Perkin Elmer, Germany). Peptide concentrations causing 50% hemolysis ($EC_{50}$) were derived from the dose–response curves. Values determined in repeat experiments differed by less than 5%.

### 2.8. Antibacterial activity

Gram-negative *Escherichia coli* (DH5α strain) and Gram-positive *Bacillus subtilis* (PY 22 strain) were used to test the antibacterial activity of the peptides. Bacteria were cultivated in LB at 37°C with shaking at 180 rpm. The inoculum was prepared from mid logarithmic phase cultures ($OD_{600} = 0.5$). Aliquots of the cell suspensions were added to the wells of a microtiter plate containing 50 μl peptide solutions of different concentrations. The final concentration of bacteria in the wells was $1.25 \times 10^6$ colony forming units (CFU)/ml. The final concentrations of peptides ranged from 0.04 to 80 μM in two-fold dilutions. Peptides were tested in duplicate. The microtiter plates were incubated 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 optical density.

Bacterial protoplasts were prepared as follows. An aliquot of an overnight culture of *E. coli* was further cultivated in LB to an $OD_{600} = 0.8$ at 37°C with gentle shaking at 120 rpm. 50 μl cell suspension was centrifuged for 10 min at $3000 \times g$ and 4°C. The pellet was resuspended in 2.5 ml ice cold sucrose solution (10% in 50 mM Tris, 10 mM $MgCl_2$, pH 8.0) and mixed with 0.5 ml lysozyme (5 mg/ml in 50 mM Tris, pH 8.0). After 5 min at 4°C 1 ml EDTA (25 mM in 50 mM Tris, pH 8.0) was added. After gentle shaking for 5 min at 4°C 1 ml Tris buffer (50 mM, pH 8.0) was added. After shaking (80 rpm) for another 15 min at 37°C 2 ml 10% sucrose solution was added and the suspension was centrifuged for 20 min at $2000 \times g$. The pelleted *E. coli* protoplasts were resuspended in 10% sucrose solution for further use. Protoplasts of *B. subtilis* were prepared by cultivation of the cells to an $OD_{600} = 0.8$. After centrifugation of 50 ml cell suspension for 10 min at $3000 \times g$ and 4°C the pellet was resuspended in 5 ml SMMPA solution and mixed with 5 ml lysozyme (4 mg/ml SMMPA). SMMPA solution was prepared from 49.5 ml SMM solution (consisting of 2 ml 1 M maleic acid, pH 6.4; 2 ml 1 M $MgCl_2$; 25 ml 2 M sucrose; 21 ml $H_2O$), 36 ml antibiotica medium 3 and 4.5 ml bovine serum albumin solution (50 mg/ml $H_2O$). After incubation for 120 min at 37°C and gentle shaking the suspension was centrifuged for 15 min at $3000 \times g$ and the pellet was resuspended in 2.5 ml SMMPA. Incubation, centrifugation and resuspension were repeated once. The concentration of protoplasts was estimated by cell counting under the microscope using a Neubauer blood counting chamber. In order to determine peptide-induced lysis aliquots of the protoplast suspension were pipetted into cuvettes containing different concentrations of peptide in the corresponding medium. The final cell content was $2 \times 10^8$ protoplasts/ml. The peptide concentration varied between 0.25 and 64 μM. After 10 min at room temperature the optical density of the samples of *B. subtilis* protoplasts was measured at 600 nm (Lambda 9, Perkin Elmer). Peptides lysed protoplasts in a concentration-dependent manner. The $EC_{50}$ was derived from dose–response curves as the peptide concentration causing half maximal reduction of optical density. Peptide-induced lysis of *E. coli* protoplasts was followed by measuring the scattering intensity at 475 nm using a fluorescence spectrophotometer (LS 50B, Perkin Elmer, Germany). The $EC_{50}$ of lysis was derived from dose–response curves as the concentration causing half maximal reduction of the scattering intensity.

*E. coli* W*1655 F*+ cells (LWF+) were cultivated in BHI (containing 100 U/l penicillin G). *B. subtilis 170* (L170) grew in BHI (containing 1% yeast extract, 3% sucrose and 100 U/l penicillin G). Bacteria suspended in 5 ml cultivation medium were incubated overnight at 37°C and at 180 rpm. An aliquot of the culture was diluted in 50 ml medium and further cultivated to an $OD_{550}$ of 0.8 corresponding to $7.8 \times 10^8$ LWF+ cells/ml and $3.2 \times 10^8$ L170 cells/ml. After dilution, the inoculum was prepared from an $OD_{550} = 0.4$ cell suspension. 180 μl cell suspension was added to wells of a microtiter plate containing 20 μl peptide solution. The final peptide concentration ranged between 0.5 and 100 μM, the cell concentration in the wells was $3.5 \times 10^8$ LWF+ cells/ml and $1.4 \times 10^8$ L170 cells/ml. After incubation overnight at 37°C and shaking at 180 rpm the absorbance was read at 600 nm (Microplate Autoreader EL 311, Bio-Tek Instruments, USA) and the MIC was determined.

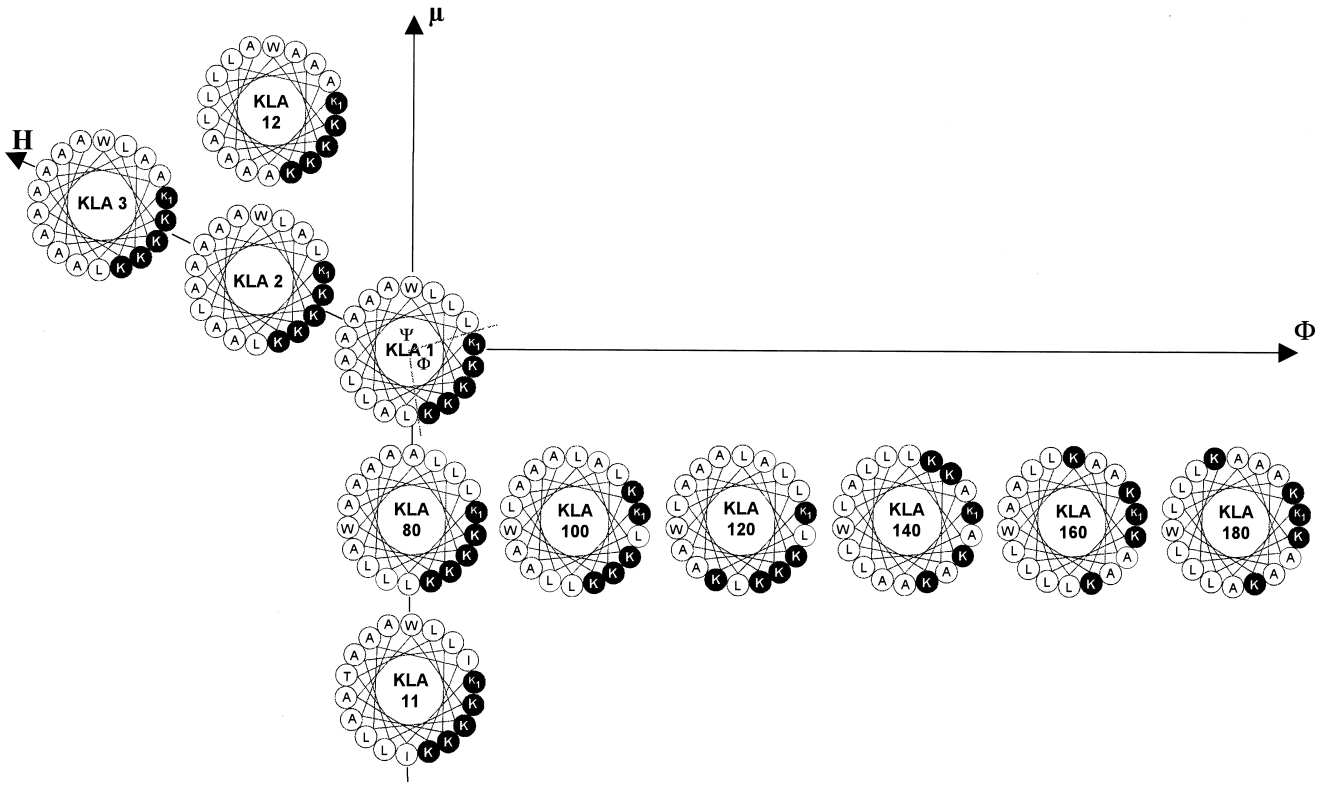


Fig. 1. Helical wheel projection of KLA model peptides of modified hydrophobicity ($H$), hydrophobic moment ($\mu$) and angle subtended by polar ($\Phi$)/hydrophobic ($\Psi$) amino acid residues.

## 3. Results

### 3.1. Peptide design and structural properties

The model peptides were designed on the basis of criteria derived for natural antimicrobial peptides: cationic charge and the ability to from an amphipathic helix. The basic sequence, KLA1, consists of 18 amino acid residues including five lysine residues and one tryptophan in addition to leucine and alanine (Fig. 1). Sets of peptides of identical charge and size but of modified hydrophobicity ($H$-set), changed hydrophobic moment ($\mu$-set) and varying angle subtended by charged ($\Phi$)/hydrophobic ($\Psi$) residues ($\Phi$-set) (Table 1) were designed on the basis of two principles: changes in the position of individual amino acid residues and minor residue exchange. The Eisenberg consensus scale of hydrophobicity was used for calculating the structural parameters [32]. $H$ is the mean residue hydrophobicity calculated as the sum of the hydrophobicities of the individual residues and $\mu$ is their vector sum. The peptides of the $H$-set were developed by reducing the number of leucine and increasing the number of alanine residues (compare KLA1 vs. KLA 2 vs. KLA3). As the result of amino acid exchange the total peptide hydrophobicity varied between $-0.025$ and $-0.087$ and the hydrophobicity of the non-polar helix surface ($H_{hd}$) decreased from 0.389 to 0.302. $H_{hd}$ was calculated as mean residue hydrophobicity of the amino acid residues on the helix surface described by the angle $\Psi$. The hydrophobic moment of KLA2 (0.33) was enhanced by changing the position of selected alanine and leucine residues (compare KLA2 vs. KLA12). Starting from the most hydrophobic KLA1 additional amino acid substitutions were necessary to reduce the hydrophobic moment from 0.33 to 0.28 (compare KLA1 vs. KLA11). $H$, $H_{hd}$ and $\Psi$ were conserved in the two $\mu$-sets. Peptides of the $\Phi$-set were designed exclusively by changing the position of the five cationic, six alanine and six leucine residues. Although the total hydrophobicity was unchanged these modification resulted in differences in $H_{hd}$, which thus lay between 0.363 and 0.389 for the peptides with a large

hydrophobic domain ($280° > \Psi > 240°$) and between 0.43 and 0.47 for peptides with $220° > \Psi > 180°$ (Table 1).

The linear peptides exhibited high conformational flexibility in aqueous solution, but assumed a helical conformation in the presence of structure inducing TFE as confirmed by CD spectroscopic studies (Table 1). Following Lehrman et al. [38], we take the helicity in the TFE environment as a measure of the ability of peptides to form a helix. Comparably high helicities were determined for vesicle-bound peptides. Conditions of binding were derived from the CD spectroscopic titration experiments presented in Section 3.3.

For potentially amphipathic peptides, it has been shown that the retention time ($t_R$) in RP-HPLC is related to the helicity of peptides bound to the hydrophobic stationary phase [39,40]. The $t_R$ of peptides of the *H*-set correlated well with the helix probability, but considering all peptides, Fig. 2A reveals no correlation between $t_R$ and $\alpha$ in TFE (correlation coefficient −0.008). An improved relationship between $t_R$ and the structural properties of the peptides was reached when taking into consideration $H_{hd}$. The correlation coefficient is 0.64 for $t_R = f(H_{hd})$ and 0.60 for the regression presented in Fig. 2B. Properties related to helicity such as the size of the hydrophobic surface and $H_{hd}$ determine the interaction of the peptides with the RP-HPLC stationary phase. A pronounced *H* which is independent of the peptide conformation, however, may restrict their binding modulating potential.

### 3.2. Membrane permeabilizing activity

Peptides of modified *H* and $\mu$ displayed minor differences in their permeabilizing activity on negatively charged POPG vesicles (Fig. 3). The peptide concentration required to induce half maximal fluorescence dequenching ($EC_{50}$) ranged between about 0.1 and 0.5 μM. In contrast, the activity modifying effect of the angle subtended by charged residues on POPG bilayers was much more pronounced. Increase of the size of the polar helix surface connected with a decrease of the hydrophobic surface area distinctly impaired the permeabilizing effect. On neutral POPC vesicles, *H* was the most effective activity modifying factor, $\mu$ was less effective and the influence of the angle $\Phi$ was negligible (Fig. 3).

Changes in the activity profile influenced the bilayer selectivity. KLA1 was slightly selective to neutral lipid membranes (Fig. 3). With decreasing *H* the selectivity shifted towards negatively charged bilayers as result of conserved activity on POPG but drasti-

Table 1
Properties of KLA peptides

| Code | *H* | $\mu$ | $\Phi/\Psi$ (°) | $H_{hd}$ | $t_R$ (min) | $\alpha$ (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | TFE | POPG $c_l/c_p = 240$ | POPC $c_l/c_p = 500$ |
| KLA1 | −0.025 | 0.329 | 80/280 | 0.389 | 20.4 | 73 | 54 | 62 |
| KLA2 | −0.0561 | 0.329 | | 0.345 | 17.8 | 68 | 46 | 25[b] |
| KLA3 | −0.0872 | 0.329 | | 0.302 | 13.1 | 59 | 56 | –[c] |
| KLA11 | −0.0267 | 0.284 | | 0.386 | 19.9 | 69 | –[a] | 53 |
| KLA12 | −0.0561 | 0.391 | | 0.345 | 20.4 | 67 | 54 | 66 |
| KLA80 | −0.025 | 0.32 | 80/280 | 0.389 | 19.7 | 61 | 68 | 68 |
| KLA100 | −0.025 | 0.300 | 100/260 | 0.377 | 19.4 | 60 | 68 | 70 |
| KLA120 | −0.025 | 0.295 | 120/240 | 0.363 | 21.1 | 59 | 58 | 69 |
| KLA140 | −0.025 | 0.299 | 140/220 | 0.43 | 22.6 | 62 | 67 | 78 |
| KLA160 | −0.025 | 0.297 | 160/200 | 0.45 | 21.6 | 60 | 59 | 63 |
| KLA180 | −0.025 | 0.291 | 180/180 | 0.475 | 22.3 | 62 | 54 | 57 |

Characteristics of KLA model peptides: hydrophobicity (*H*), hydrophobic moment ($\mu$) and angle subtended by cationic ($\Phi$)/hydrophobic ($\Psi$) residues, hydrophobicity (per residue) of the hydrophobic surface ($H_{hd}$), retention time ($t_R$) in RP-HPLC and amount of helix ($\alpha$) in TFE/buffer mixture (1/1 v/v), buffered POPG, POPC SUV suspension at a lipid to peptide molar ratio $c_l/c_p$. The peptide concentration, $c_p$ was $10^{-5}$ M.

[a]The sample was turbid at all $c_l/c_p$.

[b]All values except this represent the conformation of vesicle-bound peptides.

[c]The peptide did not bind to POPC vesicles.

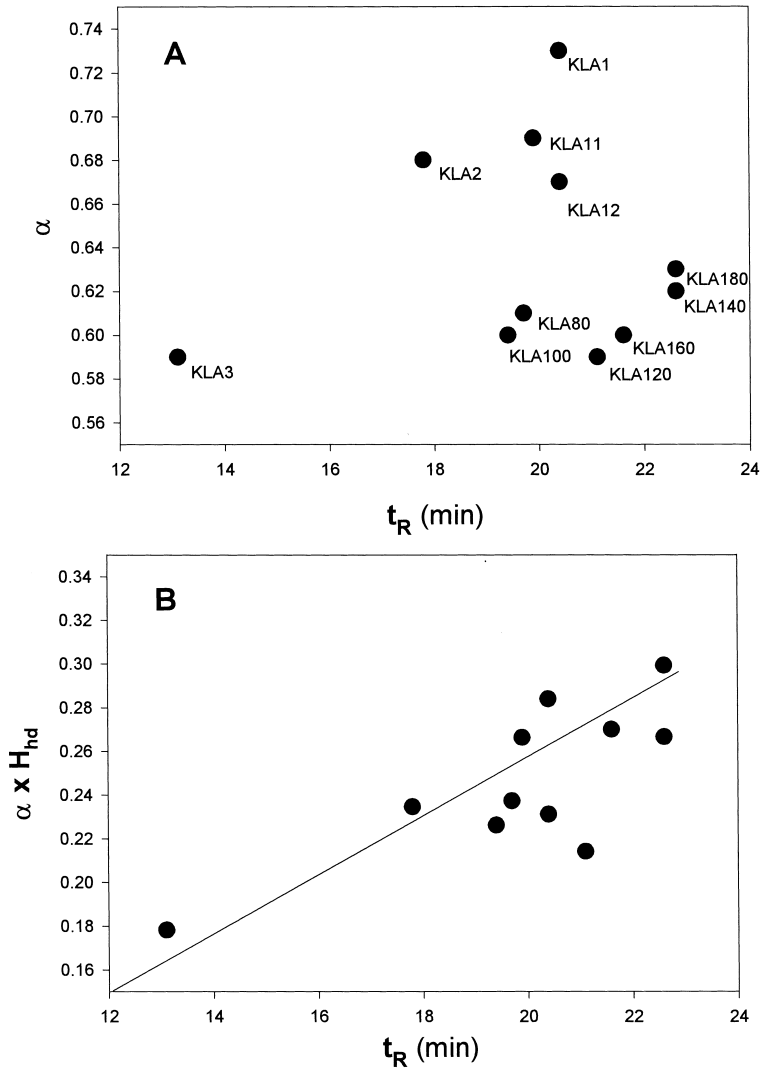


Fig. 2. Relationship between the RP-HPLC retention times ($t_R$) of KLA peptides and helical content ($\alpha$) (A) and the product of $\alpha$ and the hydrophobicity of the non-polar helix surface ($H_{hd}$) (B). $\alpha$ was determined in a TFE/buffer (1/1 v/v) mixture. The buffer was 10 mM Tris, 154 mM NaCl, 0,1 mM EDTA, pH 7.4. $H_{hd}$ was calculated as the mean residue hydrophobicity of the amino acid residues covering the hydrophobic helix surface defined by $\Psi$.

cally reduced activity on POPC vesicles. The selectivity modifying effect of $\mu$ was the higher the lower the peptide hydrophobicity (compare KLA1, KAL11 with KLA2, KLA12). On analyzing the peptides of the $\Phi$-set it became obvious that the selectivity for POPC bilayers is distinctly enhanced for analogs with a large $\Phi$ and small hydrophobic domain (KLA140, KLA160, KLA180). Here, the increased POPC specificity is mainly caused by a pronounced reduction of peptide activity against the negatively charged POPG vesicles.

### 3.3. Binding and permeabilizing efficiency

Modification of membrane activity and selectivity of the peptides can be due to variations in membrane affinity as well as changes in the ability of the bound peptide fraction to disturb the bilayer structure. CD spectroscopic studies of the peptides with POPG SUVs revealed the high affinity of all peptides to negatively charged membranes. The spectra of a given analog (peptide concentration $10^{-5}$ M) were almost identical at POPG concentrations between

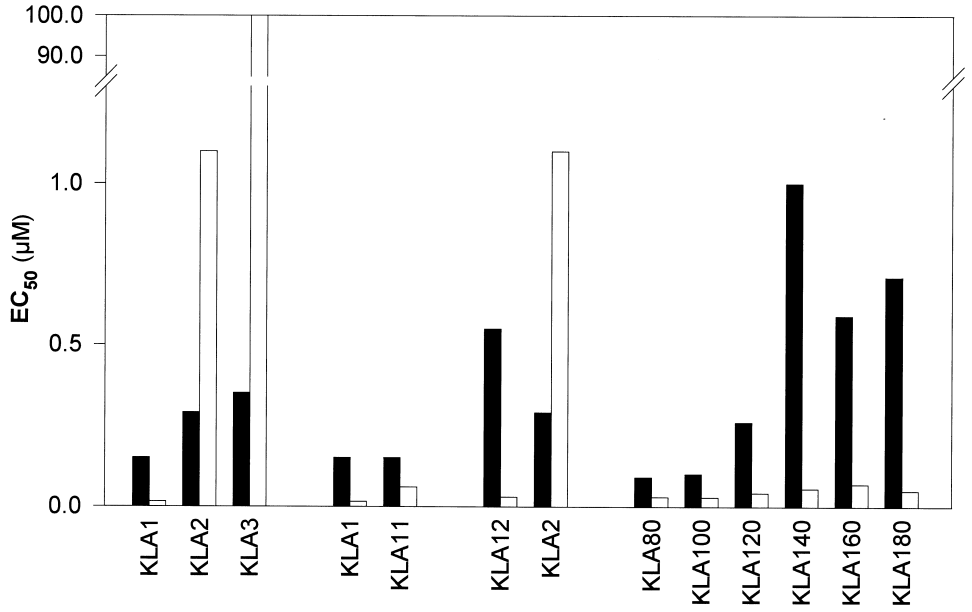


Fig. 3. Concentration of half maximal dequenching of calcein fluorescence ($EC_{50}$) induced by KLA model peptides for negatively charged POPG (black bars) and neutral POPC (white bars) LUVs. The lipid concentration, $c_l$, was 12 μM in buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH 7.4).

$2.5\times10^{-4}$ and $5\times10^{-3}$ M and reflect maximal helicity as result of binding at a low lipid/peptide ratio of 25 (data not shown). Binding studies of peptides of the $\Phi$-set using a dye release assay confirmed the high affinity. This method is suitable if membrane binding correlates directly with membrane leakage [41]. While the apparent binding constants ($K_{app}$) of peptides with a small polar domain were greater than 200 000 $M^{-1}$, the $K_{app}$ of peptides with $80° \leq \Phi \leq 120°$ ranged between 130 000 and 180 000 $M^{-1}$ (Table 2).

Peptide affinity to the neutral POPC bilayer was much lower and strongly influenced by $H$ and $\mu$ (Fig. 4). The $K_{app}$ values of KLA1 and KLA2 were determined to be 5000 and 500 $M^{-1}$, respectively, while the most hydrophilic KLA3 did not bind at all. Similarly, binding to POPC vesicles was reduced with reduction of $\mu$ in the order KLA1 ≫ KLA11 and KLA12 > KLA2. The binding constants of analogs with $80 < \Phi < 120$ were found to be about 5000 $M^{-1}$ while peptides with $140 < \Phi < 180$ bound to POPC

Table 2
Activity, affinity and efficiency of peptides of modified $\Phi$ on lipid bilayers

| Peptide | $EC_{50}$ (μM) | | $K_{app}$ (1/M) | | $r_{(F=50\%)}$ | |
|---|---|---|---|---|---|---|
| | POPC | POPG | POPC | POPG | POPC | POPG |
| KLA80 | 0.03 | 0.09 | 5100 | 250 000 | 0.0010 | 0.007 |
| KLA100 | 0.051 | 0.10 | n.d. | 200 000 | 0.0007 | 0.008 |
| KLA120 | 0.023 | 0.26 | 5500 | 220 000 | n.d. | 0.016 |
| KLA140 | 0.044 | 1.0 | 7500 | 130 000 | n.d. | 0.044 |
| KLA160 | 0.070 | 0.60 | 7500 | 180 000 | 0.0023 | 0.034 |
| KLA180 | 0.042 | 0.71 | n.d. | 130 000 | 0.0011 | 0.036 |

Activity of KLA peptides of modified angle subtended by charged residues ($\Phi$) on neutral POPC and negatively charged POPG vesicles and their affinities and permeabilizing efficiencies: half maximal concentration ($EC_{50}$) of peptide induced dye release (see also Fig. 3), CD spectroscopically derived apparent binding constants ($K_{app}$) for the binding to POPC SUVs (see also Fig. 4) and to POPG vesicles as determined by dye release assay. The ratio of bound peptide per lipid inducing half maximal dye from lipid vesicles ($r_{(F=50\%)}$) is a measure of the bilayer permeabilizing efficiency of the bound peptides. n.d., not determined.

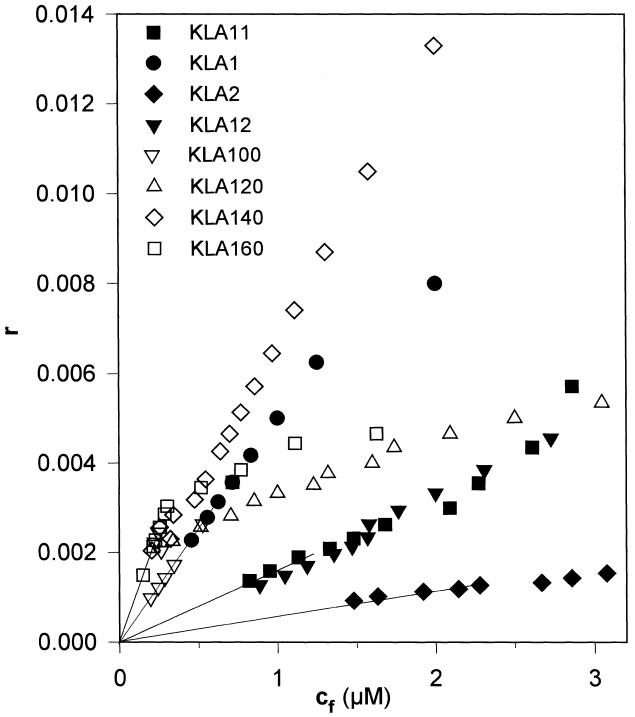


Fig. 4. Binding isotherms for the interaction of KLA model peptides with POPC SUVs. The degree of binding, $r$ (mol peptide bound per mol lipid) and the free peptide concentration, $c_f$ were determined by CD spectroscopic titration experiments (see Section 2).

with a $K_{app}$ of about 7500 $M^{-1}$ (Table 2). The affinity seems to correlate with $H_{hd}$ which is less than 0.39 for the first peptide group and over 0.43 for the second (Tables 1 and 2).

Peptide affinity to POPC correlated qualitatively with the bilayer permeabilizing activity. The differences in the binding constants of the peptides of different $H$ and $\mu$ do not, however, explain the much more pronounced changes in activity. Thus, while reduction of $H$ resulted in a 10 times reduced affinity (compare KLA1 vs. KLA2) the activity decreased by a factor of 92. Comparably, reduction of the hydrophobic moment (KLA11 vs. KLA1) reduced $K_{app}$ by a factor of about 3 but the activity was 8 times lower. Furthermore, although the binding constant of KLA1 and peptides of the $\Phi$-set was many times higher on POPG than on POPC bilayers, the peptides were much more active against POPC vesicles.

Fig. 5 and Table 2 illustrating the relationship between dye release ($F$) and the amount of bound peptide per lipid ($r$) demonstrate the parameter-dependent permeabilizing efficiency of the peptides. The ability of the peptides to permeabilize neutral POPC bilayers is high and $H$, $\mu$ and $\Phi$ exhibit only a slight modulating effect. Binding of five KLA1 and 11 KLA11 molecules per 10 000 POPC molecules induced an initial dye release of 50%, demonstrating the modest variability of the permeabilizing efficiency with reduction of the hydrophobic moment. No correlation was found between $\Phi$ and $r$. Half maximal dequenching of dye fluorescence was induced by the binding of 7–23 peptides per 10 000 lipid molecules (Fig. 5, Table 2).

In contrast, on highly negatively charged POPG bilayers the high affinity was offset by a low permeabilizing efficiency. The ability of KLA1 to permeabilize the POPG membrane ($r_{F=50\%} = 0.012$) was more than 20 times lower than their ability to disrupt neutral POPC bilayers. Peptides of modified $\Phi$ confirm the distinct differences (Table 2). Additionally, a differentiation in efficiency on POPG was found be-

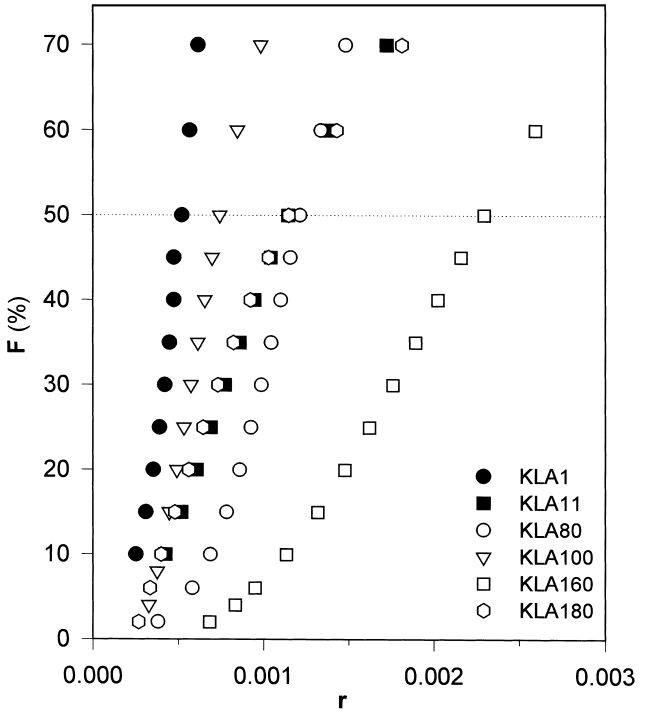


Fig. 5. Relationship between peptide-induced dye release (dequenching of calcein fluorescence, $F$) from POPC LUVs and the molar ratio of bound peptide per lipid ($r$). The $r$ value at a given $F$ is a measure for the permeabilizing efficiency of the peptides.

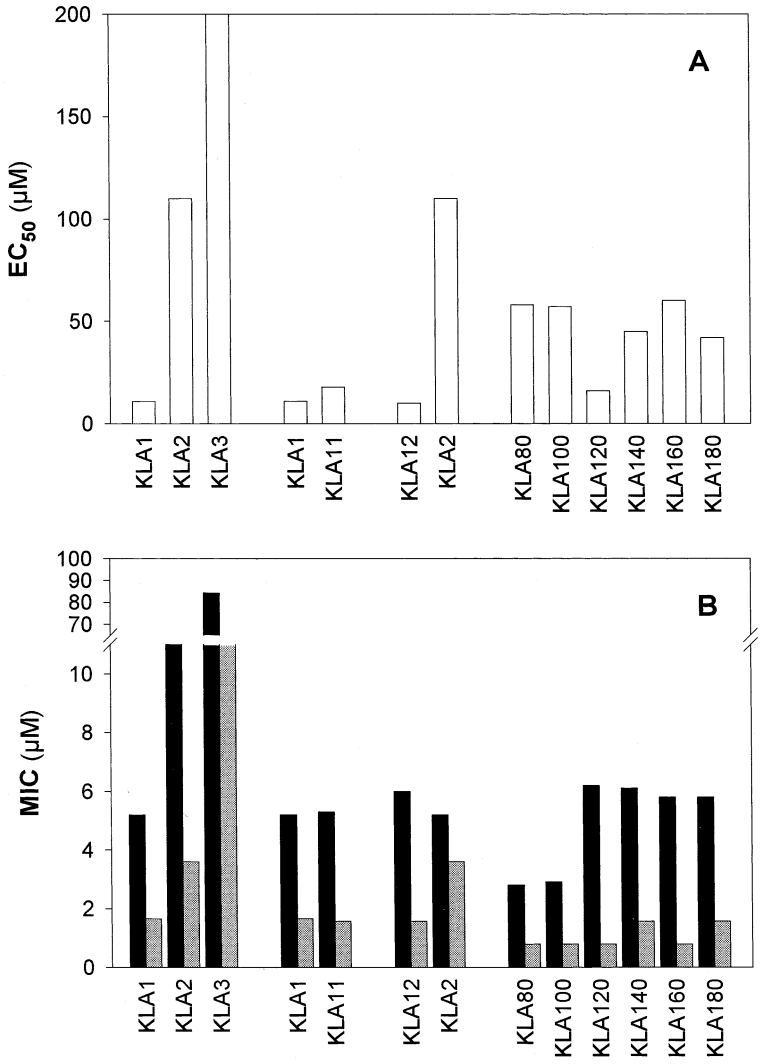


Fig. 6. Biological activity of KLA model peptides. (A) Concentration for half maximal lysis ($EC_{50}$) of human red blood cells ($2.3\times10^8$ cells/ml). Values obtained in repeat determinations differed by less than 5%. (B) MIC for the growth of *E. coli* (black bars) and *B. subtilis* (gray bars) ($1.25\times10^6$ CFU/ml).

tween peptides with a small ($80 \leq \Phi \leq 120$) and large polar surface area ($140 \leq \Phi \leq 180$). Binding of 70–160 molecules of the first group but of 340–440 molecules of the latter per 10 000 lipid molecules was necessary to induce half maximal fluorescence dequenching of POPG vesicle entrapped dye.

### 3.4. Biological activity

The investigated parameters are modulators of the antimicrobial as well as hemolytic activity (Fig. 6A,B). The peptide activity towards red blood cells decreased with reduction of $H$ and $\mu$ while there is no correlation with $\Phi$. The activity profiles were comparable to those of dye release from POPC vesicles (compare Fig. 6A and 3). Decrease of $H$ led also to a pronounced reduction of the antimicrobial activity against both *E. coli* and *B. subtilis* (Fig. 6B). $\mu$ slightly influenced the activity against *E. coli*. The effect was more pronounced for peptides of reduced $H$ (KLA2, KLA12). The growth of *E. coli* was also sensitive to changes in the size of the polar helix surface while the activity against *B. subtilis* appeared to be less $\Phi$-dependent. To further elucidate the peptide effect on bacterial cells the lytic activity of the structurally modified peptides on protoplasts and cell

wall-less L-forms of *E. coli* and *B. subtilis* was determined. Removal of the cell wall of *E. coli* was expected to eliminate the effect caused by the highly negatively charged cell envelope and thus to change the activity pattern. The results are summarized in Table 3. Decrease of $H$ distinctly reduced the activity on protoplasts, LWF+ and L170 cells. $\mu$ became an effective activity modulating parameter in the case of less hydrophobic KLA peptides (KLA2, KLA12). No common activity profile was found for peptides of the $\Phi$-set, but KLA120 seemed to be the most active analog on protoplasts and the L-form cells.

## 4. Discussion

This study demonstrates that modulation of the peptide parameters $H$, $\mu$ and $\Phi$ was sufficient to confer specific interaction with either electrically neutral or negatively charged lipid bilayers.

Increase of the parameters $H$, $\mu$ and $\Phi$ enhanced the specificity for neutral POPC bilayers, but the physical basis was different. For peptides of the $H$- and $\mu$-sets, the selectivity increase resulted from an enhanced activity against the neutral bilayer. Hydrophobic interactions between the non-polar surface of the peptide helix and the lipid acyl chains of the bilayer have been suggested to be responsible for the pronounced permeabilizing efficiency [30]. But, while the influence of $H$ and $\mu$ on the activity determining permeabilizing efficiency was modest, the activity profiles of the peptide sets correlated well with the affinity to POPC bilayers and the retention behavior on the hydrophobic HPLC stationary phase. Thus, the pronounced changes in activity reflect variations in the low binding affinity of the analogs to the neutral bilayer.

In contrast, the modulated selectivity of peptides of the $\Phi$-set is based on activity changes against highly negatively charged POPG bilayers. Interestingly, two peptide classes could be distinguished: high affinity and moderate bilayer permeabilizing ability characterize the peptides with a small $\Phi$ domain while peptides with $\Phi \geq 140°$ are somewhat less affine and much less efficient. As shown for several cationic peptides, the effect on highly negatively charged POPG bilayers is determined by high binding via electrostatic interactions [30,42] offset by a low permeabilizing efficiency which is mediated by hydrophobic interactions. The study of the $\Phi$-set shows that the properties of both the charged and the hydrophobic helix domain are important for

Table 3
Peptide activity against bacterial protoplasts and L-form cells

| Peptide | Modified parameter | *E. coli* | | *B. subtilis* | |
|---|---|---|---|---|---|
| | | Protoplasts $EC_{50}$ (μM) | LWF+ MIC (μM) | Protoplasts $EC_{50}$ (μM) | L170 MIC (μM) |
| | $H$ | | | | |
| KLA3 | −0.087 | – | >80 | – | 13.5 |
| KLA2 | −0.056 | 67.3 | 80 | 7.2 | 13.1 |
| KLA1 | −0.025 | 1.8 | 10 | 0.4 | 1.6 |
| | $\mu$ | | | | |
| KLA2 | 0.329 | 67.3 | 80 | 7.2 | 13.1 |
| KLA12 | 0.391 | 1.9 | 5.0 | 0.6 | 1.6 |
| KLA11 | 0.284 | 1.7 | n.d. | 0.4 | 1.8 |
| KLA1 | 0.329 | 1.8 | 10 | 0.4 | 1.6 |
| | $\Phi$ | | | | |
| KLA80 | 80 | 1.7 | 10 | 0.3 | 2.3 |
| KLA100 | 100 | 1.4 | 10 | 0.4 | 3.6 |
| KLA120 | 120 | 1.1 | 2.5 | 0.4 | 1.0 |
| KLA140 | 140 | 1.8 | n.d. | 0.8 | 1.9 |
| KLA160 | 160 | 3.9 | 5.0 | 0.9 | 2.3 |
| KLA180 | 180 | 2.3 | 2.5 | 1.1 | 3.4 |
| Cells/ml | | $2\times10^8$ | $7.8\times10^8$ | $2\times10^8$ | $3.5\times10^8$ |

Antibacterial activity of KLA peptides: the MIC for bacterial growth against *E. coli* derived LWF+ cells, *B. subtilis* derived L170 cells and the half maximal concentration for lysis of *E. coli* and *B. subtilis* protoplasts ($EC_{50}$).

KLA peptide effects on POPG bilayers. Since the total peptide charge was conserved, the reduction of electrostatic attraction must be associated with reduction of the cationic charge density of the polar helix surface. Affinity differences of other peptides to negatively charged lipid bilayers might also be related to variations in their charge topology [21]. The distinctly reduced permeabilizing efficiency appears to be related to the decrease in the size of the hydrophobic helix surface. Even a pronounced increase of $H_{hd}$ could not compensate for the reduced hydrophobic peptide–lipid interactions caused by a smaller $\Psi$ and reduced insertion. The results revealed a general principle of activity modification: depending on the lipid system, one of the two determinants of the bilayer permeabilization step, either binding or efficiency, dominates the activity, but its modulation is caused by the influence of the structural parameters on the second, less pronounced determinant.

The second important outcome of the studies is the observation that the activity modifying potential of the structural motifs was limited and connected with the magnitude of the parameters. The high value of hydrophobicity of the model peptides restricted the activity modulating potential of $\mu$ and $\Phi$. Threshold values of hydrophobicity dictating peptide properties and behavior in lipid bilayers have also been described for magainin peptides of modified total charge [43] and are known for transmembrane peptide sequences [44].

Our studies result in a model of peptide–lipid interaction which explains activity as function of the number of surface accumulated peptide molecules and their depth of insertion (Fig. 7). The high activity of the peptides against the POPG bilayer (Fig. 7A) derives from the concentrated surface accumulation. Insertion into the acyl chain region is inhibited because of electrostatic binding in the lipid head group region. Resultant expansion of the outer lipid layer induces tension between the outer and inner leaflets which may be released by rupture of the bilayer [4]. A lipid exchange between the layers via toroidal pores [45] in highly negatively charged bilayers has not yet been described. Decrease of the charge density of the polar helix surface reduces binding and the size-reduced hydrophobic helix surface inhibits bilayer insertion, thus reducing activity.

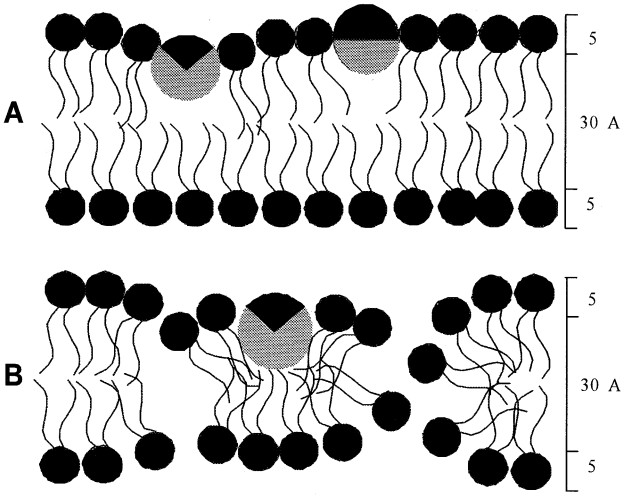


Fig. 7. Cartoon illustrating the different interactions of KLA model peptides with highly negatively charged POPG (A) and neutral POPC bilayers (B). (A) Electrostatic interactions between cationic peptide residues and the anionic lipid head groups anchor the helix in the bilayer surface. The sizes of the polar/hydrophobic domains modify the depth of insertion. (B) The highly hydrophobic peptide inserts deeply into the lipid acyl chain region thus disturbing the arrangement of a large number of molecules and inducing the release of peptide–lipid micelles.

On neutral POPC bilayers (Fig. 7B) peptides, even when weakly bound, may penetrate deeply into the hydrophobic membrane region. The high permeabilizing efficiency of the hydrophobic peptides points to drastic disturbance of the lipid bilayer arrangement, possibly by the formation of large holes as result of the release of peptide–lipid complexes. KLA peptide-induced enhancement of the phase transition temperature of bilayers composed of lipids with an intrinsic negative curvature strain (phosphatidylethanolamine; data not shown), as also described for magainin sequences [21], point to the induction of positive curvature strain in flat POPC bilayers. Positive curvature strain may promote bilayer disruption by formation of micelles. Thus, melittin induces a convex structure in phosphatidylcholine bilayers [46,47] and stimulates the release of lipids from erythrocytes [48].

It has been proposed that the main target for the killing of bacteria by antimicrobial peptides is the cytoplasmic membrane [49]. On disturbance of the lipid matrix the transmembrane potential and the pH gradient are destroyed, the osmotic regulation

is affected and respiration is uncoupled [50–52]. Comparable lytic effects are observed with erythrocytes [48].

The membrane of red blood cells is composed of electrically neutral zwitterionic lipids. Actually, the activity profiles of the investigated peptides correlated well with the effects on POPC bilayers. The highest variability in the hemolytic effect was induced by modification of $H$, but a high $H$ restricted the activity modulating potential of $\mu$ and $\Phi$.

The envelope of Gram-negative bacteria has a complex structure. To reach the inner target membrane the cationic peptides must cross the outer wall which they apparently do by utilizing the 'self promoted uptake' pathway [53]. The increase in activity towards Gram-negative bacteria is largely consistent with the demonstrated increase in binding to the highly negatively charged lipopolysaccharide and subsequent outer membrane permeabilization [54]. However, the lipid composition of the inner membrane of *E. coli* is dominated by neutral phosphatidylethanolamine. On this level, membrane damage is determined by hydrophobic interactions. This is documented by the corresponding activity profiles on neutral bilayers and on protoplasts and LWF+ cells. The differences in the susceptibility of *E. coli* protoplasts and cultured LWF+ cells might be based on slight differences in the respective membrane properties. LWF+ cells were derived from the *E. coli* K12 strain [55], protoplasts were prepared from the DH5α strain. It is well known that the membrane lipid composition in bacteria is influenced by the physiological state of the cell and by various growth factors [56]. Additionally, a comparison of the lipid composition of the LWF+ membrane and the cytoplasmic membrane of the walled parent strain showed that the contents of negatively charged phosphatidylglycerols were 8% and 17%, respectively [57]. Electrostatic interaction has also been suggested to contribute to the high activity of the peptides against the Gram-positive cell strains [19]. Comparable activities towards *B. subtilis* and the protoplasts, presented in this study, imply that the negative charges in the murein envelope [58] are of reduced importance. Thus, the low MIC values against *B. subtilis* might be explained by the increased affinity to the negatively charged membrane lipids. Additionally, hydrophobic interactions are present, consistent with the observation that the activity profile corresponds to that of neutral lipid bilayers.

In summary, we have shown that the importance of the peptide structural parameters derives from their different roles in peptide interaction with neutral and negatively charged membranes. Coulombic forces are responsible for the pronounced affinity which dominates the activity on highly negatively charged lipid bilayers. Hydrophobic interactions determine the permeabilizing efficiency which is responsible for the drastic effect of KLA peptides on neutral lipid bilayers. However, the variability of peptide activity on the two lipid systems investigated seems to be based on the parameter's influence on the second, less dominating step of the bilayer permeabilization process. Furthermore, improvement of the peptide selectivity for neutral lipid bilayers results from either increased activity against POPC or reduced activity against POPG bilayers. The first is dominated by the properties of the hydrophobic helix surface, the latter depends on the charge density of the polar and on the size of the hydrophobic helix domains. A high hydrophobicity reduces the activity modifying potential of the other investigated parameters. On the complex membranes of biological cells, parameter-dependent peptide effects on charged and neutral lipid bilayers become superimposed. As a consequence, the activity is modified by variation of the parameters but their selectivity influencing potential on cells is substantially reduced.

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