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Structural rearrangement of model membranes by the peptide antibiotic NK-2

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Abstract

We have developed a novel α-helical peptide antibiotic termed NK-2. It efficiently kills bacteria, but not human cells, by membrane destruction. This selectivity could be attributed to the different membrane lipid compositions of the target cells. To understand the mechanisms of selectivity and membrane destruction, we investigated the influence of NK-2 on the supramolecular aggregate structure, the phase transition behavior, the acyl chain fluidity, and the surface charges of phospholipids representative for the bacterial and the human cell cytoplasmic membranes. The cationic NK-2 binds to anionic phosphatidylglycerol liposomes, causing a thinning of the membrane and an increase in the phase transition temperature. However, this interaction is not solely of electrostatic but also of hydrophobic nature, indicated by an overcompensation of the Zeta potential. Whereas NK-2 has no effect on phosphatidylcholine liposomes, it enhances the fluidity of phosphatidylethanolamine acyl chains and lowers the phase transition enthalpy of the gel to liquid cristalline transition. The most dramatic effect, however, was observed for the lamellar/inverted hexagonal transition of phosphatidylethanolamine which was reduced by more than 10 °C. Thus, NK-2 promotes a negative membrane curvature which can lead to the collapse of the phosphatidylethanolamine-rich bacterial cytoplasmic membrane.

Keywords: Antimicrobial peptide; Membrane curvature; NK-lysin; Phosphatidylethanolamine; Small-angle X-ray diffraction

1. Introduction

The increasing resistance of pathogenic bacteria to conventional antibiotics has become a serious problem in health care, which requires alternatives to be developed [1]. One strategy is to modify the natural antimicrobial peptides/proteins of the innate immune system which act by the disruption of the integrity of bacterial cell walls. Over the last two decades, antimicrobial peptides have been identified for almost every organism analyzed so far (see http://www.bbcm.univ.trieste.it/~tossi/pag1.htm and [2–6]). These antimicrobial peptides are small in size, cationic, and of amphipathic structure, at least upon membrane interaction.

Different models describing the process of membrane destruction by these peptides have been developed ranging

Abbreviations: DSC, differential scanning calorimetry; FTIR, Fourier-transform infrared spectroscopy; SAXS, small-angle X-ray scattering; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol

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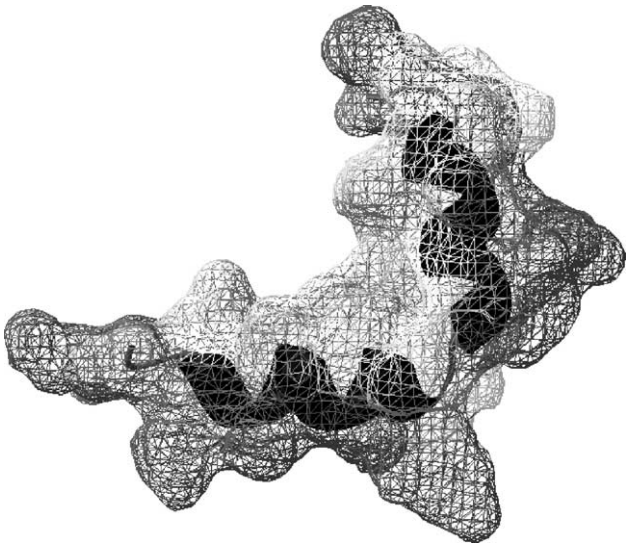

Fig. 1. Structure model of the synthetic peptide NK-2. The structure was extracted (residues 39–65) from the pdb entry of the NMR structure of NK-lysin (1NKL) [18]. The molecular surface was calculated using the Swiss-Pdb Viewer software (V3.7). A positive surface potential is indicated darker grey. The alpha-helical content of the peptide's secondary structure was confirmed by CD- and FTIR-spectroscopy [26,30].

from the 'barrel-stave model' of alamethicin [7], where transmembrane bundles of amphipathic helices should be formed, to the 'carpet' and 'toroidal-hole' models of cecropins and magainins, where peptides first cover the membrane like a carpet, and in a second step, after a certain threshold of peptide concentration has been reached, they bend the membrane and form toroidal-like lesions in a way that the peptides are always associated to the lipid head-groups [8–10]. The overall physicochemical membrane properties may be affected in different ways by those mechanisms. In particular for the formation of the torus-type pores proposed by the carpet model, a rearrangement of the lipid molecules is necessary. For magainin, the 'classical' toroidal-pore forming peptide, consistently the induction of a positive membrane curvature strain was observed, which is paralleled by a membrane rigidification and an increase of the lamellar/inverted hexagonal phase-transition temperature [11,12].

NK-lysin from porcine NK-cells [13], its human counterpart granulysin [14,15], and amoebapore from a pathogenic amoeba [16,17] are 8–9 kDa alpha-helical proteins that belong to the SAPLIP family [18–20]. All three proteins kill bacteria by permeabilizing their membranes [21–24], and also synthetic peptides derived from the core-regions of these proteins exhibited potent membranolytic activities [23,25–28]. One of these peptides, termed NK-2, representing the third and fourth helices (residues 39–65) of NK-lysin, is of particular therapeutic interest (Fig. 1). It is of low hemolytic activity and devoid of cytotoxicity against human cell lines [26,29], but exerted a potent broad spectrum activity against bacteria (Gram-positive and Gram-negative) [26], *Candida albicans* [26], and even against the protozoan parasite *Trypanosoma cruzi* [29]. In a previous paper [30], we showed that the target selectivity of NK-2 can be explained without the exploitation of a specific receptor, simply by a higher affinity of the peptide for the lipid matrix of the cytoplasmic membrane of bacteria than for human cells. The lipid composition of both cell types differ markedly ([1,31] and references therein). The bacterial plasma membrane mainly consists of zwitterionic PE combined with a considerably high amount of anionic lipids, like PG and cardiolipin, whereas the membrane of healthy human cells appears uncharged in the outer bilayer leaflet consisting almost exclusively of the zwitterionic choline phospholipids PC and sphingomyelin and the non-phospholipid compound cholesterol (Table 1). NK-2 has a clear affinity for anionic charges, as one would expect for a cationic peptide, however, it is also able to differentiate between PC and PE [30], which bear an identical charge and have a very similar chemical structure.

To gain more insights into the mechanism of selectivity and membrane destruction, we performed a detailed structural analysis of NK-2 induced changes in biomembrane mimetic systems. Four different techniques for the structural characterization of the samples were used: small-angle X-ray scattering (SAXS) addressing the supramolecular structure of the biomembrane mimetic system, while the effects of the peptide on lipid phase transition behaviour and acyl chain fluidity were further investigated by differential scanning calorimetry (DSC) and Fourier-transform infrared spectroscopy (FTIR). Finally, the influence of the peptide NK-2 on the surface charge of liposomes was determined by measuring the Zeta potential.

The liposomes were composed of phospholipids which mimic bacterial (PE and PG) and human erythrocyte plasma membranes (PC). In general, phospholipids with unsaturated and saturated acyl chains were used. In some cases, owing to the easier technical feasibility of the experiments, symmetrically substituted phospholipids with disaturated fatty acid chains were used.

Table 1
Phospholipid composition (mol%) of the cytoplasmic membranes of some bacteria and of the outer membrane leaflet of human erythrocytes ([31] and references therein)

Organism/cell type	PE	PG	CL	PC	SM
Human erythrocytes	–	–	–	57	43
Escherichia coli	82	6	12	–	–
Salmonella typhimurium	60	33	7	–	–
Pseudomonas cepacia	82	18	–	–	–

The cytoplasmic membrane of human cells also consists of cholesterol (about 46 mol% [63]) which is not found in bacterial membranes. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; SM, sphingomyelin.

2. Materials and methods

2.1. Materials

Phospholipids were purchased from Sigma (Deisenhofen, Germany) (POPC, POPE, and POPG) and from Avanti Polar Lipids Inc. (Alabaster, AL, USA) (DPPC, DMPC, DPPE, DPPG, and DMPG). Lipids (purity >99%) were used without further purification. All other chemicals were in analytical grade from Merck (Darmstadt, Germany).

2.2. Peptides and peptide analysis

The synthetic peptide NK-2 (KILRG VCKKI MRTFL RRISK DILTG KK-NH_2) was obtained from WITA GmbH (Berlin, Germany) in a purity grade >95%. Its homogeneity was confirmed by analytical RP-HPLC (Lichrospher 100 RP 18, 5 μm column, Merck, Darmstadt, Germany), MALDI-TOF (Bruker Daltonik GmbH; both performed by the manufacturer), and N-terminal Edman degradation (Procise Protein Sequencing System, Applied Biosystems, Germany). The peptide was synthesized with an amidated C-terminus.

2.3. Phospholipid analysis

Before and after the experiments, the purity of the phospholipids was checked routinely by thin layer chromatography (TLC), using silica plates (Merck) and a mixture of chloroform/methanol/25% NH_3 (13/5/1, by volume) as solvent. TLC plates were developed with molybdatophosphoric acid (Merck).

2.4. Preparation of phospholipid vesicles

Phospholipids (total 2.5 mg) were dissolved in methanol/chloroform (1/2, by volume), the solvent was slowly removed by a constant stream of nitrogen, and the resulting lipid film was dried under vacuum overnight. The lipid films were hydrated in a buffer (see the respective method) and heated for 2–4 h above the T_m of the respective lipid with repeated vortexing (1 min) every 30 min of incubation time. The samples were cooled down to room temperature and incubated for 30 min. This procedure resulted in the formation of multilamellar liposomes (MLV) for POPE, DPPE, POPC, DPPC, and DMPC, and unilamellar vesicles for DPPG, DMPG, and POPG.

2.5. X-ray diffraction

Small-angle X-ray diffraction (SAXS) measurements were performed at the HASYLAB at DESY Soft Condensed Matter beamline A2 with a wavelength λ=0.15 nm and covered a scattering vector $s=1/d=(2\sin\theta)/\lambda$ (2θ=scattering angle; d=lattice spacing) from 1×10^{-2} to 0.5 nm^{-1}. The samples were prepared on site just before the measurements. Phospholipids were hydrated in 10 mM sodium phosphate buffer, pH 7.4 (25 mg/ml) as described above. For peptide:lipid molar ratios of 1:3000, 1:1000, 1:300, and 1:100, the respective amount of NK-2 was added from a stock solution (1 mM in H_2O) after the formation of the lipid vesicles. The samples (30 μl) were filled into glass capillaries (Hilgenberg GmbH, Malsfeld, Germany; wall thickness 0.1 mm) and measured in a temperature-controlled sample holder. The temperature was varied from 10 °C to 80 °C with 2 °C/min. Data were collected for 10 s per measurement. Before and after each temperature ramp, a capillary filled with buffer was measured at 20 °C. These measurements were used to correct for solvent background. The calibration for the SAXS pattern was done by measuring rat tail tendon (repeat distance 65.0 nm, standard at beamline A2) in addition to silver-behenate ($[CH_3(CH_2)_2OCOO–Ag]$, repeat distance 5.838 nm, made available at beamline A2). The data were normalized with respect to the primary beam and the positions of the diffraction peaks were determined using the OTOKO software [32]. In the case of POPG vesicles, the data were analyzed by fitting a Gaussian distribution to the data using the program Origin (OriginLab Cooperation, Northampton, USA). Background subtraction was performed using a program supplied by A. Meyer, beamline A2, HASYLAB at DESY.

2.6. Differential scanning calorimetry (DSC)

Phospholipids were dispersed in 10 mM sodium phosphate buffer, pH 7.4 (1 mg/ml) as described above, NK-2 was added in the appropriate concentration, and the sample was degassed for 10 min. Differential scanning calorimetry (DSC) experiments were performed with a high-sensitivity adiabatic differential scanning calorimeter (VP-DSC, MicroCal, Inc., Northhampton, MA, USA). The heating and cooling rate was 0.5 °C/min. Heating and cooling curves were measured in the temperature interval from 10 °C to 80 °C. Two consecutive heating and cooling scans were performed. The data were analyzed using the Microsoft-MicroCal's Origin software.

2.7. Fourier-transform infrared spectroscopy (FTIR)

The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). Phospholipids (DMPC, DMPG, and POPE) were hydrated in 20 mM Hepes, pH 7.0 (lipid conc. 20 mM) and incubated with NK-2 for 60 min at 37 °C at the indicated peptide:lipid molar ratios. The samples were then placed in a CaF_2 cuvette with a 12.5 μm Teflon spacer. Temperature scans were performed automatically between 10 °C and 70 °C with a heating rate of 0.6 °C/min. Every 3 °C, 50 interferograms were accumulated, apodized, Fourier transformed, and converted into absorbance spectra. For

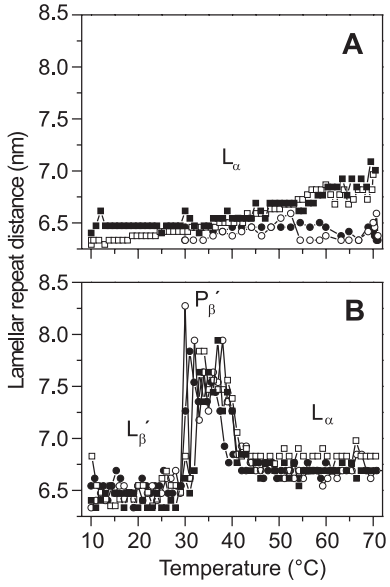

Fig. 2. Temperature-dependent influence of the synthetic peptide NK-2 on the supramolecular structure of zwitterionic phospholipids with the choline headgroup as determined by small-angle X-ray scattering. (A) POPC; (B) DPPC. Various [peptide]:[lipid] molar mixtures were prepared and the lamellar repeat distances were determined: only lipid (●), 1:1000 (○), 1:300 (□), 1:100 (■). The indicated lipid phases are L_β, $L_{\beta'}$ and L_α: (multi)-lamellar gel– and liquid–crystalline phase, respectively, and $P_{\beta'}$: ripple phase.

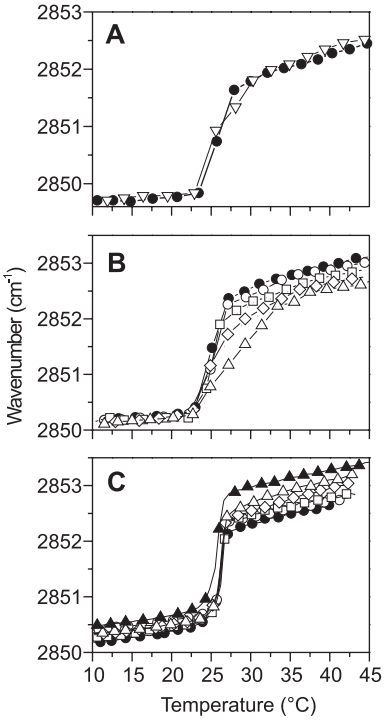

Fig. 3. Peak position of the symmetric stretching vibration of the fatty acid methylene groups $v_s(CH_2)$ of DMPC (A), DMPG (B), and POPE (C) at different [NK-2]:[phospholipid] molar ratios: only lipid (●), 1:1000 (○), 1:300 (□), 1:30 (◇), 1:10 (△), 1:5 (▲), 1:2 (▽).

strong absorption bands, the band parameters (peak position, band width, and intensity) were evaluated from the original spectra, if necessary after the subtraction of the strong water bands.

2.8. Zeta potential

Zeta potentials were determined with a Zeta-Sizer 4 (Malvern Instr., Herrsching, Germany) at room temperature at a scattering angle of 90° from the electrophoretic mobility by laser-Doppler anemometry as described earlier [33]. The Zeta potential was calculated according to the Helmholtz–Smoluchovski equation from the mobility of the aggregates in a driving electric field of 19.2 V/cm. Liposomes of DPPC, DPPE, and DPPG (0.1 mM) and NK-2 stock solutions (2 mM) were prepared in 10 mM Tris, 2 mM CsCl, pH 7.0. The peptide was added to the liposomes after the full hydration of the lipids. Data shown for the Zeta potential represent mean values from at least two independent liposome preparations. Each liposome preparation was measured five times.

3. Results

3.1. Interactions of NK-2 with PC liposomes

As a membrane mimetic for the human erythrocyte surface, we used liposomes composed of the zwitterionic

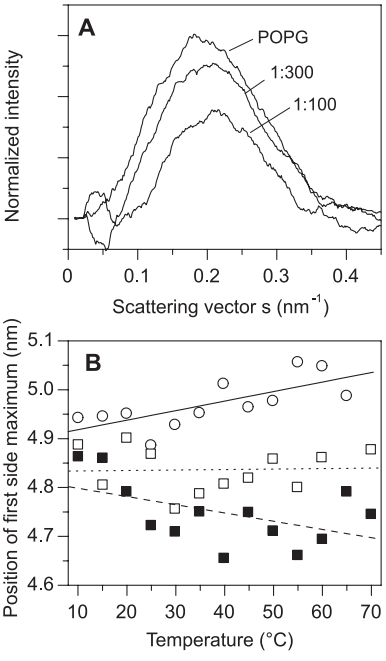

Fig. 4. (A) Small-angle X-ray scattering (SAXS) patterns for pure POPG and of [peptide]:[lipid] mixtures at the indicated molar ratios at 37 °C. (B) Influence of NK-2 at various [peptide]:[lipid] molar ratios on the position of the first side maximum of POPG vesicles in dependence on the temperature: pure POPG (○), 1:300 (□), 1:100 (■). The peak intensity decreased upon the increase of temperature (not shown). Lines represent a linear fit of the respective data series.

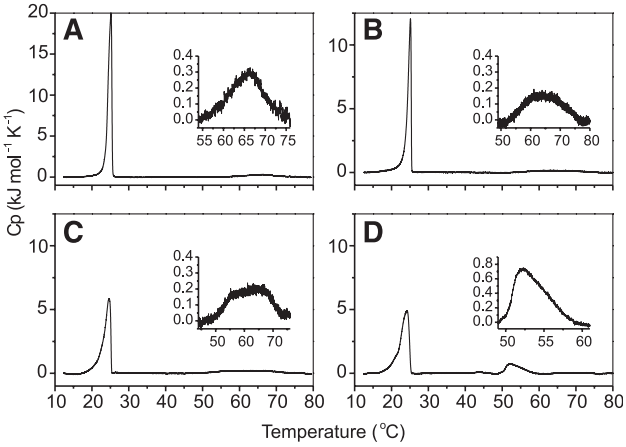

Fig. 5. DSC measurements of pure POPE (A) and of [NK-2]:[POPE] mixtures at three different molar ratios (B, 1:3000; C, 1:100; D, 1:30). The second heating scans are shown, which were fully reproducible upon further heating scans.

phospholipid phosphatidylcholine (PC) with a saturated and an unsaturated acyl moiety in positions 1 and 2 (1-palmitoyl-2-oleoyl-, POPC, 16:0, 18:1), or completely saturated fatty acids (dipalmitoyl-, DPPC, 16:0; dimyristoyl-, DMPC, 14:0). The latter lipids show interesting phase behaviors giving a gel phase ($L_{\beta'}$) ↔ ripple phase ($P_{\beta'}$) ↔ liquid crystalline phase (L_α) transition. By using SAXS, an effect of NK-2 on the lamellar repeat distance was found for POPC above 55 °C (Fig. 2A). This may be explained by an enhanced hydration of the lipid in the presence of NK-2 [34]. For DPPC no influences on the phase transition temperatures (T_{pre}, T_m) and the lamellar repeat distances were observed (Fig. 2B). Furthermore, DSC measurements of DPPC vesicles in the presence of NK-2 in the same concentration range as used for the SAXS measurements also revealed that the peptide had no effect on the phase behaviour of this lipid (data not shown). The lack of a significant action of the peptide on phospholipids with a PC headgroup was confirmed by FTIR (Fig. 3A). The peak position of the symmetric stretching vibration of the methylene groups (CH_2) for the phospholipid acyl chains was determined here as a sensor for the lipid's phase state with wave numbers around 2850 cm^{-1} in the gel and 2852.5 to 2853 cm^{-1} in the liquid crystalline phase. Again, neither the T_m of DMPC nor the general acyl chain fluidity of the lipid fatty acids was changed by NK-2 even at a peptide to lipid molar ratio of 1:2.

3.2. Interactions of NK-2 with PG liposomes

Phosphatidylglycerol (PG), besides cardiolipin, represents the main negatively charged phospholipid of the cytoplasmic membrane of bacteria and was used here with mixed saturated/unsaturated fatty acids (POPG) for the SAXS experiments, and with two saturated fatty acid chains (DMPG) for FTIR measurements. Unlike POPC and POPE, POPG exhibits no Bragg peaks characteristic for multilamellar vesicles but solely diffuse scattering, both in the L_β and in the L_α phases (Fig. 4A). The observed scattering is due to the Fourier transform of the electron density distribution within single, uncorrelated bilayers and is characteristic for unilamellar vesicles. This demonstrates that under this experimental condition of low ionic strength, negatively charged POPG forms unilamellar vesicles because of the net electrostatic repulsion of the bilayers. This is a general observation for charged lipids and has been well documented both experimentally [35,36] and theoretically [37]. Thereby, the broad diffuse peak is related to the bilayer thickness, and therefore, the shift of this peak to higher scattering angles in the presence of NK-2 indicates a decrease in bilayer thickness, i.e. membrane thinning (Fig. 4B). The value is however small and was estimated to be about 2 Å. In contrast, FTIR data indicated that in the presence of the peptide, the DMPG membrane is stabilized, T_m is shifted to higher temperature, and the rigidity of the acyl chains of DMPG increases in the fluid phase (Fig. 3B): This should be reflected by an increase in membrane thickness owing to a thicker hydrocarbon core caused by the reduced number of *trans-gauche* isomerizations. However, this may be overcompensated by changes in the headgroup orientation and/or hydration, so that overall a membrane thinning is observed.

3.3. Interactions of NK-2 with PE liposomes

Zwitterionic phosphatidylethanolamine (PE) is the major phospholipid found in bacterial membranes. Throughout our experiments, besides Zeta-potential measurements, we used the mixed chain POPE. Upon the addition of the peptide

Table 2
Thermodynamic parameters of POPE and of various [NK-2]:[POPE] mixtures determined from the second heating scans by DSC

[NK-2]:[POPE] (molar ratio)	$L_\beta \leftrightarrow L_\alpha$			$L_\alpha \leftrightarrow H_{II}$		
	T_m (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kJ/mol)	T_{LH} (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kJ/mol)
POPE	25.1	1.1	24.7	66.4	9	2.9
1:3000	25.1	1.2	15.6	62.2	11	2.2
1:1000	25.1	0.9	21.3	61.7	11	2.8
1:300	25.1	1.0	15.1	62.1	11.5	2.4
1:100	24.6	1.9	13.8	61.1	16	3.4
1:30	24.1	2.5	14.9	52.3	4.7	3.5

T_m, T_{LH}, main and lamellar-inverted hexagonal phase transition temperature, respectively; $\Delta T_{1/2}$, width at half height; ΔH, phase transition enthalpy.

NK-2 to the POPE vesicles, the samples showed a pronounced tendency to aggregate which was observed already at the lowest [NK-2]:[POPE] molar ratio of 1:3000. Using DSC, the T_m of the gel to the liquid crystalline phase transition (i.e. the acyl chain melting) was observed for pure POPE at 25 °C (Fig. 5A, Table 2). In the presence of NK-2, the maximal decrease of T_m for the main transition was only 1 °C even in the case of the highest peptide concentration ([peptide]:[lipid] molar ratio of 1:30) (Fig. 5D, Table 2). Interestingly, FTIR showed that the addition of NK-2 to POPE liposomes resulted in an enhanced acyl chain fluidity, as evidenced by an increase in the measured wave numbers in the gel as well as in the liquid–crystalline state of POPE (Fig. 3C). In contrast to the effect observed on T_m, NK-2 drastically lowered T_{LH} in a dose-dependent manner from 66.4 °C for pure POPE down to 61.1 °C at a NK-2:POPE molar ratio of 1:100 (Fig. 5C, Table 2). At higher peptide concentrations, [peptide]:[lipid] molar ratio of 1:30, T_{LH} was even further lowered to 52.3 °C (Fig. 5D, Table 2). Interestingly, at this condition the $L_\alpha \leftrightarrow H_{II}$ phase transition was characterized by an enhanced cooperativity, i.e. a sharper transition peak. The respective transition enthalpy is close to the value of pure POPE (Table 2). Thus we can conclude that this transition actually reflects a transformation from L_α to H_{II}. A lamellar to cubic phase transition can rather be excluded, because it can hardly be detected by DSC owing to its very low transition enthalpy [38]. X-ray diffraction experiments of pure POPE showed a typical Bragg diffraction pattern with a first order diffraction peak for lamellar L_β (gel) and L_α (liquid crystalline) phases with a T_m of 25 °C. Above 70 °C reflections at the periodicity of 6.25 nm, $6.25/\sqrt{3}$=3.6 nm and $6.25/\sqrt{4}$=3.1 nm, typical for an inverted hexagonal phase (H_{II}), were observed (Fig. 6A). The lamellar repeat distance of POPE was shown not to be affected by the peptide. However, concomitant to the DSC measurements, T_{LH} was reduced to 60 °C at a 1:1000 [peptide]:[lipid] molar ratio. Similar to DPPC, the influence of the peptide on the POPE $L_\beta \leftrightarrow L_\alpha$ transition was found to be negligible up to a peptide:lipid ratio of 1:1000 (Fig. 6).

3.4. Surface charges of phospholipid vesicles (Zeta potential)

The Zeta potential, as an indicator for accessible surface charges, was determined for pure phospholipids DPPC, DPPE, and DPPG to be -2.3 ± 2.0, -32.4 ± 3.4, and -56.0 ± 2.2 mV, respectively. Note, a dramatic difference exists between the two structurally very similar zwitterionic phospholipids PC and PE. The addition of NK-2 had no influence on the Zeta potential of DPPC, indicating that the peptide does not interact with this lipid matrix. However, a charge neutralization was observed for DPPE and DPPG. In particular for DPPE, the Zeta potential was compensated at very low [peptide]:[lipid] molar ratios which indicates an electrostatically driven interaction. At higher [peptide]:[lipid] ratios a charge overcompensation was observed, which suggests a hydrophobic interaction after the saturation of the negative surface charges (Fig. 7). It should be emphasized that in the case of DPPG a stronger overcompensation occurs, suggesting a higher affinity of NK-2 for PG than for PE.

4. Discussion

We have found previously that the peptide NK-2 is a very promising candidate for a novel antibiotic, because it displays a high antimicrobial activity and is non-toxic to

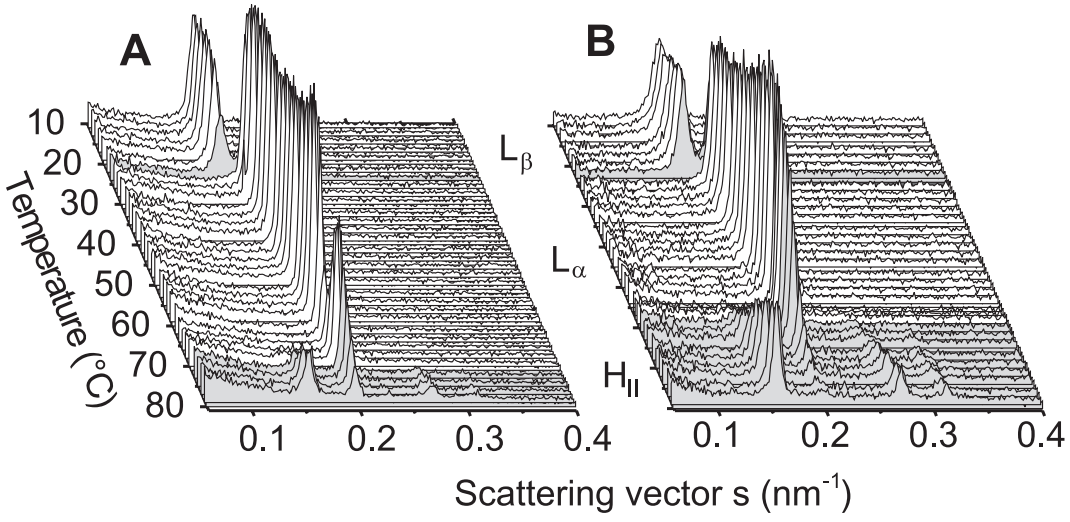

Fig. 6. Small-angle X-ray diffraction pattern of (A) POPE and of (B) a [NK-2]:[POPE] molar mixture of 1:1000. A temperature ramp was performed from 10 °C to 80 °C spanning the two phase transitions. A coexistence of phases around the transition temperatures was observed in all cases (shaded in grey), ranging from approximately 2 °C for the gel (L_β) to liquid crystalline (L_α) transition, and more than 10 °C for the L_α to inverted hexagonal phase (H_{II}) transition. In the presence of NK-2, the coexistence of the L_α and H_{II} phases was extended to a temperature range of approximately 20 °C. Moreover, at the highest temperature measured the fraction of the liquid crystalline phase was negligible in the presence of the peptide.

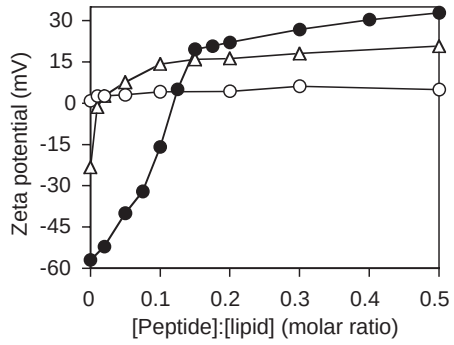

Fig. 7. Zeta potential of phospholipid liposomes (DPPC, ○; DPPG, ●; DPPE, △) with dependence on [NK-2]:[phospholipid] molar ratios from the determination of the electrophoretic mobility by laser Doppler anemometry.

human cells [26]. It binds to and permeabilizes membranes containing negatively charged PG (found in the cytoplasmic membranes of bacteria) but a negligible interaction was observed with pure zwitterionic PC model membranes (as a mimetic for human cell membranes) [30]. In addition, NK-2 binding to PE model membranes is followed by a slight insertion of the peptide as detected by biosensor and intrinsic tryptophan fluorescence measurements [30]. PE is a major phospholipid component of bacterial cell membranes. Its headgroup consists of a positively charged amino alcohol linked to the negatively charged phosphate group. This combination of charged groups is very similar to PC and suggests an overall neutral net charge for both phospholipids. Consequently, the different binding/insertion characteristics of NK-2 to apparently identical (i.e. neutral) charged PC and PE imply that the molecular basis for peptide/membrane interaction and its selectivity towards bacteria and human cells is more than simple electrostatic interactions. Interestingly, our Zeta-potential measurements indicate that the accessible surface charges of PC and PE liposomes at neutral pH are quite different with a considerable negative potential for PE, an observation which has been reported also by other groups [39]. A possible explanation may be that, in contrast to PE, in the case of PC, the negatively charged phosphate group is shielded by the voluminous trimethylamine umbrella of the choline group. PE has a smaller headgroup than PC, and adjacent PE molecules form hydrogen bonds between the amino and the phosphate groups, which favours a tight packing of the lipids. These factors affect the molecular shape of the lipids and the curvature of the formed biomembranes [40]. A cylindrically-shaped molecule (like dipalmitoyl-PC) will form a nearly planar lipid bilayer. On the contrary, conically-shaped phospholipids with a higher cross section of the fatty acid chain moiety than the headgroup (like 1-palmitoyl,-2-oleoyl-PE or dioleoyl-PE) form membranes with an intrinsic negative curvature strain (bent to the outside). These membranes are prone to convert into inverted non-lamellar supramolecular aggregate structures (cubic or hexagonal phases). In this context, we performed a detailed structural analysis of mixtures of the peptide NK-2 with phospholipids typical for the bacterial (PE, PG) and the eukaryotic (PC) cytoplasmic membrane, respectively.

In the presence of NK-2 we observed a drastic lowering of the $L_\alpha \leftrightarrow H_{II}$ phase transition of POPE and an increase of the chain fluidity in its lamellar phases even at low peptide concentrations. Also other antibacterial peptides are known to modulate the spontaneous curvature properties of phospholipid assemblies and thereby induce the formation of non-lamellar phases: gramicidin A, a β-helix forming peptide which consists of alternating L- and D-amino acid residues [41,42], and the cyclic decapeptide gramicidin S [43], both are produced by *Bacillus brevis*; the lantibiotic nisin [44], alamethicin [45], and a 17-residues β-peptide [46]. Among these, gramicidin A is the most prominent inverted phase-inducing peptide reported so far. It promotes a negative membrane curvature resulting in the formation of the inverted hexagonal H_{II} phase. Magainin, originally isolated from the skin of the African clawed frog *Xenopus laevis* [47], is the prototype of an α-helical antimicrobial peptide. It resembles much more NK-2 than the peptides described above, which are distinguished by a very unique structure and/or mode of action. Until now, several analogs of magainin with improved activity have been described and run clinical trials [48,49]. These peptides also affect the phase transition temperature of the liquid–cristalline to inverted hexagonal phase of PE lipids. However, in this case, a shift towards higher temperatures was found, most likely caused by a compensation of the intrinsic negative curvature strain [11,12], which stabilizes the membrane, and is in contrast to our findings with NK-2 but in accordance with the formation of toroidal-like pores proposed by the toroidal and the carpet model [8–10,50,51].

NK-2 does not interact with PC vesicles, as evidenced by the lack of a disordering effect in the diffraction pattern, in the FTIR spectra, or a significant change in Zeta potential of PC lipids. Moreover, biosensor assays showed no mass increase upon the interaction of NK-2 with POPC layers [30]. A possible explanation could be that no charge interaction takes place since, as indicated by a Zeta potential close to zero, the phosphate group of PC lipids seem to be completely hidden under the umbrella of the PC methyl groups.

The X-ray data analysis and interpretation of the interaction of negatively charged PG, a component of the bacterial membrane, are rather difficult because of the resulting unilamellar liposomes. Compared to POPG alone, the NK-2:lipid mixture has a tendency to decrease the virtual repeat distances. Using FTIR spectroscopy, a shift of T_m of the gel to liquid–cristalline phase transition towards higher values was detected for DMPG. This is in good agreement with the observation that upon the addition of positively charged proteins or ions the T_m value of PG increases [52], accompanied by a tightly packed structure of the lipids [53]. Additionally, biosensor measurements showed a strong binding of NK-2 to a POPG containing model membrane [30]. The results support the view that NK-2 binds via a strong electrostatic interaction to PG. It is

likely that these strong forces keep the peptide close to the membrane surface, where the charges are located, and do not allow a deep, i.e. perpendicular, penetration of NK-2 into the hydrophobic moiety.

Summarizing the interaction of NK-2 with model membranes we suggest the following model for target selectivity and membrane destruction:

(i) Negative charges will increase the probability of the presence of the peptide at the membrane surface. This will function for bacterial membranes (consisting of PE and PG), as well as for other membranes in which respective charges can be found (e.g. phosphatidylserine on the surface of aged red blood cells and certain cancer cells) [54–56], and is also underlined by the affinity of the peptide for anionic lipopolysaccharide (endotoxin, LPS), the main component of the outer membrane of Gram-negative bacteria [57].
(ii) A direct interaction of NK-2 with negatively charged lipids like POPG leads to a rigidification of the membrane without destroying the membrane structure. The peptide is fixed to the membrane surface by strong electrostatic interactions.
(iii) The interaction of NK-2 with lipids favouring the formation of inverted lipid structures (H_{II}, cubic), which are present in the bacterial cytoplasmic membrane (i.e. PE), promotes the intrinsic negative curvature strain of these lipids and also enhances the fluidity of the PE acyl chains.
(iv) Finally, the enhancement of the negative curvature of PE-enriched membrane regions by the insertion of the wedge shaped NK-2, in combination with the stiffening of other areas, for instance those which are rich in PG, results in a membrane tension at the PE/PG interface ultimately resulting in membrane disruption. Whereas the first step of the NK-2 membrane interaction resembles the carpet model, the mode of membrane destruction is definitely different.

As outlined, our data give a satisfactory explanation for the mechanism of membrane permeabilization, as well as for target selectivity of the peptide NK-2, based only on the respective composition of the lipid matrix of the target cell. Apparently, the properties of phospholipids found in the cytoplasmic membrane of bacteria differ sufficiently, by charge and their tendency to assemble to aggregates, from those found in the plasma membrane of human cells. Though not absolutely necessary, it is likely that other membrane and surface factors contribute to the target cell specificity. Lipopolysaccharides and lipoteichoic acids of Gram-negative and Gram-positive bacteria are potentially negatively charged binding sites for cationic peptides [57–59], and cholesterol, exclusively found in mammalian cell membranes, may attenuate the binding and/or pore formation of antimicrobial peptides like magainin, gramicidin, or NK-2 [60–62].

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