Volume 244, number 1, 99–102 FEB 06784 February 1989

Interaction of the pore forming-peptide antibiotics Pep 5, nisin and subtilin with non-energized liposomes

Marianne Kordel*, Friederike Schüller and Hans-G. Sahl

Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, D-5300 Bonn-Venusberg and **GBF, Mascheroder Weg 1, D-5300 Braunschweig, FRG*

Received 27 November 1988

The cationic peptide antibiotics Pep 5, nisin and subtilin depolarize bacterial and artificial membranes by formation of voltage-dependent multi-state pores. Studies with non-energized liposomes indicated that the peptides do not span the membrane in the absence of a membrane potential. The effects of Pep 5 and nisin on neutral membranes, as studied by membrane fluidity, phase transition points and carboxyfluorescein efflux, were small compared to melittin. Acidic liposomes were affected more strongly, indicative of primarily electrostatic interactions with phospholipid head groups. Subtilin may slightly enter the hydrophobic core as suggested by tryptophan fluorescence quenching and liposome fusion experiments.

Cationic peptide antibiotic; Pep 5; Nisin; Subtilin; Membrane interaction

1. INTRODUCTION

The antibiotics Pep 5, nisin, and subtilin are cationic peptides with intramolecular thioether bridges introduced by posttranslational modifications of precursor peptides [1–3]. Nisin has found considerable application as a preservative for canned food or in dairy processes [4].

The peptides are bactericidal for gram-positive bacteria. They render bacterial cytoplasmic membranes permeable to ions, amino acids, and ATP, thus causing a rapid breakdown of the membrane potential [5–8] and a complete cessation of biosynthesis [9]. The antibiotics require threshold potentials for in vivo activity ranging from 50 to 100 mV and induce voltage-dependent multi-state pores of the alamethicin type in black lipid membranes [10,11]. Formation of such short-lived channels with pore diameters of up to 1 nm requires aggregation of several peptide molecules and a transmembrane orientation of the aggregates.

At present there is no information on the interaction of the peptides with membranes in the absence of a potential, particularly if peptide aggregates span the membranes and channels are opened by voltage, or if voltage forces the peptides into the membrane from a peripheral aggregation state. Therefore, we studied the influence of the peptides on non-energized membranes and compared their effects with those of the bee venom peptide melittin, which displays similar physical properties and biological activities [12–15].

Correspondence address: H.-G. Sahl, Institut für Medizinische Mikrobiologie und Immunologie der Universität Bonn, D-5300 Bonn 1-Venusberg, FRG

Abbreviations: CF, carboxyfluorescein; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DPA, dipicolinic acid; PS, phosphatidylserine; PDA, pyrenedecanoic acid; tempol, 2,2,6,6-tetramethyl-4-piperidinole 1-oxide; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid

2. MATERIALS AND METHODS

Pep 5 and subtilin were purified from culture supernatants of *Staphylococcus epidermidis* 5 and *Bacillus subtilis* ATCC 6633 as described [1,16]. Nisin was purchased from Koch-Light and melittin from Sigma. Prior to use both were chromatographed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

on HPLC reversed-phase C-18 as described for Pep 5 [1] for final purification and removal of phospholipase. For preparation of liposomes we used DOPC (Avanti), DMPC, DMPS, and PS (Sigma). PDA, CF, 5-, 12- and 16-nitroxide stearic acid (spin labels SL-5, SL-12, SL-16), and tempol were obtained from Sigma; $TbCl_3$ and DPA were from Aldrich.

Unilamellar liposomes were prepared basically according to Oku and MacDonald [17]. We obtained unilamellar vesicles of diameter 0.3–1 μm as checked by negative-stain electron microscopy. To entrap CF, $TbCl_3$, or DPA into the lumen of freeze-thawed vesicles, dried lipid films were resolved in 2 mM Tes/2 mM histidine, pH 7.4, containing the corresponding substances at 15, 150 and 100 mM. The fusion experiments were performed according to Wilschut et al. [18].

All fluorescence measurements were taken with a Shimadzu RF-540 spectrofluorometer equipped with magnetic stirring and a temperature-controlled cuvette holder. Tryptophan fluorescence spectra were recorded between 300 and 400 nm (excitation wavelength 280 nm). The spectra were corrected by subtraction of baseline fluorescence in the absence of subtilin. Tryptophan fluorescence quenching by fatty acid spin labels or tempol was performed according to Luisetti et al. [19].

The influence of the peptides on phase transition temperature and membrane fluidity was investigated by measuring the formation of excited dimers (excimers) of PDA according to Galla and Hartmann [20]. PDA at concentrations of 3–15 mol% of total lipid was incorporated into the membranes during the formation of unilamellar liposomes.

3. RESULTS AND DISCUSSION

3.1. *Influence on membrane fluidity and phase transition temperature*

We measured the influence of the peptide antibiotics on physical properties of membranes by monitoring the formation of excited dimers of PDA incorporated into membranes of different composition. All experiments were conducted well above the phase transition temperatures of the respective phospholipids.

The fluidity of DOPC vesicles containing 3 mol% PDA was not influenced by Pep 5 and subtilin, whereas nisin had a significant, but small effect as compared to melittin (fig.1A). With DOPC/DMPS (4:1) vesicles the influence of Pep 5 and nisin became stronger while subtilin was still not very effective. Pure DMPS vesicles agglutinated heavily upon addition of one of the four peptides, which prevented exact measurements. In a series of experiments with 10–15% PDA and PS instead of DMPS we obtained similar results, but vesicle agglutination did not occur. In this case the fluidity of pure PS membranes was drastically reduced by Pep 5 and nisin (fig.1B). These results indicate that for the membrane interaction of Pep 5 and nisin the presence of negatively charged phospholipids is essential.

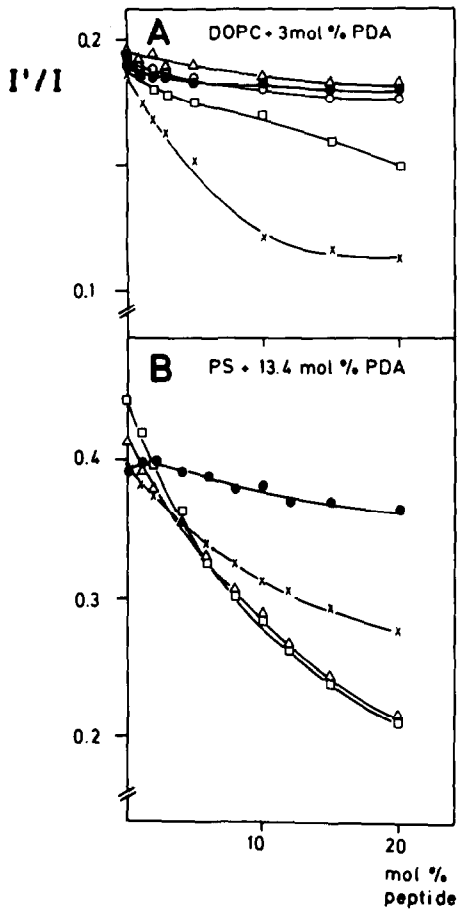

Fig.1. Dimer/monomer ratio (I'/I) of PDA in liposome membranes as a function of peptide antibiotic concentration. The peptide concentrations are given in mol% referring to the lipid content in the assay, which was 30 μM in A and 100 μM in B. Temperatures were 30°C (A) and 40°C (B). (I,I') Fluorescence intensity at 395 and 478 nm, respectively; (△) Pep 5, (□) nisin, (×) melittin, (○) subtilin, (●) untreated control.

We investigated the influence of the peptides on the phase transition temperature (T_i) of DMPC/DMPS (4:1) membranes containing 3 mol% PDA (fig.2). For control membranes a clear T_i of 23.5°C was resolved. Melittin exerted the strongest effect; no transition point was obtained even when the temperature range was extended to 5–40°C. Also for subtilin a transition point could not be clearly detected, but with Pep 5 and nisin T_i was approx. 20 and 21°C, respectively. In experiments with DMPC/PS (4:1) and 10%

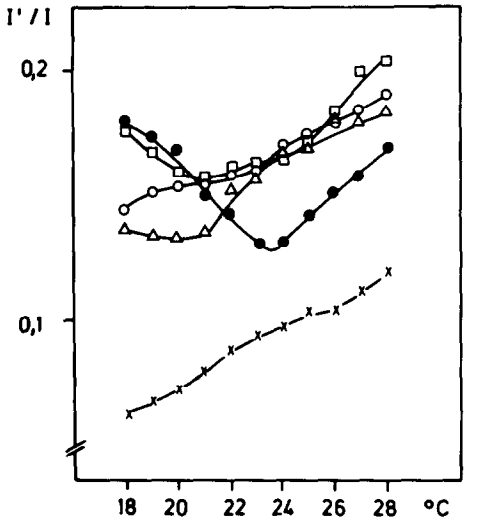

Fig.2. Phase transition curves of DMPC/DMPS (4:1) membranes containing 3 mol% PDA in the presence of cationic peptide antibiotics. Lipid concentration in assays, 100 μM; peptide concentrations, 20 μM. (I,I') Fluorescence intensity at 395 and 478 nm, respectively; (△) Pep 5, (□) nisin, (×) melittin, (○) subtilin, (●) untreated control.

PDA the results were similar, though more pronounced, perhaps due to the higher content of charges introduced by the increased amount of PDA. In this case T_i shifted upwards from 19.5°C (control) to 25°C with nisin and 28.5°C with Pep 5. Generally, the peptide antibiotics, in particular Pep 5, interacted more strongly with the rigid membrane, i.e. below ~20°C, than with the fluid membrane.

3.2. *Localization of the tryptophan residue of subtilin in the membrane*

In contrast to Pep 5 and nisin, subtilin contains a tryptophan residue. Its fluorescence spectrum in aqueous solution showed a maximum at 353 nm, which is typical for a polar environment of the tryptophan residue (e.g. [21]). Stepwise addition of DOPC vesicles at 21°C induced a blue-shift of maximally 8 nm at a peptide/lipid ratio of 1:400. The blue-shift reflects decreased polarity of the tryptophan environment, thus indicating transfer of the indole ring to the vicinity of the aliphatic chains. The affinity of subtilin for membranes was not as pronounced as with melittin, where larger shifts (up to 15 nm) at lower ratios (< 1:100) were observed [22]. Quenching of the tryptophan fluorescence with fatty acid spin labels in positions 5, 12, and 16 showed that SL-5 was the most effective quencher. However, the water-soluble tempol also decreased the tryptophan fluorescence intensity in the presence of liposomes. From these experiments we can conclude that the tryptophan residue of subtilin is located close to the water-lipid interface slightly entering the hydrophobic core of the membrane with its indole ring.

3.3. *CF efflux and fusion of liposomes*

The greatest extent of liposome fusion was obtained with subtilin at 70 μM, corresponding to a lipid/peptide molar ratio of approx. 8:1 (fig.3). Pep 5 and nisin did not promote significant fusion up to 100 μM, and melittin-induced fusion did not exceed 15% of the rate obtained with 70 μM subtilin. However, monitoring the CF efflux from vesicles indicated that melittin caused an almost complete leakage of liposomal contents at 1 μM (fig.4). Only a residual 2% of CF was released by addition of 0.1% Triton X-100. This residual amount of intact vesicles after melittin treatment may account for the 15% relative fusion in comparison to 70 μM subtilin.

Melittin was reported to fuse efficiently acidic

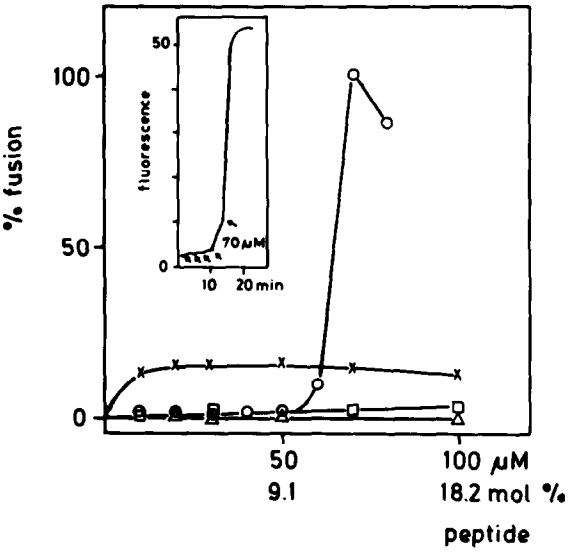

Fig.3. Relative fusion potential of Pep 5 (△), nisin (□), melittin (×), and subtilin (○) for DOPC vesicles. The degree of fusion obtained with 70 μM subtilin was set as 100%. Lipid concentration, 0.55 mM. (Inset) Time course of subtilin-induced vesicle fusion; subtilin added in 10 μM steps (↑) to a final concentration of 70 μM.

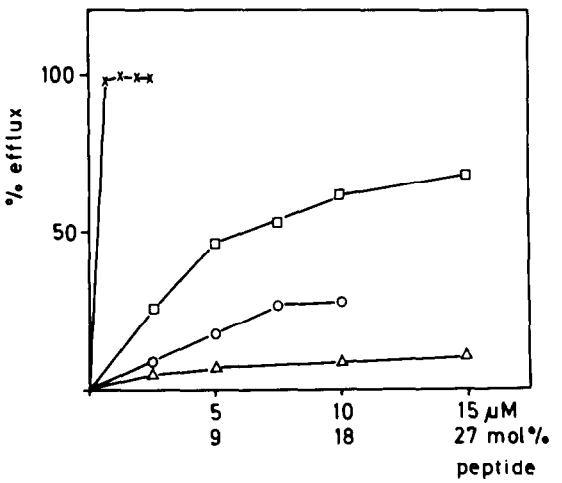

Fig.4. CF efflux from DOPC vesicles at increasing peptide antibiotic concentrations. 100% efflux was determined after lysing the vesicles with 0.1% Triton X-100. Lipid concentration, 0.55 mM. (△) Pep 5, (□) nisin, (×) melittin, (○) subtilin.

liposomes over the concentration range 1–50 μM and to cause rapidly disruption at higher concentrations [23]. Possibly, for DOPC vesicles the effective melittin concentrations are below 1 μM, a concentration range which was not of concern with the peptide antibiotics described here.

The high fusion potential of subtilin may be explained by its unique hydrophobic central domain (amino acids 4–31). Assuming that both charged termini are located at the membrane surface it seems likely that the apolar domain forms a wedge in the outer monolayer, thereby facilitating fusion.

The effect of the peptide antibiotics on the integrity of liposomes was low compared to melittin (fig.4). PS vesicles were not fused by any of the four peptides. Instead, we observed liposome aggregation which, however, did not result in membrane leakage as no CF efflux could be measured.

3.4. *Conclusions*

The results show that the affinity of the antibiotics for liposome membranes is considerably lower than that of melittin, although they equally form voltage-dependent multi-state pores in black lipid membranes. The affinity of the more basic Pep 5 and nisin is strongly dependent on the presence of negatively charged phospholipids and primarily based on electrostatic interactions, while subtilin may partially penetrate into membranes with its hydrophobic central domain. The results indicate that the peptide antibiotics do not span bilayer membranes in the absence of a membrane potential and therefore that the potential should be necessary to force the peptides into a transmembrane orientation during pore formation.

Acknowledgement: This work was supported by the Deutsche Forschungsgemeinschaft (Sa 292/5-1, 5-2).

REFERENCES

[1] Sahl, H.-G., Großgarten, M., Widger, W.R., Cramer, W.A. and Brandis, H. (1985) Antimicrob. Agents Chemother. 27, 836–840.
[2] Gross, E. and Morell, J.L. (1971) J. Am. Chem. Soc. 93, 4634–4635.
[3] Gross, E., Kiltz, H.H. and Nebelin, E. (1973) Hoppe Seyler's Z. Physiol. Chem. 354, 810–812.
[4] Hurst, A. (1981) Adv. Appl. Microbiol. 27, 85–123.
[5] Sahl, H.-G. and Brandis, H. (1983) FEMS Microbiol. Lett. 16, 75–79.
[6] Kordel, M. and Sahl, H.-G. (1986) FEMS Microbiol. Lett. 34, 139–144.
[7] Ruhr, E. and Sahl, H.-G. (1985) Antimicrob. Agents Chemother. 27, 841–845.
[8] Sahl, H.-G. (1985) J. Bacteriol. 162, 833–836.
[9] Sahl, H.-G. and Brandis, H. (1982) Zentralbl. Bakteriol. Mikrobiol. Hyg. I. Abt. Orig. A 252, 166–175.
[10] Kordel, M., Benz, R. and Sahl, H.-G. (1988) J. Bacteriol. 170, 84–88.
[11] Sahl, H.-G., Kordel, M. and Benz, R. (1987) Arch. Microbiol. 149, 120–124.
[12] Habermann, E. (1972) Science 177, 314–322.
[13] Kempf, C., Klausner, R.D., Weinstein, J.N., Van Renswoude, J., Pincus, M. and Blumenthal, R. (1982) J. Biol. Chem. 257, 2469–2476.
[14] Vogel, H., Jähnig, F., Hoffmann, V. and Stümpel, J. (1983) Biochim. Biophys. Acta 733, 201–209.
[15] Tosteson, M.T. and Tosteson, D.C. (1984) Biophys. J. 45, 112–114.
[16] Sahl, H.-G. and Brandis, H. (1981) J. Gen. Microbiol. 127, 377–384.
[17] Oku, N. and MacDonald, R.C. (1983) Biochemistry 22, 855–863.
[18] Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021.
[19] Luisetti, J., Möhwald, H. and Galla, H.J. (1979) Biochim. Biophys. Acta 552, 519–530.
[20] Galla, H.J. and Hartmann, W. (1980) Chem. Phys. Lipids 27, 199–219.
[21] Surewicz, W.K. and Epand, R.M. (1984) Biochemistry 23, 6072–6077.
[22] Dufourcq, J. and Faucon, J.-F. (1977) Biochim. Biophys. Acta 467, 1–11.
[23] Eytan, G.D. and Almary, T. (1983) FEBS Lett. 156, 29–32.