Biochimica et Biophysica Acta 1788 (2009) 1656–1666

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Biological activity and structural aspects of PGLa interaction with membrane mimetic systems

Karl Lohner *, Florian Prossnigg

Institute of Biophysics and Nanosystems Research, Austrian Academy of Sciences, Schmiedlstraβe 6, A-8042 Graz, Austria

ARTICLE INFO

Article history:
Received 10 April 2009
Received in revised form 21 May 2009
Accepted 22 May 2009
Available online 29 May 2009

Keywords:
Amphibian skin
Antimicrobial peptide
Lipid discrimination
Membrane thinning/thickening
Non-bilayer structure

ABSTRACT

Peptidyl-glycine-leucine-carboxyamide (PGLa), isolated from granular skin glands of *Xenopus laevis*, is practically devoid of secondary structure in aqueous solution and in the presence of zwitterionic phospholipids, when added exogenously, but adopts an α-helix in the presence of anionic lipids. The peptide was shown to exhibit antifungal activity and to have antimicrobial activity towards both Gram-negative and Gram-positive bacteria. As a broad variety of peptides is found in the secretions of amphibian skin combinatorial treatment of PGLa and magainin 2 was studied showing enhanced activity by a heterodimer formation. Thus production of mutually recognizing peptides seems to be an effective way in nature to increase selective membrane activity. Biophysical studies on membrane mimics demonstrated that PGLa can discriminate between different lipid species, preferentially interacting with negatively charged lipids, which are major components of bacterial but not mammalian cell membranes. This emphasizes the role of electrostatic interactions as a major determinant to trigger the affinity of antimicrobial peptides towards bacterial membranes. PGLa induced the formation of a quasi-interdigitated phase in phosphatidylglycerol bilayers below their chain melting transition, which is due to the creation of voids below the peptide being aligned parallel to the membrane surface. In the fluid phase of phosphatidylglycerol the peptide inserts perpendicularly into the bilayer above a threshold concentration, which results in a hydrophobic mismatch of the peptide length and bilayer core for lipids ≤ C16. This mismatch is compensated by stretching of the acyl chains and in turn thickening of the bilayer demonstrating that membrane thinning cannot be taken generally as the hallmark of pore formation by antimicrobial peptides. Furthermore, PGLa was shown to affect membrane curvature strain of phosphatidylethanolamine, another main lipid component of bacterial membranes, where a cubic phase coexists with the fluid bilayer phase. Investigations on living *Escherichia coli* showed distinct changes in cell envelope morphology, when treated with the peptide. In a first stage loss of surface stiffness and consequently of topographic features was observed, followed in a second stage by permeabilization of the outer membrane and rupture of the inner (cytoplasmic) membrane supposedly by the mechanism(s) derived from model studies.

Contents

Nowadays, we face a world-wide re-emergence of infectious diseases and a rapid increase in pathogenic bacteria that are multi-resistant to commercially available antibiotics. Hence the World

* Corresponding author. Tel.: +43 316 4120 323; fax: +43 316 4120 390.
E-mail address: Karl.Lohner@oeaw.ac.at (K. Lohner).

0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2009.05.012

Health Organization ranked antibiotic resistance as a priority disease and published a comprehensive document "Global Strategy for the Containment of Antimicrobial Resistance" suggesting some guidance on the implementation of interventions such as improving the use of antibiotics, enforcing regulations, strengthening health-care systems and encouraging the development of novel antibiotics. Latter is a pressing need, because the number of new antibiotics markedly decreased within the last decades. However, new drugs with similar structures relative to existing antibiotics will remain highly vulnerable to bacterial resistance mechanisms and will have only a limited life span. Therefore, alternative agents with novel mechanisms of action have to be developed. One emerging strategy is based on host defence peptides, which have evolved in nature to contend with invaders as an active system of defence (see also Zasloff this special issue).

This review focuses on one of the antimicrobial peptides found in skin secretions of frogs, namely PGLa (peptidyl-glycine-leucine-carboxyamide). In the first part of the review it will be reported about the discovery of this peptide and its biological activities. It will be described, how this peptide acts synergistically with magainin, another antimicrobial peptide component of frog, to combat pathogenic fungi, which are one cause of the world-wide decline of the population of this animal species. The potential of this peptide to be used for the development of a novel antimicrobial therapeutic will be also briefly addressed. As this peptide act on the cell membrane level a large extent of this review is devoted to studies using membrane mimetic systems, which have shed light on the mode of action of PGLa. It will be shown that the mechanism of membrane perturbation strongly depends on the phospholipid matrix and that PGLa is able to discriminate between different lipid species. These biophysical studies suggest that the peptide is capable of forming pores in lipid bilayers. However, it was also shown that PGLa affects membrane properties such as curvature strain and induces lipid–peptide domain formation owing to the preferential interaction of the cationic peptide with the anionic membrane lipids. Both perturbations may alter the environment of membrane proteins leading to membrane dysfunction. The final part of the review deals with microscopy experiments on living *Escherichia coli* cells showing that indeed membrane damage may be considered as the mode of killing by PGLa.

1. Discovery and characterization of PGLa

The skin of frog is a rich source of peptides [1], which are present in large quantities, in particular in the *Xenopus* species, as well as in *Bombina* as outlined by Simmaco and co-authors as well as by Zasloff within this special issue. A comprehensive analysis performed for the South African clawed frog, *Xenopus laevis* [2] showed that major components are peptidyl-glycine-leucine-carboxyamide or PGLa, consisting of 21 amino acid residues, magainins (23 residues) and a 25 residue peptide derived from the xenopsin precursor, which differ in their primary structure. The existence of PGLa was predicted through screening of a c-DNA library for clones encoding the precursor of caerulein by Kreil et al. [3], when searching in amphibian skin secretions for peptides closely related to mammalian hormones and neurotransmitters. The novel predicted peptide comprised 24 amino acids starting with tyrosine and ending with an amidated leucine and thus was designated PYL^a. In this study it was concluded that this peptide can form a membrane-active amphipathic helix similar to peptides with bacteriostatic, cytotoxic and/or lytic properties. The natural counterpart was isolated two years later from skin secretion of *X. laevis* by the same group [4], which was very similar to PYL^a except for the absence of the first three N-terminal amino acids (Tyr-Val-Arg). This truncated peptide, corresponding to the amino acids 39 to 59 of the precursor protein, can be generated by additional processing at the single arginine residue, another proteolytic cleavage in addition to the predicted processing events. The peptide can undergo further fragmentations producing at least eight different peptides [5]. The most abundant fragments related to amino acids 1–11 and 12–21, obtained through cleavage at the Gly-Lys site of PGLa. The actual peptide was characterized by HPLC and mass spectroscopic analysis [4,5] yielding the primary structure:

GMASKAGAIAGKIAKVALKAL-carboxyamide

now termed PGLa with a molecular weight of 1968.5. At neutral pH this peptide, ultimately shown to be antimicrobial [6] has a positive net charge of 5 because of the four lysine residues and the amino group at the N-terminal glycine. Furthermore, the peptide possesses, as does melittin, an amidated C-terminus, which supposedly increases its resistance to proteases as shown for magainin [7]. PGLa has little homology to other peptides found in the skin secretions of *X. laevis*, but owing to the general similarities, it is often included in the magainin family.

In aqueous solution, PGLa is less soluble than magainin 2a [8]. CD spectra of the peptide dissolved in physiological buffer indicated that the peptide is practically devoid of secondary structure at concentrations below 1 mg/ml [9]. At higher peptide concentrations the percentage of random structure decreased and of β-structure increased. Additionally a small amount of α-helix was detected. Similarly, IR experiments with PGLa in D_2O solution showed at acidic and neutral pD a maximum at 1643 cm^{-1}, which is characteristic for peptides with little or no well-defined secondary structure [10]. Raman [8] and CD studies [11,12] also showed little regular structure in aqueous solution at neutral pD for magainins. In contrast, Raman data [8] obtained at low temperatures in saline solution at pH 7.4 indicated that PGLa formed reversibly a precipitate containing β-sheet (63%) and reverse turn structure (21%). Jackson et al. [10] explained this observation by the different experimental conditions, which probably reflects aggregation of the peptide in the Raman study. As peptide aggregation is often accompanied by intermolecular H-bonds, the formed intermolecular β-sheets may be mistaken for intramolecular β-sheets.

2. Tissue distribution and biological activities of PGLa

The tissue distribution and cellular localization of PGLa and peptides of the magainin family in *X. laevis* were early on studied. Two genes from this family, magainin and PGLa, are expressed at high level in the skin and throughout the gastrointestinal tract as well as in the small intestine [13–16]. Initial studies on the biosynthesis of PGLa demonstrated that it is located in the granular skin glands of *X. laevis* and proceeds through a pathway that involves discrete morphological rearrangements of the entire secretory compartment [13]. However, the peptide was found not only being stored at high concentration in the skin but being also abundant in the gastrointestinal system of the frog [14]. The antimicrobial activity detected in extracts of stomach tissue led to the purification of nine antimicrobial peptides, shown to undergo the same processing as their dermal counterparts. In the gastric mucosa the peptides are stored in granular cells, which have a spherical, syncytial multinucleated structure containing a cytoplasmic space filled with dense rice-shaped granules [15]. This enteric peptide-producing cell is strikingly similar both morphologically and biochemically to the granular gland of the amphibian skin. Finally, PGLa and magainin gene expression was also found in large, eosinophilic granular cells of the *X. laevis* small intestine, which share some common feature with the mammalian Paneth cell, a site of expression of antimicrobial peptides [16]. Thus the biological activity of PGLa was tested on a number of bacteria and fungi, but also on viruses and human cells in order to gain information on the potential of the peptide for therapeutic development and to understand first line defence mechanism.

In this respect, the capability of amphibians to defend themselves against pathogens gained some major interest because of the worldwide decline of the population of these animals. Pathogens associated with the mass mortality of amphibians are the fungi, *Batrachochytrium dendrobatidis* and *Basidiobolus ranarum*, respectively, as well as the bacterium *Aeromonas hydrophila* [17 and refs. therein]. *A. hydrophila* is an opportunistic bacterium found on the skin and in the digestive tracts of healthy amphibians, but capable to induce diseases as shown for the South African clawed frog *X. laevis* and American toad *Bufo americanus*. All these pathogens can infect the skin of the animals and thus it was of interest to study the protective effect of antimicrobial peptides isolated from the granular glands of skin. Six peptides originating from three different frogs were tested: PGLa, caerulein precursor fragment (CPF), magainin 1 and 2 from *X. laevis*, ranalexin from the bullfrog *Rana catesbeiana* and dermaseptin from *Phyllomedusa sauvagii* [17]. All peptides were able to kill or inhibit the growth of both fungi but showed different efficacy. At least one peptide of each species was effective against both fungi at a concentration of about 10–20 μM. PGLa was highly active against *B. ranarum* inhibiting growth at a concentration of 3.1 μM. However, higher peptide concentrations were necessary to prevent significant growth at later time points (after 48 h) and to completely eliminate growth during 4 days of culture, a pattern also observed with other peptides. All six peptides, however, were ineffective against the bacterium *A. hydrophila*. Because these peptides are released in the skin together, an equimolar mixture of PGLa and magainin 2 was tested at concentration as high as 50 μM each, but failed to inhibit growth of the bacterium. In contrast, a synergistic effect was observed on the fungi, e.g. complete growth inhibition of *B. ranarum* at a concentration of 0.4 μM of each peptide was achieved. This suggests that antimicrobial peptides in the skin of amphibians can act as a first line of defence against fungi.

Antifungal activity of cationic amphiphilic peptides is also of interest because of the rise in number of immunocompromised patients, which has led to an increase in mucosal and systemic fungal infections, and concomitantly to the development of antifungal drug resistance owing to the prophylactic use of antifungal agents [18]. A number of pathogenic *Candida* strains as well as *Cryptococcus neoformans* were shown to be highly susceptible to PGLa with IC_{50}-values lower than amphotericin B [17] (Table 1). Only *Candida glabrata* was less sensitive to cationic antifungal agents like PGLa most likely due to the regulation of the expression of drug efflux pumps, which affects negatively the efficacy and intracellular accumulation of cationic molecules [19]. In some pilot experiments, PGLa was added to dilution series of amphotericin B, fluconazole, and 5-flucytosine to assess the effects of the peptide on the minimal inhibitory concentration of these agents [20]. Addition of PGLa to amphotericin B showed a synergistic effect against several *Aspergillus*, *Candida* and *Cryptococcus* strains, while no enhanced activity was found in combination with fluconazole or 5-flucytosine. *Aspergillus fumigatus* being resistant to amphotericin B was shown to be also sensitive to sole PGLa [17]. Nevertheless, Helmerhorst et al. [21] pointed out that the candicidal activity of cationic peptides may strongly depend on the ionic strength of the test media. Therefore, the authors compared growth inhibition of *Candida albicans* and lysis of human erythrocytes at low and high ionic strength for several peptides including peptides of the magainin family. Subtle differences of biological activities between these peptides were only detectable at low ionic strength, while all peptides showed weak haemolytic and antifungal activity at physiological buffer conditions. PGLa, when tested under low salt concentration (buffer supplemented with 297 mM glucose to prevent osmotic lysis of erythrocytes) was highly active against both human erythrocytes (HC_{50} = 0.6 μM) and *C. albicans* (IC_{50} = 1.1 μM) and thus is poorly selective. In contrast, magainin 2 exhibited a reasonable selectivity also at low ionic strength.

Table 1
Killing activities of PGLa and amphotericin B against pathogenic yeasts.

Strain	IC_{50} [μM][a]	
	PGLa	Amphotericin B
Candida pseudotropicalis 311	1.3 ± 0.7	7.2 ± 1.9
Candida albicans 315	1.0 ± 0.3	2.2 ± 0.18
Candida krusei 355	0.8 ± 0.3	>70
Candida parapsilonis 356	0.8 ± 0.4	6.0 ± 1.0
Candida glabrata 359	8.5 ± 1.0	1.9 ± 0.2
Cryptococcus neoformans 316	0.4 ± 0.2	1.0 ± 0.1

[a] Data are taken from Helmerhorst et al. [44] and represent means ± standard deviations from two independent experiments.

Table 2
Spectrum of antimicrobial activity of the *Xenopus* granular gland peptides PGLa and magainin 2 (Mag2).

Bacteria	Species	Minimal inhibitory concentration [μg/ml]	
		PGLa	Mag2
Gram-negative	*Escherichia coli*	32[a]	64[a]
	Pseudomonas aeruginosa	128[a]	128[a]
		200–500[b]	50–100[b]
Gram-positive	*Staphylococcus aureus*	64[a]	256[a]
		50–100[b]	>500[b]
	Streptococcus pyogenes	10–50[b]	10–50[b]

[a] Data taken from Blazyk et al. [23].
[b] Data taken from Soravia et al. [6].

Antimicrobial peptides have also been strongly considered for the development of novel antibiotics owing to their strong growth inhibitory and microbicidial activity against bacteria in *in vitro* assays. Table 2 summarizes the antimicrobial activity of PGLa and magainin 2 on some representative bacterial species of Gram-negative and Gram-positive bacteria demonstrating that both peptides exhibit a similar spectrum and range of minimal inhibitory concentration (MIC). In a more recent study a lower MIC-value of 8 μg/ml was reported for *E. coli* and growth inhibition of the Gram-positive bacteria *B. subtilis* and *M. luteus* was observed in the same concentration range (MIC = 4 μg/ml) [22]. Therefore, PGLa has served as a template for synthetic peptides, which exhibited improved antimicrobial activity, but also increased potential towards haemolytic activity [e.g. 23,24]. A development towards a therapeutic agent requires however that these peptides have low cytotoxicity towards the host cell. Comparing the haemolytic and antimicrobial activity of a peptide yields an estimate for the cell selectivity of the investigated peptides as described above. Sometimes it is difficult to compare the reported haemolytic activity of a given peptide owing to different experimental protocols, which often differ regarding cell density. For PGLa e.g. 7% hemolysis at 1000 μg/ml peptide [6] and 18% hemolysis at 500 μg/ml peptide were reported [24].

As mentioned earlier the broad variety of peptides found in the secretions of amphibian skin has suggested to investigate whether combinatorial treatment can also enhance antimicrobial activity. In fact, the equimolar mixture of PGLa and magainin 2 showed a significant improvement of antimicrobial activity in comparison to the sole peptides [25], but also a marked functional synergism in tumor cells [26] and model membranes [8,26,27,28]. Cross-linking experiments suggested the formation of a heterodimer composed of parallel helices [29]. This was confirmed by a chemically fixed heterodimer by adding a Gly-Gly-Cys group to each peptide linked via a disulfide bond [25]. This hybrid peptide showed membrane permeabilizing activity against negatively charged liposomes and antimicrobial activity similar to the physical mixture of PGLa and magainin 2 (Table 3). In addition, this synthetic heterodimer showed enhanced activity against zwitterionic liposomes and human erythrocytes as compared to the physical mixture and the individual peptides (Table 3). These

Table 3
Comparison of antimicrobial activities and hemolysis of PGLa and magainin 2 (Mag2) with their equimolar mixture and hybrid peptide, respectively.

	L18W-PGLa	Mag2	Mixture	Hybrid[a]
	Minimal inhibitory concentration [μM][b]			
Escherichia coli	20	20	2.5	1.25
Staphylococcus epidermidis	5	20	2.5	5
	% hemolysis[c]			
Human erythrocytes	2.0	1.2	13.4	77.9

[a] Concentrations are expressed as concentrations reduced to monomers.
[b] Data taken from Nishida et al.[25], the Try-analog (L18W-PGLa) showing the same activity as PGLa.
[c] After 3 h incubation of erythrocytes (1% v/v) with 15.6 μM (L18W-PGLa or Mag2) or 31.2 μM (mixture and hybrid) peptides.

observations suggest that the production of mutually recognizing peptides seems to be an effective way in nature to increase selective membrane activity [25].

3. Interaction of PGLa with membrane mimetic systems

3.1. Lipid discrimination and membrane selectivity of PGLa

As addressed briefly in the previous chapter antimicrobial peptides can exhibit selective activity towards bacterial and mammalian cells, which is thought to be strongly related to the different membrane architecture and lipid composition of eukaryotic and bacterial cell membranes [30,31 and refs. therein]. The general differences in the lipid composition between these cell membranes relates to the increased amount of negatively charged lipids in the outer membrane leaflet of bacterial cell membranes, which serves as a primary target of the cationic peptides. Thus, zwitterionic lipids such as PC and sphingomyelin (SM) are characteristic for mammalian cell membranes, while the negatively charged lipids PG and diPG or cardiolipin are main components that comprise – together with PE – bacterial cytoplasmic membranes. Moreover, the cell envelope of Gram-negative bacteria is a complex structure consisting of the cytoplasmic or inner membrane and a unique outer membrane with an intervening layer of peptidoglycan (Fig. 1). The outer membrane has a distinctive, highly asymmetric composition with anionic lipopolysaccharides (LPS or endotoxin) located exclusively in its outer leaflet, which is of particular interest in respect of interaction of cationic antimicrobial peptides with these bacteria (see Shai and Mangoni within this issue). In contrast, Gram-positive bacteria have a simple lipid bilayer membrane protected by a lipoteichoic acid layer, which confers the bacterial surface a negative charge too. Although recent studies showed that the lipid net charge is not the decisive factor determining the activity and membrane-perturbing mechanism(s) of antimicrobial peptides [32], electrostatic interaction will govern adsorption of these peptides on the membrane surface [31]. In addition, upon interaction with the negatively charged lipid headgroup conformational changes can be induced, which transform antimicrobial peptides that are unstructured in aqueous solution into a membrane-active, mostly α-helical conformation (see below). Thereby, induction of the active conformation upon interaction with the target membrane represents one possibility to control membrane selectivity.

Early biophysical studies on membrane model systems clearly demonstrated that indeed a number of antimicrobial peptides can distinguish between different lipid species, which led to the concept of lipid discrimination [9,30,33,34]. For example, preferential interaction with negatively charged phospholipids was reported for the β-sheet peptides such as tachyplesin from horseshoe crab [35], human neutrophil peptide [34,36] or protegrin-1 from porcine leukocytes [37]. Furthermore, magainin killed more effectively Gram-negative bacteria containing an inner membrane with higher amounts of phosphatidylglycerol [38], which emphasizes the role of electrostatic interactions as a major determinant to trigger the affinity of antimicrobial peptides towards bacterial membranes.

The same general observation was made for PGLa. Biophysical studies on the interaction of PGLa with liposomes consisting of dipalmitoylphosphatidylcholine (DPPC) and egg sphingomyelin, a simple mimic for mammalian cell membranes, and of the acidic dipalmitoylphosphatidylglycerol (DPPG), representative of bacterial membranes, demonstrated that PGLa also discriminates between these lipids [9]. Microcalorimetric experiments showed that PGLa had no effects on the thermotropic phase behavior of liposomes composed of the choline phosphatides, while separation of a distinct peptide-rich domain was observed for phosphatidylglycerol liposomes (Fig. 2).

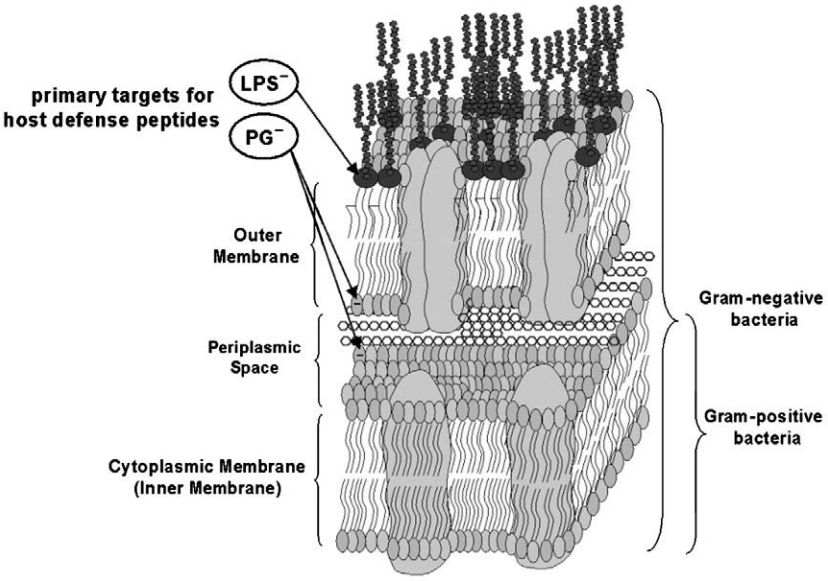

Fig. 1. Schematic representation of the membrane architecture of Gram-negative bacteria consisting of an outer membrane with an asymmetric distribution of lipopolysaccharides (LPS) and phospholipids, and a cytoplasmic or inner membrane. Phosphatidylglycerol is the most abundant negatively charged phospholipid species found in both Gram-negative and Gram-positive bacteria. Latter has only a cytoplasmic membrane. Predominant lipid targets of antimicrobial peptides are indicated.

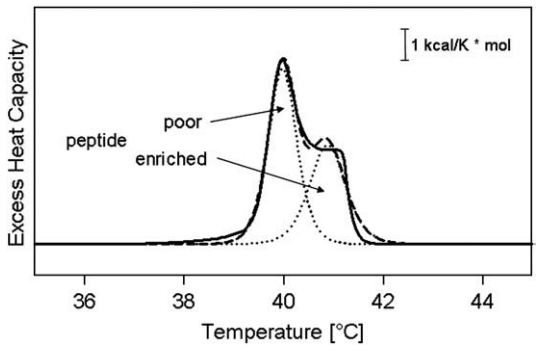

Fig. 2. Thermogram of DPPG in the presence of PGLa at a lipid/peptide molar ratio of 50. Lipid and peptide were co-dispersed in 10 mM NaPi, pH 7.4. Experimental data (solid line), fitted data (broken line), individual components of peptide poor and enriched domains (dotted line).

In addition to the main transition of pure DPPG at 40 °C a second narrow transition owing to the peptide perturbed lipid domains was found at 41 °C, which enthalpy increased upon increasing concentrations of PGLa. The assumption of a peptide-enriched domain was supported by X-ray diffraction experiments, which indicated that PGLa penetrates into the hydrophobic core of the bilayer inducing an untilting of the hydrocarbon chains as observed in the gel phase of the pure lipid. The nature of the peptide induced lipid domains was revealed only recently [39] and will be described in the following chapter.

Discrimination between neutral and negatively charged lipid species is further supported by monolayer studies, where PGLa was found to mix with PG at a molecular level, but to form separate islands in zwitterionic PC monolayers [40,41]. Structural details of the phospholipid monolayers in the presence and absence of PGLa were obtained from Synchrotron experiments (Fig. 3) showing that the peptide strongly perturbed the lipid acyl chain order of distearoylphosphatidylglycerol (DSPG) [40]. An increase of bending rigidity of the DSPG monolayer was observed, while adding PGLa to a DSPC monolayer resulted in a reduction of the overall bending rigidity [41]. One can envisage that changes of membrane bending rigidity owing to peptide insertion into the lipid bilayer may result in domain and/or defect structures, which will affect membrane integrity.

Surface pressure/potential-area isotherms showed that the antimicrobial frog skin peptide, PGLa, formed a very stable monolayer with 2 PGLa molecules per kinetic unit and a collapse pressure of ~22 mN/m, which is below the lateral pressure of biological membranes [40]. X-ray grazing incidence diffraction indicated that the peptide-dimer formation did not lead to self-aggregation with subsequent crystallite formation. The scattering length density profile derived from X-ray reflectivity measurements yield information on the PGLa monolayer that protrudes into the air phase by about 0.8 nm suggesting that the peptide is aligned parallel to the air/water interface [40]. In bilayers different orientations of PGLa were reported depending on peptide concentration, phase state and synergistic interactions with magainin-2, ranging from a surface aligned monomeric state, via tilted dimers, to an upright inserted state [e.g. 28,39,42,45,48], which are described in detail by Ulrich and coworkers within this special issue. The process of PGLa binding and insertion was studied thermodynamically using PC/PG mixtures (3/1 mol/mol) showing an exothermic binding of the peptide to the membrane surface, followed by a slower endothermic process above a threshold concentration [43]. Latter was related to pore formation, which was also deduced from other biophysical approaches [27,39].

Information on the secondary structure of PGLa upon interaction with lipids was obtained by various spectroscopic techniques. CD spectra of PGLa in the presence of the zwitterionic phosphatidylcholine and sphingomyelin, closely resembled the features of the spectrum gained for the peptide in buffer solution indicating that the peptide adopts a random structure [9]. A different behavior was observed in the presence of DPPG, where two defined minima at 210 and 222 nm were detected [9], characteristic for an α-helical structure in agreement with IR-data [10]. A helical structure to about 60% was calculated, which suggests an α-helical C-terminal and a more or less unstructured N-terminal part. This would be consistent with the primary structure of PGLa, from where it can be inferred that several Gly residues, known as helix breakers, are located in the more hydrophobic N-terminal part of the sequence, while the remainder consists predominantly of helix stabilizing amino acids, namely Ala, Leu and Lys. This was confirmed by calculating a helix probability profile, which shows a marked increase in helicity for the C-terminal half in the presence of anionic lipids [9] and is in agreement with multidimensional solution NMR spectroscopy data showing that PGLa is helical between residues 6 and 21, when associated with detergent micelles [46]. This study also revealed that the amino-terminal residues are highly mobile and that the fluctuations of backbone sites decrease from Ala at position 6 towards the C-terminus with Ala at position 20 being essentially rigid. If PGLa, however, is not added exogenously but co-dispersed from a dry lipid/peptide film, and if there is no excess of water present in the sample into which the cationic peptide can escape, it will form an α-helix even in neutral phosphatidylcholine [47] and phosphatidylethanolamine bilayers [68]. A helical wheel projection of PGLa indicates the lateral amphipathic character of the peptide clustering the positively charged Lys residues on one side of the helix (Fig. 4). It should be noted however that induction of an α-helix is not necessarily sufficient to induce antimicrobial activity. For example, an alanine substitution analog of PGLa, where the bulky Ile residues, supposed to act as membrane anchor, were replaced by Ala, exhibited even a slightly higher content of α-helix in the presence of bacteria mimetic vesicles, but did not show any appreciable antimicrobial activity against *E. coli*

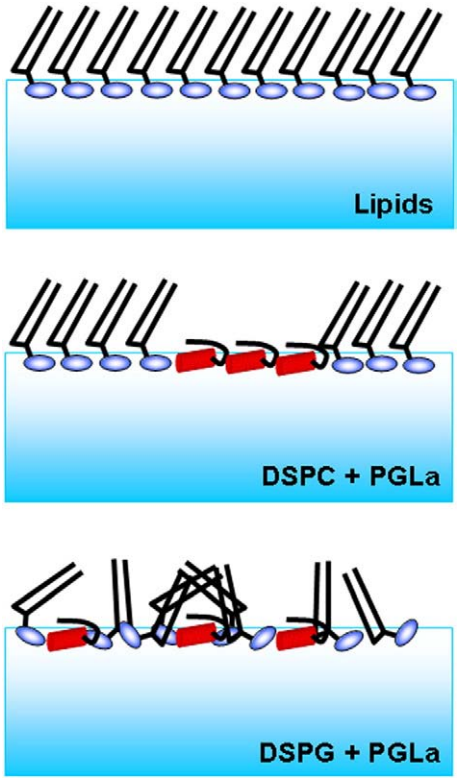

Fig. 3. Models of lipid organization in a monolayer with and without PGLa based on X-ray reflectivity and grazing incidence diffraction data. Molecular mixing of PGLa and DSPG with perturbed hydrocarbon chain order as well as formation of separate domains of PGLa and DSPC is shown.

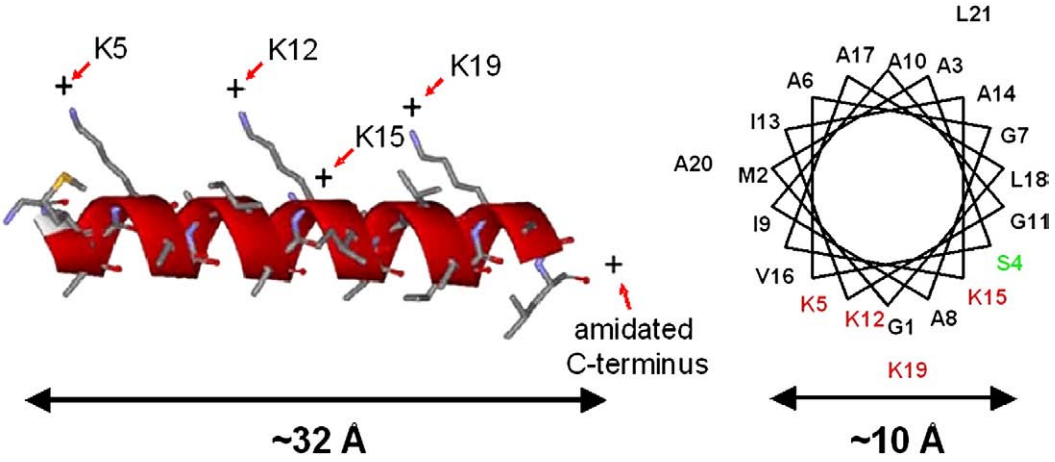

Fig. 4. α-helical structure of PGLa assuming a full helix (coordinates kindly provided by A. Ulrich, Forschungszentrum Karlsruhe, Germany) and helical wheel presentation. Position of lysine residues and amidated C-terminal (amide is not shown) as well as length and diameter of the helix are indicated in the panel.

and *Staphylococcus aureus* [49]. This observation can be attributed to the lower hydrophobic moment per residue and total hydrophobicity of the alanine substitution analog as compared to PGLa affecting membrane partitioning.

3.2. PGLa has distinct effects on the bilayer thickness of PG

Recently it was demonstrated, how profoundly the mechanism of membrane disruption depends on the nature of the membrane lipid composition, varying not only with lipid headgroup charge but also with hydrocarbon chain length [32,50]. Based on systematic studies on LL-37 a phase diagram for PG and PC showing different lipid/peptide arrangements as a function of hydrocarbon chain length and peptide concentration was established. Further, it had been suggested that this phase diagram is generally applicable to membrane-active peptides localized parallel to the membrane surface, as similar effects were observed for PGLa and melittin [50].

A follow-up study using X-ray diffraction, solid-state 2H-NMR, differential scanning calorimetry, and dilatometry, investigated in detail the structural changes occurring in PG bilayers by addition of PGLa as a function of the acyl chain length (C14 to C18) [39]. The effects of the peptide varied strongly with the phase state of the lipid in a concentration dependent manner. In the gel phase, PGLa induced a quasi-interdigitated phase (Fig. 5), which coexists in various proportions with the original lamellar gel phase, manifested as two separate phase transition, as had been observed in initial DSC studies

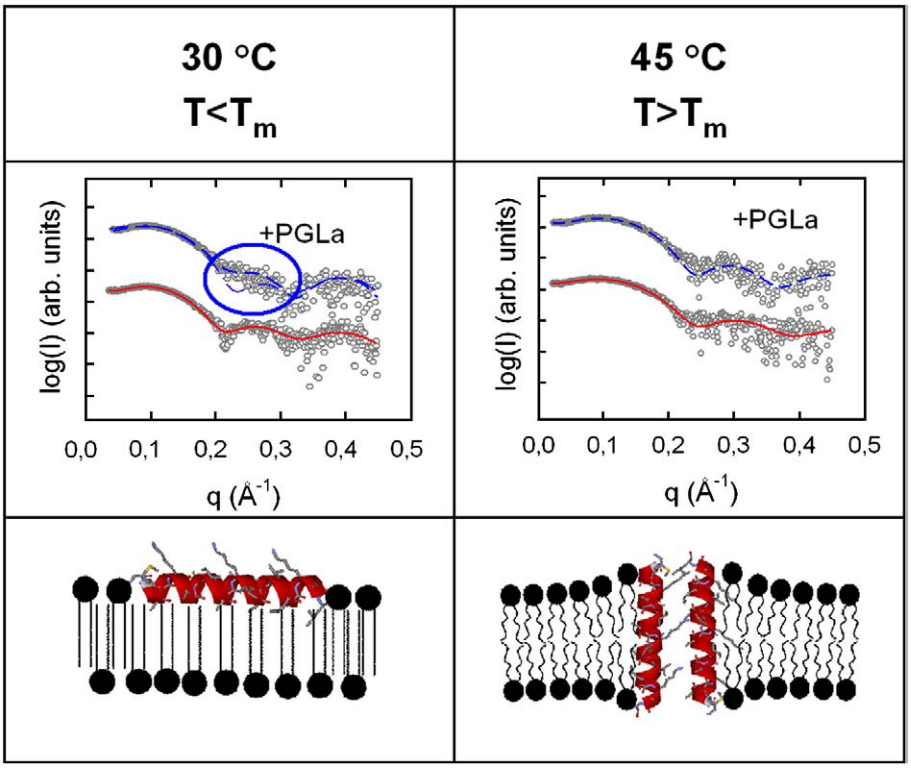

Fig. 5. Schematic representation of structural changes induced by PGLa, when incorporated in DPPG bilayers. In the lamellar gel phase ($T<T_m$) PGLa aligns parallel to the membrane surface inducing an averaged thinning of the bilayer, while in the fluid phase ($T>T_m$) it inserts perpendicular to the membrane plane inducing a thickening of the bilayer. Typical small-angle scattering curves of DPPG in the gel and fluid phase in the absence and presence of PGLa are presented. Solid lines show the model fits to the data (red line: pure DPPG, blue line: DPPG/PGLa). The circle indicates the deviation of model fits assuming no and the presence of a quasi-interdigitated phase. Lipid/peptide molar ratio is 25.

[9]. Thereby the higher melting transition is due to the conversion of the quasi-interdigitated phase into the fluid L_α phase. The presence of this structure was most prominent for the longest chain PG studied, i.e. DSPG. The occurrence of a quasi-interdigitated structure was also reported for LL-37 for these PGs [50]. While PGLa had no profound effect on DPPC, LL-37 led to a disintegration of the lamellar organization of this zwitterionic lipid into disk-like micelles. However, interdigitation was observed for the longer-chain C18 and C20 PC. This dual behavior of LL-37 was attributed to a balance between electrostatic interactions reflected in different penetration depths of the peptide and hydrocarbon chain length as outlined below [50]. These observations clearly indicate that there is a tight coupling between the peptide properties and those of the lipid bilayer.

Induction of interdigitation of hydrocarbon chains in the gel phase can be understood in terms of the "free volume" (void) model, as described earlier for small amphiphilic molecules such as e.g. alcohols, anesthetics or surfactants [51,52]. It has been suggested that the lateral expansion of a phospholipid bilayer caused by the intercalation of amphiphilic molecules between phospholipid molecules and the mismatch between their hydrocarbon chain lengths results in the creation of voids in the hydrophobic region of the bilayer. In analogy, when peptides get embedded into the gel phase bilayer at the water/lipid interface aligned parallel to the membrane surface, it will create a void below the peptide. The extent of void formation will depend on the size and penetration depth of the peptide. It was shown that cationic peptides penetrate less deeply into the hydrophobic core of anionic as compared to neutral bilayers [53]. Formation of free volume within the hydrophobic core of the bilayer is energetically unfavourable and hence the system will rearrange in a way to maximize the hydrophobic interactions. Elimination of the free volume can be achieved via increased *trans-gauche* isomerisation of the hydrocarbon chains of the neighboring lipids or by moving the methyl ends of the opposing lipid monolayer close to the peptide, thereby generating the interdigitated lipid structure. Both will result in a decrease of the bilayer thickness though to various degree. A compensation of the free volume by an elastic monolayer deformation is not possible due to the high bending rigidity in the gel phase [54]. An increased acyl chain disorder was observed in DPPG/PGLa mixtures both from wide-angle X-ray diffraction and NMR data arising from phospholipids at the borderlines of the defect zones between the coexisting gel phases. This phenomenon was not observed for DSPG indicating that PGLa very effectively transforms the bilayer into an interdigitated phase, which is consistent with the larger void induced in the hydrophobic core of the bilayer and the propensity of DSPG to form an interdigitated phase even in the absence of the peptide [55].

In the biologically more relevant fluid phase PGLa forms pores in PG membranes above a critical threshold concentration [39]. Thus the authors addressed the question, how this translates into structural changes of the fluid phase, as previous experiments had shown that LL-37 leads to a thinning of PC and PG bilayers [32]. Membrane thinning has been also observed in several other lipid/peptide interaction studies [56 and refs. therein]. In the fluid phase the bilayer may respond elastically to a peptide embedded in the water/lipid interface, because the bending rigidity is about one order of magnitude lower than in the gel phase, which can lead to a local membrane deformation and thinning on the global average over the membrane upon insertion of the peptides [56–58]. In contrast to these findings PGLa induced a membrane thickening of DMPG and DPPG bilayers, while no changes in the overall bilayer structure were detected for DSPG [39]. Latter observation was explained by considering the length of the α-helix of PGLa and the thickness of the lipid bilayer core showing that PGLa remarkably fits into the hydrophobic core of DSPG, when inserted perpendicular to the bilayer plane. However, in case of DPPG the peptide cannot be accommodated with its full length and thus the lipid responds to the insertion of the peptide by stretching of the hydrocarbon chains leading to an increase of membrane thickness by about 2 Å (Fig. 5). Such a behavior was reported earlier for PC bilayers in the presence of hydrophobic model peptides of various length with N- and C-terminal interface anchors [59]. In the case of DMPG, the increase in bilayer thickness was less pronounced than for DPPG due to tilting of the peptide with respect to the bilayer normal, which has been observed in DMPC/DMPG [47]. These results demonstrate that membrane thinning cannot be taken generally as the hallmark of pore formation by antimicrobial peptides.

3.3. Cubic phase formation — effect on membrane curvature

Microorganisms such as e.g. *E. coli* or *Acholeplasma laidlawii* have been shown to precisely regulate their lipid composition in a narrow window close to a bilayer to non-bilayer phase boundary, thereby conferring upon these membranes a degree of non-lamellar structure forming propensity [60–62]. The presence of non-lamellar phase forming lipids such as PE, cardiolipin or monoglucosyldiacylglycerol leads to an increase in the lateral pressure in the center of the bilayer because of their cone-shaped molecular geometry (smaller headgroup area as compared to the cross sectional area at the methyl region of the acyl chains). This in turn results in a bilayer state, where the desire for monolayer curvature is physically frustrated, which is in contrast to lamellar phase forming lipids such as PG or choline phospholipids exhibiting a cylindrical molecular shape and therefore a uniform lateral pressure throughout the hydrocarbon chain region of the bilayer. Thus, it was proposed that antimicrobial peptides may lower the bilayer to non-bilayer phase boundary leading to membrane rupture in case of bacteria with high content of non-lamellar phase forming lipids [31].

Formation of non-bilayer structures may be understood qualitatively in terms of this lateral pressure profile [63] balancing the repulsive and attractive interactions between the individual lipid molecules within the compound of the lipid bilayer. Insertion of a membrane-active compound affects this balance and may lead to a long-range effect in form of a globally curved membrane [64]. Such effects are particularly pronounced, if lipids exhibit a propensity to negative curvature such as phosphatidylethanolamines, which make up more than 80% of the total phospholipids of *E. coli* [30]. This amino phospholipid can adopt non-bilayer structures, such as the inverted hexagonal (H_{II}) and cubic (Q_{II}) phases [65,66], whereby the L_α-H_{II} phase transition temperature depends on the acyl chain composition being around 75 °C for palmitoyloleoyl-PE (POPE) [65,67]. A recent differential scanning calorimetry and small-angle X-ray diffraction study showed that pure POPE partially converts into cubic phases upon cooling from the H_{II} phase [68]. Thereby, two Q_{II} phases of space group Pn3m and Im3m, respectively, coexist with the L_α phase and vanish upon the transition into the L_β gel phase (Fig. 6). Adding PGLa (4 mol%) to POPE caused an increase of the bilayer to non-bilayer phase transition temperature by about 9 °C and a significant broadening of the transition range [68]. Small-angle X-ray diffraction showed that a pure L_α phase exists up to 70 °C. Upon further heating the intensity of the Bragg peak being characteristic for the lamellar phase continuously decreased on account of peaks that can be ascribed to a cubic phase belonging to the space group Pn3m with a lattice spacing of 131 Å at 90 °C, which coexists with the fluid L_α phase. Upon cooling the Q_{II} phases vanished and at 30 °C only the fluid L_α phase was observed (Fig. 6).

In the presence of melittin (4 mol%) the L_α-H_{II} transition was abolished and only a bilayer structure was observed in the temperature range studied, which was explained by electrostatic repulsion due to the charged peptide [68]. Seemingly this does not play a role for PGLa, which has the same net charge as melittin, but exhibited a strongly reduced bilayer separation as compared to melittin. This is most likely due to shielding of the positive charges of PGLa by insertion into the lipid bilayer supported by the observation that PGLa significantly affected the chain melting transition of POPE.

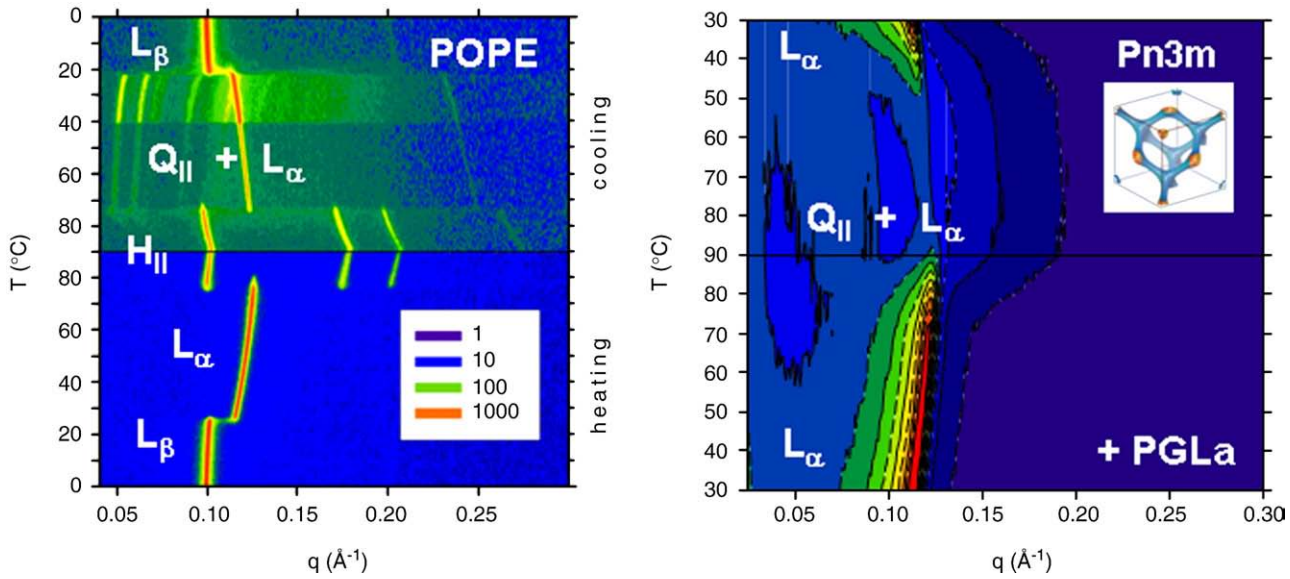

Fig. 6. Structural phase transitions of pure POPE (left) and in the presence of PGLa (right) as a function of temperature. A contour plot of the small-angle X-ray diffraction data obtained upon heating and cooling is shown. Phase assignment is indicated in the panel (lamellar gel phase, L_β, fluid lamellar phase, L_α, inverted hexagonal phase, H_{II} and cubic phases, Q_{II}). The insert shows the water channels within the unit cell of Pn3m (adapted from http://www.msri.org/about/sgp/jim/geom/surface/global/skeletal/index.html).

In conjunction with NMR data that indicated that PGLa inserts into PC membranes above a critical threshold concentration [47] and permeability experiments that suggested pore formation [27] it was proposed that PGLa is present in the PE bilayer as lipid/peptide pores as well as inserted as monomers [68]. It was suggested that the monomeric fraction of PGLa is responsible for the increase of transition temperature by stabilization of a negative Gaussian curvature modulus but facilitates at higher temperature the formation of the Q_{II} phases similar to fusion peptides by creating local defects. The complete stabilization of the L_α phase, i.e. the fraction which did not convert into a cubic phase, was attributed to be due to lipid/PGLa pores, as the formation of a fusion pore in such a case would require that the lipid/peptide pores first come into registry and then promote trans-membrane contact. In the absence of any driving force for this mechanism the authors concluded that such a scenario remains highly unlikely.

A different behavior was observed for gramicidin S, which led to a complete transformation of the fluid L_α phase into Q_{II} phases [68]. The capability of gramicidin S to strongly promote cubic phase formation was shown earlier using lipid extracts from *E. coli* and *A. laidlawii* [69]. It was proposed that the formation of the bicontinous cubic lipid phases is due to the limited flexibility of the β-turn of gramicidin S as well as to the clustered location of the ornithine side chains that strongly promotes negative curvature. A significant increase in monolayer curvature stress is likely to be of major importance for the formation of non-lamellar structures [33]. Promotion of such structures in PE model systems has been also shown for other antimicrobial peptides [31 and refs. therein]. Huang and coworkers [56 and refs. therein] proposed that membrane thinning, related to the elastic response of the lipid bilayer, is compensated by an increase of the hydrophobic cross sectional area of the lipid acyl chains. In case of PE this lateral expansion would further enhance the existing mismatch between the cross sectional areas of the headgroup and hydrocarbon side chains, inducing the lipid monolayer to curl.

Finally, it should be mentioned that some antimicrobial peptides such as nisin [70] or a 17 β-amino acid oligomer [71] can stabilize the inverse hexagonal phase. In case of nisin this was explained in analogy to hydrophobic molecules such as squalene [72] being due to insertion of the large hydrophobic section of nisin (segments 1–19), which will lead to an increase of the hydrophobic volume in the bilayer interior. This in turn will promote negative curvature and hence formation of inverted non-lamellar structures. Similarly it was argued that owing to the penetration depth of a 17 β-amino acid oligomer H_{II}-structures were facilitated by expanding the regions of the bilayer below the pivotal plane more than regions close to the interface. Again this will result in an increase of negative curvature strain [71].

The different packing properties may have also implications for membrane function. For example, it has been suggested that the high lateral hydrocarbon chain pressure, exhibited by such lipids, controls the conformation of integral membrane proteins. In accordance with this assumption are observations that non-lamellar lipids are often required for functional reconstitution of membrane proteins [73] and that PE is found in protein rich membrane domains [74]. Therefore, one may speculate that antimicrobial peptides that affect the lateral hydrocarbon chain pressure upon insertion may lead to conformational changes of integral membrane proteins and hence to impairment of membrane function.

4. Images on the impact of PGLa on living *E. coli* cells

The influence of PGLa on living *E. coli* was investigated by imaging the cells with atomic force microscopy [75,76] showing distinct changes in cell envelope morphology, when treated with the peptide. AFM images obtained under physiological aqueous conditions [75], where the mica was pre-treated with polylysine for better immobilization of the bacteria, revealed that PGLa induces formation of micelles within 5 min of treatment accompanied by a morphological change of the bacteria from a rod-shape to a fluid flattened structure. After 30 min only the lower parts of cellular membrane was left attached to the substrate, which had lengths and widths that correspond to the size of the fixed bacterial cell. Another important observation relates to the complete removal of the pilar structure attached to the bacteria indicating that the peptide leads to disruption of the outer membrane. However, under these conditions the resolution of the images were low most likely due to the deformation of the bacteria during scanning and therefore observation of bacteria in air was also performed giving a better resolution than in water.

These experiments demonstrated that interaction of PGLa with *E. coli* strongly reduced the cell stiffness and increased the surface roughness [75,76], which was also observed in the presence of magainin 2a and the cytolytic peptide melittin [76]. In general the roughness of the outer membrane increased with the increase in

peptide concentration, with melittin having the most pronounced effect. This morphological change may be caused by exposure of the peptidoglycan layer and/or peptide incorporation into the LPS-containing outer membrane causing a "crumbling" effect due to an increase of the lateral monolayer area. Incorporation of cationic antimicrobial peptides into the LPS layer was also suggested in earlier studies [e.g. 77,78]. Such a damage to the outer membrane may in turn enhance the penetration of peptides to the highly sensitive inner membrane. Peptide transfer across the outer membrane to the periplasmic space, which involves the direct binding of the cationic peptides to LPS, was proposed by Hancock and termed the so-called self-promoted uptake system [79]. The packing of LPS molecules, which is essential for the permeability control and membrane integrity, strongly depends on divalent cations [80]. Interaction of the cationic peptides may displace Mg^{2+} ions from the LPS layer of the cell surface leading to insertion of the peptide into the outer membrane cross binding the negative charges of LPS as has been shown for example for the antimicrobial peptide polymyxin B [81,82]. In fact, addition of 10 mM Mg^{2+} ions prior to PGLa treatment partially inhibited the effects of the peptide [75]. Under these experimental conditions PGLa showed less damage to *E. coli* cells owing to the competition for the negative charges present in LPS molecules. Interestingly, bacteria treated with the chelating agent EDTA did neither show substantial damage of the membrane nor measurable differences in stiffness as compared to the untreated bacteria. Furthermore, the treated bacteria retained their overall morphology. The effect simply seems to be restricted to a roughening of the outer membrane. This observation is in agreement with the suggestion that EDTA removes Mg^{2+} ions from the LPS layer of *E. coli* increasing the electrostatic repulsion within the LPS molecules [84]. The observed roughness can then be explained by an increase in the area of the LPS monolayer and partial loss of LPS without formation of holes or discontinuities. Although EDTA increases the permeability of the outer membrane, treatment of *E. coli* cells with this chelating agent during short periods is not lethal, but can be used to introduce macromolecules through the outer membrane without significant loss in viability [81,83]. The fact that *E. coli* retained its rigidity and morphology during the EDTA treatment suggested that it obviously does not affect the peptidoglycan layer, which serves as a strong mechanical structure as well as diffusion barrier.

AFM images obtained in the presence of magainin 2a and melittin showed similar but in some aspects different effects on *E. coli* cells as compared to PGLa [76]. These experiments, performed below and above their LC_{50}-value, gave additional interesting details upon the action of membrane-active peptides on bacterial cells. Images obtained below LC_{50} demonstrated that all three peptides tested induced the most damage at the apical ends of the cell. This is of interest, because results from experiments with a cardiolipin sensitive dye demonstrated that cardiolipin domains were located at the apical ends of *E. coli* inner membrane [73]. Therefore it is tempting to speculate that these peptides may preferentially interact with the negatively charged cardiolipin.

In fact, it has been shown that the antimicrobial peptide protegrin-1 can distinguish between different negatively charged lipids [85]. This is further supported by recent observation on a synthetic antimicrobial peptide, which induced demixing of a cardiolipin and phosphatidylglycerol mixture [86]. In this respect it is also interesting to note that leakage experiments showed that in the case of rabbit defensin, cardiolipin seems to be essential for creating lesions as no leakage was observed for liposomes composed of phosphatidylglycerol and -ethanolamine [36]. On the other hand, phase segregation in this model system, which is a mimic of the bacterial cytoplasmic membrane, was reported for PGLa [33] and also observed for other antimicrobial peptides such as HNP-2 [32,34] or alpha/beta antimicrobial peptides [87] emphasizing the preferential interaction of the cationic peptides with negatively charged lipids. Formation and segregation of lipid domains may influence membrane properties and in turn membrane function by creating defect zones of increased permeability and membrane domains of changed fluidity.

Melittin was most effective in cell rupture, as can be expected from its low MIC and non-selective membranolytic activity. Already below LC_{50} melittin induced the formation of grooves and pore-like lesions as well as the collapse of the cell structure at the apical ends, with one end being more elastic [76]. In addition, a large amount of released vesicles or peptide aggregates, which may include LPS and membrane-associated compounds, was clearly visible. Above LC_{50} in addition to a collapse of the apical ends, the outer membrane showed severe damage with large lesions. Furthermore, a very pronounced leakage of cytosolic fluid was observed clearly indicating the damage of the inner membrane. Below LC_{50} magainin 2a induced blebbing (surface-bound vesiculation) of the LPS-rich outer membrane as well as some lesions in agreement with an earlier electron microscopy study [88]. Above LC_{50} it almost completely destructed the bacterial cell, where vesiculation, deep lesions as well as a collapse of the apical and the septal region of the cell were observed. A possible exposure of parts of the more rigid peptidoglycan layer was also suggested. The effects observed for PGLa below LC_{50} were intermediate between the changes induced by melittin and magainin 2a, while above LC_{50} PGLa had similar but less visual effects as compared to magainin 2a.

In summary, the first stage of action can be characterized by the loss of surface stiffness and consequent loss of bacteria topographic features owing to the incorporation of the peptides into the LPS layer. In a second stage after permeabilization of the outer membrane the peptides also interact with the inner (cytoplasmic) membrane finally leading to total cell rupture as suggested by the loss of cytoplasmic material. Biophysical studies have shown that a number of relevant parameters have to be considered to understand the effect of antimicrobial peptides on membranes and thus caution has to be taken to derive the molecular mechanism(s) from model studies. Nevertheless, the studies described within this review suggest that disruption of the inner membrane by PGLa will most likely occur by pore formation but changes in membrane fluidity due to lipid segregation and membrane curvature strain may be considered as well.

Acknowledgements

The authors would like to thank Georg Pabst and Andrea Hickel for stimulating discussions and providing material for the manuscript. Part of the work was supported by the Austrian Science Funds P18100-B10.

References

[1] V. Erspamer, Half a century of comparative research on biogenic-amines and active peptides in amphibian skin and molluscan tissues, Comp. Biochem. Biophysiol. C- Comp. Pharmacol. Toxicol. 79 (1984) 1–7.
[2] G. Giovannini, L. Poulter, B.W. Gibson, D.H. Williams, Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones, Biochem. J. 234 (1987) 113–120.
[3] W. Hoffmann, K. Richter, G. Kreil, A novel peptide designated PYLa and its precursor as predicted from cloned mRNA of *Xenopus laevis* skin, EMBO J. 2 (1983) 711–714.
[4] D. Andreu, H. Aschauer, G. Kreil, R.B. Merrifield, Solid-phase synthesis of PYLa and isolation of its natural counterpart, PGLa [PYLa-(4-24)] from skin secretion of *Xenopus laevis*, Eur. J. Biochem. 149 (1985) 531–535.
[5] B.W. Gibson, L. Poulter, D.H. Williams, J.E. Maggio, Novel peptide fragments originating from PGLa and the caerulein and xenopsin precursors from *Xenopus laevis*, J. Biol. Chem. 261 (1986) 5341–5349.
[6] E. Soravia, G. Martini, M. Zasloff, Antimicrobial properties of peptides from *Xenopus* granular gland secretions, FEBS Lett. 228 (1988) 337–340.
[7] D. Juretic, H.C. Chen, J.H. Brown, L. Morell, R.W. Hendler, H. Westerhoff, Magainin 2 amide and analogues. Antimicrobial activity, membrane depolarization and susceptibility to proteolysis, FEBS Lett. 249 (1989) 219–223.
[8] R.W. Williams, R. Starman, K.M. Taylor, K. Gable, T. Beeler, M. Zasloff, D. Covel, Raman spectroscopy of synthetic antimicrobial frog peptides magainin 2a and PGLa, Biochemistry 29 (1990) 4490–4496.

[9] A. Latal, G. Degovics, R.F. Epand, R.M. Epand, K Lohner, Structural aspects of the interaction of peptidyl-glycylleucine-carboxyamide, a highly potent antimicrobial peptide from frog skin, with lipids, Eur. J. Biochem. 248 (1997) 938–946.
[10] M. Jackson, H.H. Mantsch, J.H. Spencer, Conformation of magainin-2 and related peptides in aqueous solution and membrane environments probed by Fourier transform infrared spectroscopy, Biochemistry 31 (1992) 7289–7293.
[11] H.C. Chen, J.H. Brown, J.L. Morell, C. Huang, Synthetic magainin analogues with improved antimicrobial activity, FEBS Lett. 236 (1988) 462–466.
[12] K. Matsuzaki, M. Harada, T. Handa, S. Funakoshi, N. Fujii, H. Yajima, Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles, Biochim. Biophys. Acta 981 (1989) 130–134.
[13] B.E. Flucher, C. Lenglachner-Bachinger, K. Pohlhammer, H. Adam, C.J. Mollay, Skin peptides in *Xenopus laevis*: morphological requirements for precursor processing in developing and regenerating granular skin glands, J. Cell Biol. 103 (1986) 2299–2309.
[14] K.S. Moore, C.L. Bevins, M. Brasseur, N. Tomassini, K. Turner, H. Eck, M. Zasloff, Antimicrobial peptides in the stomach of *Xenopus laevis*, J. Biol. Chem. 266 (1991) 19851–19857.
[15] K.S. Moore, C.L. Bevins, N. Tomassini, K.M. Huttner, K. Sadler, J.E. Moreira, J. Reynolds, M. Zasloff, A novel peptide-producing cell in *Xenopus*: multi-nucleated gastric mucosal cell strikingly similar to the granular gland of the skin, J. Histochem. Cytochem. 40 (1992) 367–378.
[16] D.S. Reilly, N. Tomassini, C.L. Bevins, M. Zasloff, A Paneth cell analogue in *Xenopus* small intestine expresses antimicrobial peptide genes: conservation of an intestinal host-defense system, J. Histochem. Cytochem. 42 (1994) 697–704.
[17] L.A. Rollins-Smith, J.K. Doersam, J.E. Longcore, S.K. Taylor, J.C. Shamblin, C. Carey, M. Zasloff, Antimicrobial peptide defenses against pathogens associated with global amphibian declines, Dev. Comp. Immunol. 26 (2002) 63–72.
[18] T. White, K.A. Marr, R.A. Bowden, Clinical, cellular, and molecular factors that contribute to antifungal drug resistance, Clin. Microbiol. Rev. 11 (1998) 382–402.
[19] E.J. Helmerhorst, C. Venuleo, A. Beri, F.G. Oppenheim, *Candida glabrata* is unusual with respect to its resistance to cationic antifungal proteins, Yeast 22 (2005) 705–714.
[20] W. van't Hof, I.M. Reijnders, E.J. Helmerhorst, E. Walgreen-Weterings, I.M. Simoons-Smit, E.C. Veerman, A.V. Amerongen, Synergistic effects of low doses of histatin 5 and its analogues on amphotericin B anti-mycotic activity, Antonie Van Leeuwenhoek 78 (2000) 163–169.
[21] E.J. Helmerhorst, I.M. Reijnders, W. van 't Hof, E.C. Veerman, A.V. Nieuw, A. Amerongen, A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides, FEBS Lett. 449 (1999) 105–110.
[22] E. Strandberg, D. Tiltak, M. Ieronimo, N. Kanithasen, P. Wadhwani, A.S. Ulrich, Influence of C-terminal amidation on the antimicrobial and hemolytic activities of cationic α-helical peptides, Pure Appl. Chem. 79 (2007) 717–728.
[23] J. Blazyk, R. Wiegand, J. Klein, J. Hammer, R.M. Epand, R.F. Epand, W.L. Maloy, U.P. Kari, A novel linear amphipathic beta-sheet cationic antimicrobial peptide with enhanced selectivity for bacterial lipids, J. Biol. Chem. 276 (2001) 27899–27906.
[24] E.N. Kanithasen, D. Tiltak, J. Burck, P. Wadhwani, O. Zwernemann, A.S. Ulrich, Solid-state NMR analysis comparing the designer-made antibiotic MSI-103 with its parent peptide PGLa in lipid bilayers, Biochemistry 47 (2008) 2601–2616.
[25] M. Nishida, Y. Imura, M. Yamamoto, S. Kobayashi, Y. Yano, K. Matsuzaki, Interaction of a magainin-PGLa hybrid peptide with membranes: insight into the mechanism of synergism, Biochemistry 46 (2007) 14284–14290.
[26] H.V. Westerhoff, M. Zasloff, J.L. Rosner, R.W. Hendler, A. De Waal, A. Vaz, A. Gomes, P.M. Jongsma, A. Riethorst, D. Juretic, Functional synergism of the magainins PGLa and magainin-2 in *Escherichia coli*, tumor cells and liposomes, Eur. J. Biochem. 228 (1995) 257–264.
[27] K. Matsuzaki, Y. Mitani, K.Y. Akada, O. Murase, S. Yoneyama, M. Zasloff, K. Miyajima, Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa, Biochemistry 37 (1998) 15144–15153.
[28] P. Tremouilhac, E. Strandberg, P. Wadhwani, A.S. Ulrich, Synergistic transmembrane alignment of the antimicrobial heterodimer PGLa/magainin, J. Biol. Chem. 281 (2006) 32089–32094.
[29] T. Hara, Y. Mitani, K. Tanaka, N. Uematsu, A. Takakura, T. Tachi, H. Kodama, M. Kondo, H. Mori, A. Otaka, F. Nobutaka, K. Matsuzaki, Heterodimer formation between the antimicrobial peptides magainin 2 and PGLa in lipid bilayers: a cross-linking study, Biochemistry 40 (2001) 12395–12399.
[30] K. Lohner, The role of membrane lipid composition in cell targeting of antimicrobial peptides, in: Ed. K. Lohner (Ed.), Development of Novel Antimicrobial Agents: Emerging Strategies, Horizon Scientific Press, Wymondham, Norfolk, U.K, 2001, pp. 149–165.
[31] K. Lohner, S.E. Blondelle, Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics, Comb. Chem. High Throughput Screen 8 (2005) 239–255.
[32] E. Sevcsik, G. Pabst, W. Richter, S. Danner, H. Amenitsch, K. Lohner, Interaction of LL-37 with model membrane systems of different complexity — influence of the lipid matrix, Biophys. J. 94 (2008) 4688–4699.
[33] K. Lohner, E.J. Prenner, Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems, Biochim. Biophys. Acta 1462 (1999) 141–156.
[34] K. Lohner, A. Latal, R.I. Lehrer, T. Ganz, Differential scanning microcalorimetry indicates that human defensin, HNP-2, interacts specifically with biomembrane mimetic systems, Biochemistry 36 (1997) 1525–1531.
[35] T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao, Y. Shimonishi, Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Isolation and chemical structure, J. Biol. Chem. 263 (1988) 16709–16713.
[36] S.H. White, W.C. Wimley, M.E. Selsted, Structure, function, and membrane integration of defensins, Curr. Opin. Struct. Biol. 5 (1995) 521–527.
[37] A. Latal, R.I. Lehrer, S.S.L. Harwig, K. Lohner, Interaction of enantiomeric protegrins with liposomes, Prog. Biophys. Mol. Biol. 65 (1996) 121.
[38] K. Matsuzaki, K. Sugishita, M. Harada, N. Fujii, K. Miyajima, Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria, Biochim. Biophys. Acta 1327 (1997) 119–130.
[39] G. Pabst, S. Grage, S. Danner-Pongratz, W. Jing, A.S. Ulrich, A. Watts, K. Lohner, A. Hickel, Membrane thickening by the antimicrobial peptide PGLa, Biophys. J. 95 (2008) 5779–5788.
[40] O. Konovalov, I. Myagkov, B. Struth, K. Lohner, Lipid discrimination in phospholipid monolayers by the antimicrobial frog skin peptide PGLa. A synchrotron X-ray grazing incidence and reflectivity study, Eur. Biophys. J. 31 (2002) 428–437.
[41] O. Konovalov, S.M. O'Flaherty, E. Saint-Martin, G. Deutsch, E. Sevcsik, K. Lohner, The bending rigidity of phospholipid monolayers in presence of an antimicrobial frog peptide studied by X-ray grazing incidence diffraction, Physica B 357 (2005) 185–189.
[42] P. Tremouilhac, E. Strandberg, P. Wadhwani, A.S. Ulrich, Conditions affecting the re-alignment of the antimicrobial peptide PGLa in membranes as monitored by solid state 2H-NMR, Biochim. Biophys. Acta 1758 (2006) 1330–1342.
[43] T. Wieprecht, O. Apostolov, M. Beyermann, J. Seelig, Membrane binding and pore formation of the antibacterial peptide PGLa: thermodynamic and mechanistic aspects, Biochemistry 39 (2000) 442–452.
[44] E.J. Helmerhorst, I.M. Reijnders, W. van't Hof, I. Simoons-Smit, E.C. Veerman, A.V. Amerongen, Amphotericin B- and fluconazole-resistant *Candida* spp., *Aspergillus fumigatus*, and other newly emerging pathogenic fungi are susceptible to basic antifungal peptides, Antimicrob. Agents Chemother. 43 (1999) 702–704.
[45] R.W. Glaser, C. Sachse, U.H. Durr, P. Wadhwani, A.S. Ulrich, Orientation of the antimicrobial peptide PGLa in lipid membranes determined from 19F-NMR dipolar couplings of 4-CF3-phenylglycine labels, J. Magn. Reson. 168 (2004) 153–163.
[46] B. Bechinger, M. Zasloff, S.J. Opella, Structure and dynamics of the antibiotic peptide PGLa in membranes by solution and solid-state nuclear magnetic resonance spectroscopy, Biophys. J. 74 (1998) 981–987.
[47] E.P. Strandberg, P. Wadhwani, U.H. Tremouilhac, A. Durr, A.S. Ulrich, Solid-state NMR analysis of the PGLa peptide orientation in DMPC bilayers: structural fidelity of 2H-labels versus high sensitivity of 19F-NMR, Biophys. J. 90 (2006) 1676–1686.
[48] R.W. Glaser, C. Sachse, U.H. Durr, P. Wadhwani, S. Afonin, E. Strandberg, A.S. Ulrich, Concentration-dependent realignment of the antimicrobial peptide PGLa in lipid, Biophys. J. 88 (2005) 3392–3397.
[49] S.E. Blondelle, K. Lohner, Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure–activity relationship studies, Biopolymers 55 (2000) 74–87.
[50] E. Sevcsik, G. Pabst, A. Jilek, K. Lohner, How lipids influence the mode of action of membrane-active peptides, Biochim. Biophys. Acta 1768 (2007) 2586–2595.
[51] K. Lohner, Effects of small organic molecules on phospholipid phase transitions, Chem. Phys. Lipids 57 (1991) 341–362.
[52] P. Balgavý, F. Devínsky, Cut-off effects in biological activities of surfactants, Adv. Colloid Interface Sci. 66 (1996) 23–63.
[53] M. Dathe, J. Meyer, M. Beyermann, B. Maul, C. Hoischen, M. Bienert, General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides, Biochim. Biophys. Acta 1558 (2002) 171–186.
[54] R. Dimova, B. Pouligny, C. Dietrich, Pretransitional effects in dimyristoylphosphatidylcholine vesicle membranes: optical dynamometry study, Biophys. J. 79 (2000) 340–356.
[55] G. Pabst, S. Danner, S. Karmakar, G. Deutsch, V.A. Raghunathan, On the propensity of phosphatidylglycerols to form interdigitated phases, Biophys. J. 93 (2007) 513–525.
[56] H.W. Huang, Molecular mechanism of antimicrobial peptides: the origin of cooperativity, Biochim. Biophys. Acta 1758 (2006) 1292–1302.
[57] C. Li, T. Salditt, Structure of magainin and alamethicin in model membranes studied by X-ray reflectivity, Biophys. J. 91 (2006) 3285–3300.
[58] G. Pabst, S. Danner, R. Podgornik, J. Katsaras, Entropy-driven softening of fluid lipid bilayers by alamethicin, Langmuir 23 (2007) 11705–11711.
[59] M.R. de Planque, D.V. Greathouse, R.E. Koeppe, H. Schafer, D. Marsh, J.A. Killian, Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, Biochemistry 37 (1998) 9333–9345.
[60] S. Morein, A. Andersson, L. Rilfors, G. Lindblom, Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures, J. Biol. Chem. 271 (1996) 6801–6809.
[61] L. Rilfors, A. Wieslander, G. Lindblom, Mycoplasma cell membranes, in: S. Rottem, I. Kahane (Eds.), Subcellular Biochemistry, Vol. 20, Plenum Press, New York, 1993, pp. 109–166.
[62] R.N. McElhaney, Membrane structure, in: J. Maniloff, R.N. McElhaney, L.R. Finch, J.B. Baseman (Eds.), Mycoplasmas: Molecular Biology and Pathogenesis, American Society for Microbiology, Washington DC, 1992, pp. 113–155.
[63] J.M. Seddon, R.H. Templer, Polymorphism of lipid water systems, in: R. Lipowsky, E. Sackmann (Eds.), Structure and Dynamics of Membranes, North-Holland, Amsterdam, 1995, pp. 97–160.
[64] P. Laggner, K. Lohner, Liposome phase systems as membrane activity sensors for peptides, in: J. Katsaras, T. Gutberlet (Eds.), Lipid Bilayers. Structure and Interactions, Springer, Berlin, 2000, pp. 233–264.
[65] M. Rappolt, A. Hickel, F. Bringezu, K. Lohner, Mechanism of the lamellar/inverse hexagonal phase transition examined by high resolution X-ray diffraction, Biophys. J. 84 (2003) 3111–3122.

[66] D.P. Siegel, J.L. Banschbach, Lamellar/inverted cubic (Lalpha/QII) phase transition in N-methylated dioleoylphosphatidylethanolamine, Biochemistry 29 (1990) 5975–5981.
[67] K. Lohner, Is the high propensity of ethanolamine plasmalogens to form non-lamellar lipid structures manifested in the properties of biomembranes? Chem. Phys. Lipids 81 (1996) 167–184.
[68] A. Hickel, S. Danner, H. Amenitsch, G. Degovics, M. Rappolt, K. Lohner, G. Pabst, Influence of antimicrobial peptides on the formation of nonlamellar lipid mesophases, Biochim. Biophys. Acta 1778 (2008) 2325–2333.
[69] E. Staudegger, E.J. Prenner, M. Kriechbaum, G. Degovics, R.N.A.H. Lewis, R.N. McElhaney, K. Lohner, X-ray Studies on the interaction of Gramicidin S with microbial lipid extracts: evidence for cubic phase formation, Biochim. Biophys. Acta 1468 (2000) 213–230.
[70] R. El Jastimi, M. Lafleur, Nisin promotes the formation of non-lamellar inverted phases in unsaturated phosphatidylethanolamine, Biochim. Biophys. Acta. 1418 (1999) 97–105.
[71] R.F. Epand, N. Umezawa, E.A. Porter, S.H. Gellman, R.M. Epand, Interactions of the antimicrobial beta-peptide beta-17 with phospholipid vesicles differ from membrane interactions of magainins, Eur. J. Biochem. 270 (2003) 1240–1248.
[72] K. Lohner, G. Degovics, P. Laggner, E. Gnamusch, F. Paltauf, Squalene promotes the formation of non-bilayer structures in phospholipid model membranes, Biochim. Biophys. Acta 1152 (1993) 69–77.
[73] E. Mileykovskaya, W. Dowhan, Visualization of phospholipid domains in *Escherichia coli* by using cadiolipin specific fluorescent dye 10-nonyl acridine orange, J. Bacteriol. 128 (2000) 1172–1175.
[74] S. Vanounou, A.H. Parola, I. Fishov, Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. A study with pyrene-labelled phospholipids, Mol. Microbiol. 49 (2003) 1067–1079.
[75] A. da Silva Jr., O. Teschke, Effects of the antimicrobial peptide PGLa on live *Escherichia coli*, Biochim. Biophys. Acta 1643 (2003) 95–103.
[76] M. Meincken, D.L. Holroyd, M. Rautenbach, Atomic force microscopy study of the effect of antimicrobial peptides on the cell envelope of *Escherichia coli*, Antimicrob. Agents Chemother. 49 (2005) 4085–4092.
[77] L. Ding, L. Yang, A.J. Waring, R.I. Lehrer, H.W. Huang, Interaction of antimicrobial peptides with lipopolysaccharides, Biochemistry 42 (2003) 12251–12259.
[78] K. Matsuzaki, K. Sugishita, K. Miyajima, Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of gram-negative bacteria, FEBS Lett. 449 (1999) 221–224.
[79] R.E.W. Hancock, The bacterial outer membrane as a drug barrier, Trends Microbiol. 5 (1997) 37–42.
[80] S. Snyder, D. Kim, T.J. McIntosh, Lipopolysaccharide bilayer structure: effect of chemotype, core mutations, divalent cations, and temperature, Biochemistry 38 (1999) 10758–10767.
[81] M. Vara, Agents that increase the permeability of the outer membrane, Microbiol. Rev. 56 (1992) 395–411.
[82] R.E.W. Hancock, Alterations in outer membrane permeability, Annu. Rev. Microbiol. 38 (1984) 237–264.
[83] H. Nikaido, Prevention of drug access to bacterial targets: permeability barriers and active efflux, Science 264 (1994) 382–388.
[84] A. Aspedon, E.A. Groisman, Antimicrobial peptide resistance mechanism in bacteria, in: K. Lohner (Ed.), Development of Novel Antimicrobial Agents: Emerging Strategies, Horizon Scientific Press, Wymondham, Norfolk, U.K., 2001, pp. 31–44.
[85] W. Jing, E.J. Prenner, H.J. Vogel, A. Waring, R.I. Lehrer, K. Lohner, Headgroup structure and fatty acid chain length of the acidic phospholipids modulate the interaction of membrane mimetic vesicles with the antimicrobial peptide protegrin-1, J. Pept. Sci. 11 (2005) 735–743.
[86] A. Arouri, M. Dathe, A. Blume, Peptide induced demixing in PG/PE lipid mixtures: a mechanism for the specificity of antimicrobial peptides towards bacterial membranes? Biochim. Biophys. Acta 1788 (2009) 650–659.
[87] R.F. Epand, M.A. Schmitt, S.H. Gellman, R.M. Epand, Role of membrane lipids in the mechanism of bacterial species selective toxicity by two alpha/beta-antimicrobial peptides, Biochim. Biophys. Acta 1758 (2006) 1343–1350.
[88] K. Matsuzaki, K. Sugishita, M. Harada, N. Fujii, K. Miyajima, Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria, Biochim. Biophys. Acta 1327 (1997) 119–130.