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Biochimica et Biophysica Acta 1372 (1998) 78–90

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Membrane permeabilisation and antimycoplasmic activity of the 18-residue peptaibols, trichorzins PA

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Received 19 November 1997; revised 4 March 1998; accepted 11 March 1998

Abstract

The membrane permeabilisation properties of six linear natural 18-residue peptaibols, termed trichorzins PA, have been assessed on liposomes and on mollicutes (trivial name, mycoplasmas), a class of parasitic bacteria characterized by a small genome, the lack of a cell wall, a minute cell size, and the incorporation in their plasma membrane of exogenously supplied cholesterol. The trichorzins PA used in this study (PA II, PA IV–VI, PA VIII, and PA IX) differ between them by amino acid or amino alcohol substitutions at positions 4, 7, and 18, and form slightly amphipathic α-helices. They proved bactericidal for mollicutes belonging to the genera *Acholeplasma*, *Mycoplasma*, and *Spiroplasma*, with minimal inhibitory concentrations ($3.12 \leq \text{MICs} \leq 50\ \mu\text{M}$) generally 2 to 4 fold higher than those of alamethicin F50, a related 20-residue peptide ($1.56 \leq \text{MICs} \leq 12.5\ \mu\text{M}$). Spiroplasma cells were apparently not protected by the presence of spiralin on their surface. The activities of the six trichorzins PA were not influenced by their sequence variations and no synergistic effect was observed. Consistent with the marginal effect of cholesterol on the incorporation of the trichorzins PA into liposome bilayers, the antibiotic activity was independent of the amount of cholesterol in the membranes of the different mollicutes. The trichorzins PA and alamethicin inhibited the motility of *Spiroplasma melliferum*, the helical cells being deformed and split into coccoid forms. Membrane potential measurements in *Acholeplasma laidlawii* and *S. melliferum* showed that trichorzin PA V and alamethicin F50 very efficiently depolarized the plasma membrane of mollicutes. This was consistent with fluorescence and ^{23}Na NMR measurements on liposomes that revealed the permeabilisation of the lipid bilayer and the nonselective ionophoric activity of the trichorzins PA. These data suggest that the bactericidal activity exhibited by the

Abbreviations: Alm, alamethicin; A_{600}, light absorbance at 600 nm; CD, circular dichroism; CF, 5,6-dicarboxyfluorescein; Chol, cholesterol; CFU, colony forming unit; $diSC_3(5)$, 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide; ePC, egg phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicles; MIC, minimal inhibitory concentration; MDC, minimal deforming concentration; MLC, minimal lethal concentration; OG, *n*-octyl-β-D-glucopyranoside; R_i, lipid/peptide molar ratio; R_i^{-1}, peptide/lipid molar ratio; SUV, small unilamellar vesicles; Tris, tris-(hydroxymethyl)-aminomethane; ΔI, change in fluorescence intensity; $\Delta\Psi$, membrane electrical potential ($\Delta\Psi = \Psi_{in} - \Psi_{out}$); The standard three-letter code has been used for L-amino acids; Aib, α-aminoisobutyric acid; Iva, D-isovaline; Pheol, L-phenylalaninol; Trpol, L-tryptophanol

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PII S0005-2736(98)00045-5

Keywords: Alamethicin; Amphipathic helix; Cell deformation; Growth inhibition; Ionophoric activity; Membrane potential; Mollicute; Peptaibol; Trichorzins PA

1. Introduction

The efficiency of antibacterial chemotherapy is increasingly challenged by the emergence of pathogenic strains exhibiting high levels of antibiotic resistance [1]. In this context, three main strategies may be considered for the discovery and characterization of new antibiotics: (i) the design of new antibiotics, (ii) the improvement by chemical modification of already known antibiotics, and (iii) the search for novel antibiotics synthesized by living organisms. Regarding the third strategy, peptide antibiotics are actively investigated for their properties and potential utilization as drugs against pathogens [2–4].

Mollicutes (trivial name, mycoplasmas) are wall-less bacteria characterized by a small genome and a high structural and functional simplicity [5,6]. Though negatively stained by the Gram technique, they belong to the Gram-positive branch of the eubacterial phylogenetic tree [7]. The main consequence of the limited coding capacity of their genome is that all presently known species of mollicutes are parasites of animals, notably vertebrates and arthropods, or plants [6]. Several species are pathogenic agents for man, animals, or plants, and are responsible in some cases for serious diseases. These characteristics make mollicutes good targets for antimicrobial peptides and convenient experimental cell models [8,9].

Among the antibiotics of fungal origin, peptaibols form a class of linear peptides with blocked N- and C-termini and containing 7 to 20 residues, including a high proportion of α,α-dialkylated amino acids such as α-aminoisobutyric acid (Aib) and isovaline (Iva) [10–12]. Peptaibols including the well-known alamethicin (Fig. 1) [10] form amphipathic helices which interact with membranes, increasing their permeability and forming voltage-gated channels at low concentrations (for reviews, see Refs. [13,14]). We have recently isolated and determined the sequences and solution conformations of seven such 18-residue peptides, the trichorzins PA, which differ between them by Aib → Iva substitutions at positions 4 and 7, and by Trpol → Pheol replacements at the C-terminus (Fig. 1) [15]. Both kinds of modifications result in slight variations of the global hydrophobicity, without altering the helical structure. We have also described the voltage-dependent properties of these peptides which result from the formation of transmembrane peptide aggregates [16].

In this paper, we report the membrane permeabilisation properties of the trichorzins PA investigated on different species of mollicutes and on liposomes. The

Alm F50 I	Ac	Aib	**Pro**	**Aib**	Ala	**Aib**	**Ala**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Val**	Aib	Aib	**Gln**	Gln	**Pheol**
Alm F50 II	Ac	Aib	**Pro**	**Aib**	Ala	**Aib**	**Aib**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Val**	Aib	Aib	**Gln**	Gln	**Pheol**
PA II	Ac	Aib	**Ser**	Ala	**Aib**	**Iva**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Trpol**	
PA IV	Ac	Aib	**Ser**	Ala	**Aib**	**Iva**	Gln	**Iva**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Trpol**	
PA V	Ac	Aib	**Ser**	Ala	**Iva**	**Iva**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Trpol**	
PA VI	Ac	Aib	**Ser**	Ala	**Aib**	**Iva**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Pheol**	
PA VIII	Ac	Aib	**Ser**	Ala	**Aib**	**Iva**	Gln	**Iva**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Pheol**	
PA IX	Ac	Aib	**Ser**	Ala	**Iva**	**Iva**	Gln	**Aib**	Val	Aib	Gly	Leu	Aib	Pro	**Leu**	Aib	Aib		Gln	**Pheol**	

Fig. 1. Sequences of alamethicin F50 I, F50 II and of trichorzins PA II, IV–VI, VIII, and IX (Aib, α-aminoisobutyric acid; Iva, isovaline; Trpol, tryptophanol; Pheol, phenylalaninol). Bold letters indicate amino acid sequence differences.

properties of the trichorzins PA were compared with those of alamethicin which is the most widely studied member within this class of antibiotics and is thus considered the archetype of the peptaibol family [10]. Studies with mollicutes were focused on the growth inhibition activity of the peptides and their effects on motility, cell morphology, and membrane potential. Liposomes were used to investigate the interactions of the trichorzins PA with lipid bilayers and to analyse membrane permeabilisation to ions by these antibiotics.

2. Materials and methods

2.1. Chemicals and peptides

Egg phosphatidylcholine (ePC, type V–E), cholesterol (Chol), *n*-octyl-β-D-glucopyranoside (OG), and alamethicin F50 (Alm) from *Trichoderma viride* were purchased from Sigma (St. Louis, MO). ePC and OG were used without further purification, whereas cholesterol was recrystallized from methanol as previously described [17]. Carboxyfluorescein (CF) from Eastman Kodak was recrystallized from ethanol after removal of hydrophobic contaminants, as previously described [17]. The fluorescent dye 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide ($diSC_3(5)$) was obtained from Molecular Probes (Eugene, OR). The natural alamethicin F50 mixture was analysed by reversed-phase HPLC (Nucleosil C18/5 μm (AIT, France); 4.6 × 250 mm; CH_3OH/H_2O 85:15; flow rate 1 ml min^{-1}; UV 220 nm); the chromatogram showed four peaks, with the neutral alamethicins F50 I (Rt 23 min) and F50 II (Rt 29 min) as major components (55% and 34%, respectively). Six trichorzins PA (PA II and PA IV–VI, VIII, and IX) were purified from the natural mixture excreted by *Trichoderma harzianum* (strain M-902608, Muséum National d'Histoire Naturelle, Paris) by semi-preparative reversed-phase HPLC (Kromasil C18/5 μm (AIT, France); 7.5 × 300 mm; $MeOH/H_2O$ 84:16; flow rate 2 ml min^{-1}; UV 220 nm). The retention times for PA II, PA IV, PA V, PA VI, PA VIII, and PA IX were 74, 89, 95, 106, 127, and 136 min, respectively. The trichorzins PA were shown to be at least 98% pure by analytical HPLC in the same system. HPLC analyses were carried out with a Waters liquid chromatograph (6000A and M45 pumps, 680 automated solvent programmer, WISP 701 automatic injector, 481 UV–visible detector).

2.2. Bacterial strains

Eight strains of mollicutes: *Acholeplasma laidlawii* A-PG8, *Mycoplasma gallisepticum* S6, *M. genitalium* G37, *M. mycoides* ssp. *mycoides* SC KH3J, *Spiroplasma apis* B31, *S. citri* R8A2, *S. floricola* BNR1, and *S. melliferum* BC3 were used in this study and grown as previously described [8,18].

2.3. Circular dichroism (CD) spectroscopy

The CD spectra of trichorzins PA V and PA IX (0.12 mM in methanol, 0.1-mm path length cell, $T \approx 23°C$) were recorded on a Mark V Jobin Yvon (France) dichrograph. PAV, λ ([θ]M in deg cm^2 $dmol^{-1}$): 193 (608,000), 208 (−208,500), 223 (−171,500); PA IX, λ ([θ]M in deg cm^2 $dmol^{-1}$): 193 (910,200), 208 (−332,600), 223 (−265,600).

2.4. Preparation of small unilamellar vesicles (SUV), carboxyfluorescein (CF)-entrapped SUV and large unilamellar vesicles (LUV)

SUV were prepared by sonication to clarity of 0.5 mM ePC (or ePC/Chol 7:3 mol/mol) in 1 mM cacodylate buffer (pH 7.2, 1 mM NaCl, 0.2 mM EDTA) at 0°C under nitrogen (45 min, power 30 W, Model W-225 R Ultrasonics sonicator equipped with a microtip probe). CF-entrapped SUV were prepared by sonication of a 8.5 mM ePC/Chol (7:3, mol/mol), 56 mM CF solution in 5 mM Hepes buffer (pH 7.42, 1 mM NaCl), at 0°C under nitrogen (20 min, power 30 W). Unencapsulated CF was removed by chromatography through a 1 × 25-cm Sephadex G75 column (Pharmacia Biotechnology, Uppsala, Sweden) eluted with Hepes buffer, and the dilution factor was determined.

LUV were prepared by dialysis removal of detergent [19]. Typically, 69 μmol of ePC and 1 mmol of OG were dissolved in 3 ml of 100 mM NaCl. The solution was dialyzed for 60 h, against 5 × 2 l of 100 mM NaCl at 40°C for ^{23}Na experiments and at 4°C for ^{35}Cl experiments. The resulting LUV (average diameter, 600 nm for ^{23}Na and 400 nm for ^{35}Cl experiments) had 100 mM NaCl internally and externally.

2.5. ^{23}Na and ^{35}Cl NMR measurements

NMR spectra were acquired at 132.29 MHz (^{23}Na) or 49.00 MHz (^{35}Cl) at 293 K, either on a Bruker MSL 500 or a Varian Unity 500 spectrometer equipped with a 10-mm probe; ^{23}Na NMR: spectral width 3000 Hz, 1 K data points, 2000 free induction decays from 90° pulses, and 0.2 s relaxation delay; ^{35}Cl NMR: spectral width 10,000 Hz, 8 K data points, 20,000 free induction decays from 90° pulses, and 0.2 s relaxation delay. Typically, for ^{23}Na measurements, 6 μl of 1 M dyspronium chloride ($DyCl_3$) were added to 1 ml of LUV suspension and 1 ml of a 100 mM choline chloride in 20 mM $Na_5P_3O_{10}$ solution [20], to induce a chemical shift difference of 9.8 ppm between the in and out ^{23}Na signals; for ^{35}Cl measurements, 2 μl of 1 M manganese bromide ($MnBr_2$) were added to 2 ml of LUV suspension to induce a 200 times line broadening of the out ^{35}Cl signal [21]; 10 to 60 μl of a 0.23 mM methanolic PA VI solution were then added to the liposome suspensions in both kinds of experiments. The field-frequency ratio was locked on the ^2H resonance of D_2O contained in the inner compartment of a 4-mm insert. For each peptide concentration, 15 to 30 spectra were recorded over a period of 1 h and the areas of the peaks due to internal ^{23}Na or ^{35}Cl were measured and expressed as the percentage of the total ^{23}Na (or ^{35}Cl) concentration, with $[Na^+_{int}] = (\text{Area } Na^+_{int}/\text{Area } Na^+_{int} + \text{Area } Na^+_{ext}) \times 100$.

2.6. Fluorescence measurements on liposomes

Fluorescence data on SUV were obtained at 20°C on an Aminco SPF 500 spectrofluorometer. The peptides were added as methanolic solutions and the methanol concentration in the final solutions was kept below 0.5% by volume. For tryptophan fluorescence measurements, 5 μl of a 0.25 mM methanolic trichorzin PA V solution was added to the freshly prepared SUV suspension diluted in cacodylate buffer in order to give lipid/peptide molar ratios (R_i) in the range 0–200. The spectra were recorded after 15 min incubation and corrected for the vesicular emission. The excitation wavelength was set at 280 nm (4-nm band pass) and emission spectra were recorded between 200 and 500 nm (5-nm band pass) [22].

For permeabilisation measurements, excitation and emission wavelengths were set at 488 and 520 nm (1-nm band pass), respectively. Aliquots of methanolic peptide solutions were added to a mixture of 0.2 ml of vesicular suspension and 1.2 ml of Hepes buffer. The peptide concentrations were calculated to give peptide/lipid molar ratios (R_i^{-1}) ranging between 0 and 3×10^{-3}. The kinetics were monitored after rapid stirring and stopped at 20 min; 50 μl of 10% (vol/vol) Triton X-100 were then added to determine the total fluorescence (I_T) by disrupting the SUV. Percentages of released CF at 20 min were determined as $(I_{20} - I_0)/(I_T - I_0) \times 100$, with I_0 and I_{20} the fluorescence intensities in the absence and in the presence of peptide, respectively.

2.7. Antimicrobial assays

The mollicutes were cultured under microaerobic conditions in appropriate liquid media as described previously [8,18]. Minimal inhibitory concentrations (MICs) were determined by culturing the cells in 96-well microtitration plates, in the presence of 2-fold serial dilutions of the peptides. Organisms were considered resistant when their growth was not prevented by peptide concentrations up to 100 μM. The starting cell concentration in each well was 10^6 colony-forming units (CFU) ml^{-1}. To distinguish bacteriostatic effects from bactericidal ones, cells were incubated for 2 h in liquid medium containing or not peptides, and plated on solid culture medium without peptide. The plates were subsequently incubated and examined daily for the apparition of colonies.

2.8. Measurement of membrane potential

Exponentially growing *A. laidlawii* cells were stored at 0°C for 30 min. They were subsequently harvested, washed once and dispersed in 5 mM Hepes buffer (pH 7.0, 150 mM NaCl, 50 mM D-glucose), at 0°C, to obtain a cell density of 10^9 CFU ml^{-1} ($A_{600} = 0.06$, 20 μg of cell protein ml^{-1}). Similarly, *S. melliferum* cells were washed and dispersed in 5 mM Hepes buffer (pH 7.0, 128 mM NaCl, 50 mM D-glucose) to obtain a cell density of 10^9 CFU ml^{-1} ($A_{600} = 0.10$, 30 μg of cell protein ml^{-1}). In both cases, 3 μl of 0.15 mM $diSC_3(5)$ solution in methanol was added to 3 ml of cell suspension and the fluores-

cence signal (ΔI) was measured at 25°C. Excitation and emission wavelengths were set at 625 and 660 nm, respectively. When steady-state fluorescence levels were obtained, 3 μl of peptide solutions in methanol were added to the cell suspensions at final concentrations ranging from 0 to 15 μM. The calibration of dye fluorescence change ($\%\Delta I$) vs. change of membrane electrical potential ($\Delta\Psi$) was performed using valinomycin-induced K^+ diffusion potentials [23]. Intracellular volumes were determined as described previously [24]. A full description of these procedures is given in a recent publication [9].

2.9. Light microscopy

Exponentially growing spiroplasmas were sedimented by centrifugation at $10{,}000 \times g$ for 10 min at 4°C and dispersed in 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM D-glucose, 549 mM D-sorbitol. The suspension of helical and motile cells (10^{10} CFU ml^{-1}, $A_{600} = 1.0$) was subjected without delay to the effect of peptides and 10-μl samples were deposited on glass slides ($1 \times 25 \times 75$ mm) and covered with glass coverslips of $0.15 \times 24 \times 36$ mm. The cells were immediately observed with a Leitz Diaplan microscope equipped with a 100-W halogen lamp, Leitz oil immersion dark-field condenser (NA 1.2 to 1.4), Leitz PL Fluotar 100 × objective (NA 0.6 to 1.32), and Periplan GF 12.5 × /18 M eyepieces. Micrographs were taken with a Nikon F-801 camera using Kodak T-Max 35 mm film (ISO 3200).

2.10. Protein and cholesterol determination

Protein concentration was determined in cell suspensions by the method of Lowry et al. [25] modified by Markwell et al. [26], using bovine serum albumin as standard. Total cholesterol was colorimetrically determined in the chloroform/methanol membrane lipid fraction using the Sigma (Sigma, St. Louis, MO) cholesterol reagent kit. The percentages of cholesterol in the whole membrane lipid fractions of the different strains were: 2.1 (*A. laidlawii* A-PG8), 7.2 (*M. gallisepticum* S6), 3.5 (*M. mycoides* ssp. *mycoides* SC KH3J), 21.2 (*S. apis* B31), 25.2 (*S. citri* R8A2), 16.8 (*S. floricola* BNR1), and 22.2 (*S. melliferum* BC3).

3. Results

3.1. Effect of the trichorzins PA on the growth of mollicutes

The natural trichorzins PA mixture, the six purified trichorzins PA (PA II, IV–VI, VIII, and IX) and the natural alamethicin F50 mixture containing mainly two homologous peptides (Fig. 1) inhibited the growth of the eight strains of mollicutes, with MICs ranging

Table 1
Antibiotic activity of the trichorzins PA on eight species of mollicutes, as compared with alamethicin F50, melittin and magainin 2

Peptide	Al	Mgl	Mgn	Mm	Sa	Sc	Sf	Sm
PA	3.12	12.5	12.5	25	12.5	6.25	12.5	12.5
PA II	6.25	25	12.5	25	25	12.5	25	25
PA IV	6.25	25	12.5	25	25	12.5	50	25
PA V	3.12	25	12.5	25	12.5	6.25	25	25
PA VI	3.12	6.25	ND	12.5	12.5	12.5	25	12.5
PA VIII	3.12	25	6.25	25	12.5	6.25	12.5	12.5
PA IX	6.25	25	6.25	50	25	12.5	25	25
Alm F50	1.56	6.25	6.25	12.5	6.25	3.12	6.25	6.25
Melittin	0.78	6.25	6.25	12.5	0.20	0.78	3.12	1.56
Magainin 2	*R*	*R*	*R*	*R*	100	100	100	100

The data listed in the table are the minimal concentrations (μM) required for the total inhibition of mollicutes growth. Determination of surviving bacteria on agar plates (see Section 2) revealed that all of the peptides were bactericidal at concentrations $\geq$ MIC, except magainin 2 which was bacteriostatic. Abbreviations: PA, the natural trichozins PA mixture; Al, *A. laidlawii*; Mgl, *M. gallisepticum*; Mgn, *M. genitalium*; Mm, *M. mycoides* ssp. *mycoides* SC; Sa, *S. apis*; Sc, *S. citri*; Sf, *S. floricola*; Sm, *S. melliferum*; Alm, alamethicin; *R*, resistance to concentrations up to 100 μM; ND, not determined.

from 1.56 to 50 μM, alamethicin being slightly more potent than the trichorzins PA (Table 1). In the same conditions, the bee-venom melittin was about three to five times more active than the trichorzins PA, whilst the amphibian defence peptide, magainin 2 was quite ineffective, a 100 μM concentration only inhibiting the spiroplasma growth. Furthermore, all the peptaibols and melittin were bactericidal for the eight mollicutes, whereas magainin 2 was bacteriostatic. Indeed none of the strains was capable of resuming growth on agar plates after a 2-h treatment with mellitin, alamethicin F50 or the trichorzins PA in liquid culture medium. The minimal lethal concentrations (MLCs) proved to be identical to the corresponding MICs.

Among the mollicutes used in this study, *A. laidlawii* was the most sensitive to alamethicin F50, the trichorzin PA natural mixture, and the purified trichorzins PA, whereas *M. mycoides* ssp. *mycoides* SC was the most resistant. Finally, the absence of synergism between the different trichorzins PA was observed, as the MIC values of the natural PA mixture were in the same range as those observed for the pure trichorzins PA for the eight species of mollicutes.

3.2. Effect of alamethicin F50 and trichorzin PA V on spiroplasma motility and cell morphology

Consistent with previous observations [8], exponentially growing *S. melliferum* BC3 cells exhibited an average helix length of 6.5 ± 1.6 μm, with a pitch of about 1 turn μm^{-1}. Treatment with trichorzin PA V, alamethicin F50, melittin or magainin 2 resulted in a loss of motility followed by cell shape deformation (Fig. 2). The phenomenon occurred continuously through the five successive steps previously described for surfactin-treated cells [8]: (i) loss of helix flexibility and twitching concomitant with loss of motility, (ii) helix stretching, (iii) loss of helicity and formation of aneurisms, (iv) swelling of the aneurisms, and (v) splitting of the cell into several rounded vesicles. Table 2 shows that the minimal concentrations necessary for deforming at least 95% of the cells in less than 1 h (MDC_{100}) were lower than the corresponding MICs. However, the relative efficiencies were similar, i.e., melittin > alamethicin F50 > trichorzin PA V $\gg$ magainin 2.

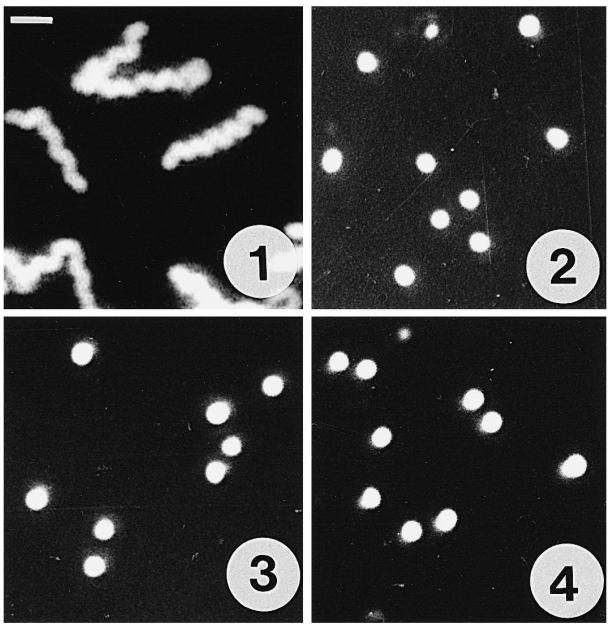


Fig. 2. Dark-field light microscopy observation of spiroplasma cell deformation. Untreated *S. melliferum* BC3 cells (1) are compared with cells treated with 0.1 μM melittin (2), 1 μM alamethicin F50 (3), or 1 μM trichorzin PA V (4). See text for details. Bar (top left corner in 1), 2 μm.

A time-course analysis of the phenomenon (Fig. 3) revealed that even in a very dense cell suspension (10^{10} CFU ml^{-1}), the cells underwent the different deformation steps almost synchronously, with slight cross-overs between the different steps. When *S. melliferum* BC3 was treated either with 1 μM trichorzin PA V or alamethicin, the percentage of intact cells dropped from 100 to below 10 within 1 min,

Table 2
Spiroplasma cell deforming activity of the peptides

Peptide	MDC_{100} (μM)	Delay (min)
Melittin	0.05	20
Magainin 2	50	20
Alamethicin F50	0.1	40
Trichorzin PA V	0.25	40

MDC_{100} is the minimal peptide concentration capable of deforming 95 to 100% of the cells in suspensions containing 10^{10} CFU/ml of 50 mM sodium phosphate buffer (pH 7.0, 50 mM D-glucose, and 549 mM D-sorbitol), in less than 1 h. In all cases, the cells were deformed and finally split into rounded vesicles (see Fig. 2).

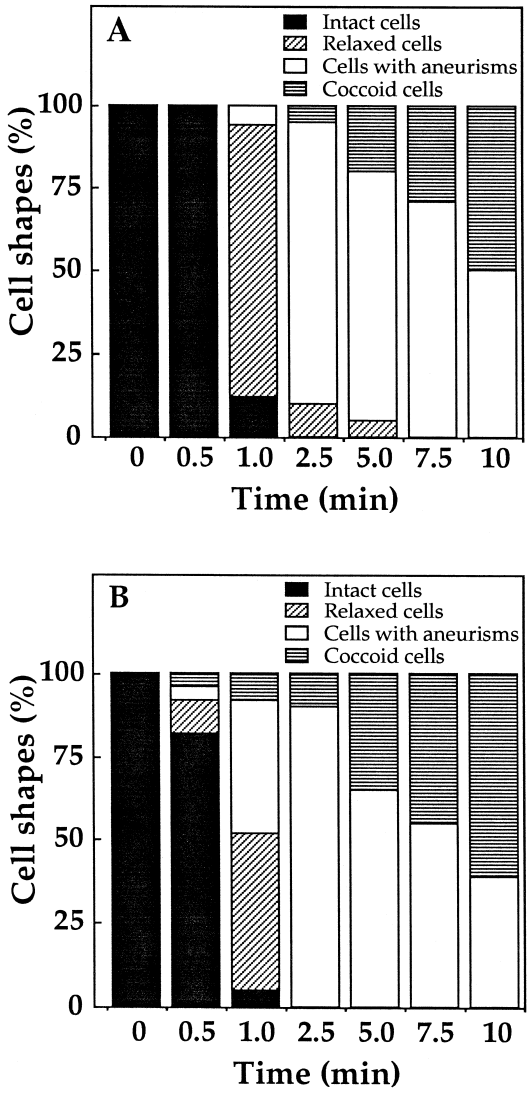


Fig. 3. Time-course of *S. melliferum* cell deformation by trichorzin PA V (A) and alamethicin F50 (B). The cells were treated with 1 μM alamethicin F50 or trichorzin PA V as described under Section 2 and observed for 10 min by dark-field light microscopy. Cells were considered intact when they were still helical but not necessarily motile. The data were obtained from three distinct determinations (SD $\leq \pm 5\%$). Each bar of the histogram is the sum (100%) of the relative percentages of the different cell shapes observed at a given time.

whilst that of relaxed filamentous cells increased concomitantly. Aneurisms started to form before all the cells lost their helical shape and, after 10 min, half of the trichorzin PA V-treated cells were in the form of round vesicles and half were in the form of relaxed filaments containing aneurisms (Fig. 3A). For the alamethicin-treated cells, the relative proportions of these two forms were 60% and 40%, respectively (Fig. 3B).

Since the MDCs and the peptide concentrations required to collapse membrane potential were lower than the corresponding MICs, controls were performed in order to explain these differences. These controls revealed that 5% horse serum (as in the culture media) decreased about 10 fold the apparent activity of the peptides.

3.3. Effect of alamethicin F50 and trichorzin PA V on membrane potential

This effect was examined by using the potential-sensitive carbocyanine dye di$SC_3(5)$. The relation-

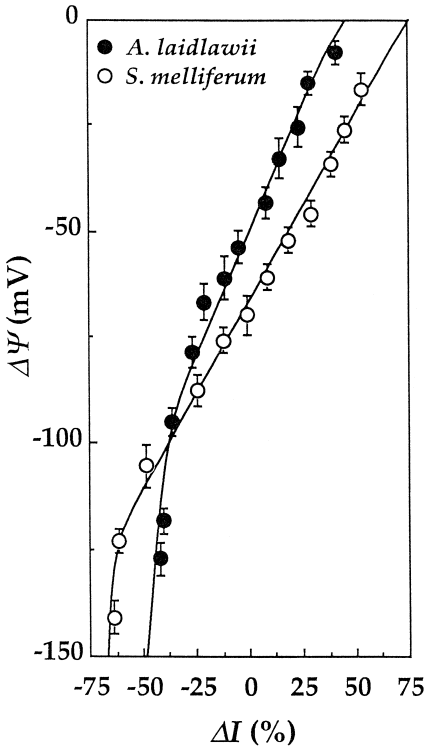
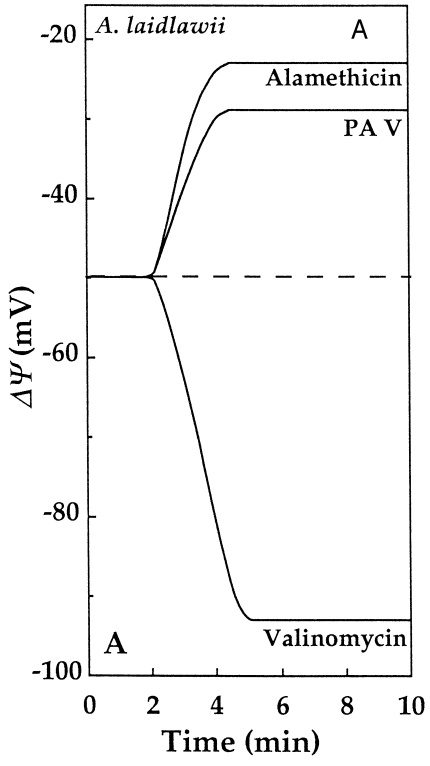
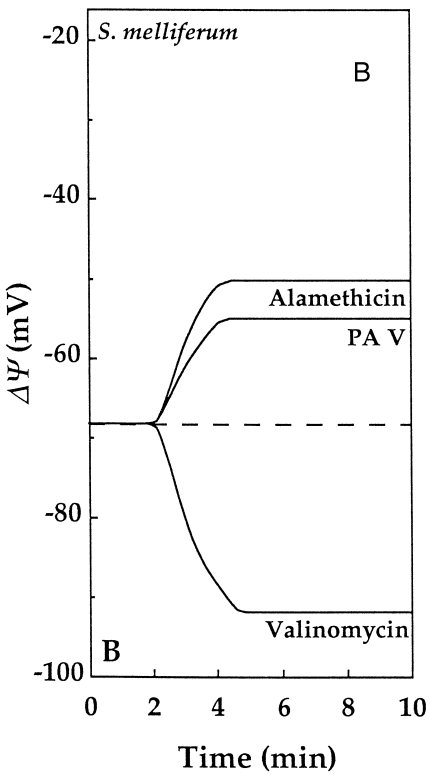


Fig. 4. $\Delta\Psi$ (mV) vs. ΔI (%) calibration curves for *A. laidlawii* (●) and *S. melliferum* (○). The cells were dispersed in 5 mM Hepes buffer (pH 7.0) containing 150 mM (NaCl + KCl) (*A. laidlawii*) or 128 mM (NaCl + KCl) (*S. melliferum*). In both cases the cell density was of 10^9 CFU/ml. The external KCl concentration was varied from 0 to 150 or 128 mM, respectively, and the membrane potential was calculated as described under Section 2 and Ref. [9].





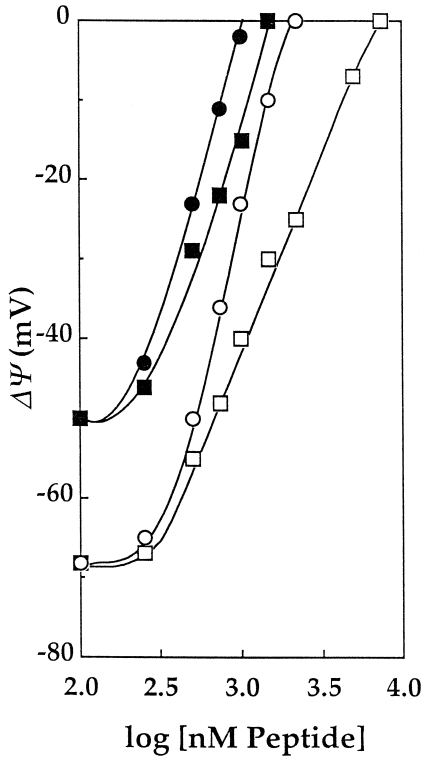


Fig. 6. Effect of peptaibol concentration on membrane potential ($\Delta\Psi$). Same conditions as indicated in Fig. 4 except that fluorescence was recorded 3 min after peptide addition to *A. laidlawii* or *S. melliferum* cell suspensions. The points on the curve are the average of at least three distinct determinations (SD $\leq \pm 8\%$). Symbols: ● and ■, *A. laidlawii*; ○ and □, *S. melliferum*; ● and ○, alamethicin F50; ■ and □, trichorzin PA V.

ship between the fluorescence change of $diSC_3(5)$ ($\Delta I\%$) and the membrane potential ($\Delta\Psi$) proved to be linear between -10 and -100 mV for *A. laidlawii* and between -20 and -125 mV for *S. melliferum*, corresponding to a relative fluorescence

Fig. 5. Effect of peptides on the membrane potential ($\Delta\Psi$) of *A. laidlawii* (A) and *S. melliferum* (B) cells. The arrow indicates the time at which the peptides were added to the cell suspensions (0.5 μM alamethicin, 0.5 μM trichorzin PA V, or 1 μM valinomycin). Each curve is the average of at least three independent measurements (SD $\leq \pm 8\%$). Cell deformation but not cell lysis occurred in the conditions of this experiment. K^+ leakage in the absence of peptide was prevented by storing the cells in the buffer at 0°C for 40 min before potential determination.

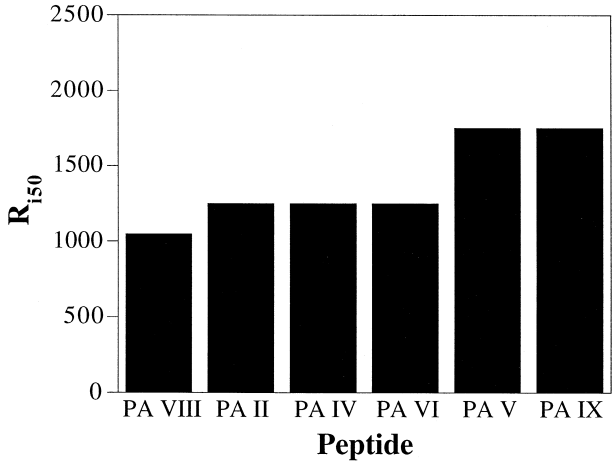


Fig. 7. Lipid/peptide molar ratios (R_{i50}) allowing 50% leakage of the entrapped CF out of PC-cholesterol SUV (PC/Chol molar ratio, 7:3; total lipid concentration 0.6 mM).

change in the range −35% to +40% and −60% to +50%, respectively (Fig. 4).

When the cells were dispersed in potassium-free buffer containing 50 mM glucose (see experimental section), *A. laidlawii* and *S. melliferum* displayed constant membrane potentials of -50 ± 4 mV and -68 ± 5 mV, respectively. As previously observed by others [27], storing the cells at 0°C for 30 to 40 min before measurement prevented the leakage of K^+ ions. Treatment of the cells with 1 μM valinomycin (Fig. 5) resulted, as expected, in a hyperpolarisation of the membranes of *A. laidlawii* ($\Delta\Psi = -93 \pm 10$ mV) and *S. melliferum* ($\Delta\Psi = -92 \pm 8$ mV) resulting from the increased membrane permeability to K^+, relative to the permeability of other ions. In contrast, 0.5 μM alamethicin or trichorzin PA V depolarized the membranes, reducing the membrane potential in *A. laidlawii* to -23 ± 3 mV and -29 ± 3 mV, respectively (Fig. 5A). Similarly, the potential was reduced to -50 ± 5 mV and -55 ± 5 mV in *S. melliferum* by alamethicin F50 and trichorzin PA V, respectively (Fig. 5B). The dose-dependence of the observed effects is illustrated in Fig. 6. In comparison, a 30 μM concentration of magainin 2 was required to obtain similar effects, whereas melittin proved to be much more potent, since a 0.1 μM concentration completely abolished the membrane potential of *A. laidlawii* and decreased that of *S. melliferum* down to -37 ± 3 mV.

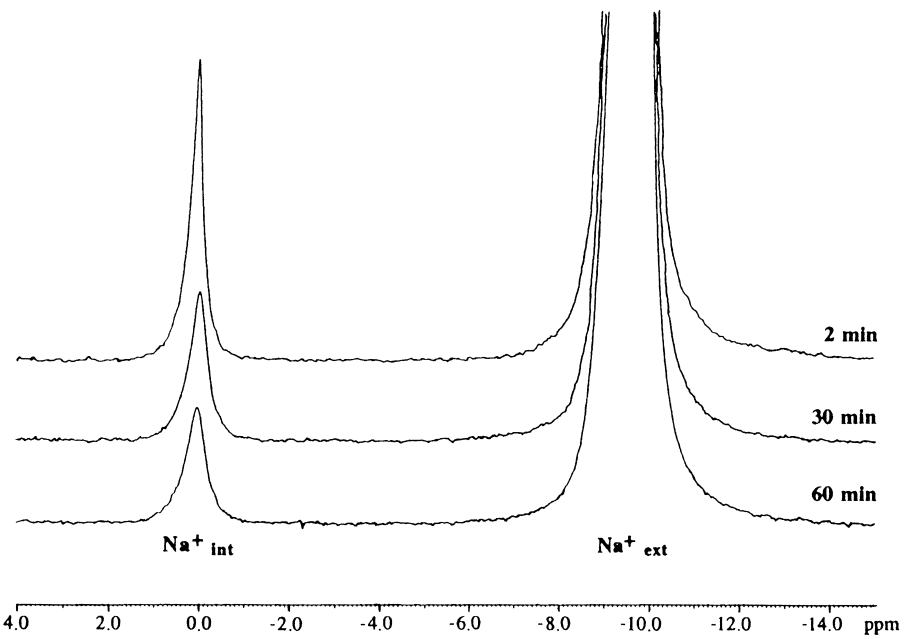


Fig. 8. Changes in ^{23}Na NMR spectra (132.29 MHz, 303 K) of LUV containing 100 mM NaCl, upon the addition of 2.3 μM trichorzin PA VI and in the presence of 3 mM $DyCl_3$ added to the suspension to create a shift difference of approximately 9.8 ppm; the [Na^+_{int}] was determined as indicated in Section 2.

3.4. Interaction of trichorzin PA V with small unilamellar vesicles (SUV)

The intrinsic C-terminal tryptophanol fluorophore of trichorzin PA V was used to probe the trichorzin/lipid bilayer interaction by fluorescence spectroscopy [16]. In aqueous solution, the fluorescence maximum of the PA V Trpol residue was at 355 nm, a wavelength characteristic of a polar environment. When adding ePC SUV till a lipid/peptide molar ratio (R_i) of 150 was reached, a 25-nm shift of the fluorescence maximum to shorter wavelengths was observed, accompanied by an enhancement of the relative fluorescence intensity. The observed modifications were characteristic of an increase in the hydrophobicity of the Trpol microenvironment, indicating that trichorzin PA V was incorporated into the lipid bilayer. The presence of 30% cholesterol in the SUV did not affect much the fluorescence changes, as it only reduced the wavelength shift to 20 nm, indicating a slight effect of cholesterol on the trichorzin PA/lipid interaction.

3.5. Permeabilisation measurements

Lipid bilayer permeabilisation and perturbation by the trichorzins PA were monitored by following the leakage of carboxyfluorescein previously entrapped in ePC/Chol (70/30) SUV at a self-quenched concentration [28]. Leakage kinetics were recorded for the six trichorzins PA and for different peptide/lipid ratios (R_i^{-1}). The R_{i50} lipid/peptide molar ratio allowing 50% leakage of the entrapped probe after 20 min, was taken as a measure of the peptide efficiency. The R_{i50} values ranged from 1000 to 2000 for the six trichorzins PA (Fig. 7) and were consistent with those previously found for related long-sequence peptaibols [12,17,29,30], indicating a high permeabilizing activity.

3.6. Ionophoric activity of trichorzin PA VI by ^{23}Na and ^{35}Cl NMR

The ionophoric activity and ion selectivity of the trichorzins PA were examined by NMR spectroscopy, using contrast reagents [21,31]. The exchange of Na^+ and Cl^- ions across the bilayer of LUV prepared with similar concentrations of NaCl inside and outside the vesicles, was followed by ^{23}Na- and ^{35}Cl NMR, respectively. The $^{23}Na^+$ ions were observed after adding dysprosium chloride ($DyCl_3$) to the suspension, which induces an upfield shift of the extravesicular Na^+_{ext} ions [20]. For ^{35}Cl observations, manganese bromide ($MnBr_2$) was used as relaxation reagent [21], at such a concentration that the $^{35}Cl^-_{ext}$ signal linewidth was 200 times larger than the $^{35}Cl^-_{int}$. Addition of aliquots of trichorzin PA VI to the vesicular suspensions resulted in a decrease in the intravesicular $^{23}Na^-_{int}$ (Fig. 8) and $^{35}Cl^-_{int}$ signals, indicating a marked ionophoric activity of trichorzin PA VI, without selectivity for anions or cations.

4. Discussion

The six 18-residue trichorzins PA (Fig. 1) proved bactericidal for the eight species of mollicutes used in this study (Table 1). The MICs were independent of the slight sequence variations, indicating that the Aib for Iva substitutions at positions 4 and 7, and the C-terminal Trpol for Pheol substitutions are without noticeable effect on the antibiotic activity. The permeabilisation of liposomes by the trichorzins PA was slightly dependent on the replacements of residues at specific sites by more hydrophobic ones, such as Aib for Iva. The strong dependence of liposome bilayer perturbations on peptide global hydrophobicity and chain length, the longer, 19/20-residue peptaibols being more potent [16,29,30], is consistent with the lower MICs noticed for alamethicin. The presence of two additional residues in the alamethicin sequence (i.e., a proline towards the N-terminus and a glutamine towards the C-terminus) might thus be responsible for the higher activity of the longer peptaibol. Finally, it should be noted that there was no synergism between the trichorzins PA, as the growth inhibition activity of the natural mixture of these peptides was similar to that of the purified molecules.

It has often been speculated that the differential sensitivity of procaryotes and eucaryotes towards some membranotropic peptides might be due to the presence of sterols in the plasma membranes of eucaryotes in contrast to the membranes of bacteria. However, although being procaryotes, mollicutes incorporate in their membranes substantial amounts cholesterol. This is notably the case of mycoplasmas

and spiroplasmas which need exogenously supplied cholesterol (or analogous sterols) for their growth [5,6]. Acholesplasmas are not cholesterol-dependent for their growth, but they also incorporate this lipid into their membranes when it is present in the culture medium [6]. Interestingly, *Acholeplasma* spp. are much more sensitive to the bacteriocin nisin than *Mycoplasma* spp., apparently owing to higher amounts of cholesterol in the membranes of the latter [32]. In the case of peptaibols, we have not observed significant differences in the sensitivities to alamethicin or trichorzins PA of mollicute spp. exhibiting cholesterol concentrations in the membrane total lipid fraction from 2.1% for *A. laidlawii* to 25.2% for *S. citri*. Although the compositions of mollicute membranes differ also for lipids other than sterols, the differences in membrane cholesterol contents are large enough to suggest that the susceptibility of these bacteria to peptaibols is not modulated by cholesterol. This is consistent with the fact that the incorporation of trichorzin PA V into the lipid bilayer of SUVs was not significantly modified by the presence or the absence of cholesterol in the bilayer. Furthermore, liposome permeabilisation induced by related 19-residue neutral peptaibols is independent of cholesterol concentrations ranging from 0 to 30% of the total lipid [17].

Under favourable physiological conditions, spiroplasmas possess a helical cell shape and exhibit a characteristic motility based upon screw-like, flexing and twitching movements [33]. Having recently shown that membranotoxic peptides inhibit motility and deform spiroplasma cells [8,9], we have compared the effects of trichorzin PA V and alamethicin F50 on *S. melliferum*. The results (Figs. 2 and 3, and Table 2) confirm growth inhibition data, since both peptaibols inhibit cell motility and efficiently deform the shape of the cells. They also indicate that the high concentration of the surface-anchored lipoprotein spiralin in *S. citri* and *S. melliferum* [34–37] cells does not protect the cells against peptaibols. Furthermore, these experiments show that spiroplasma deformation and motility inhibition are simple, sensitive, fast, and reliable tests to assess the toxicity of membranotropic peptides.

Previous CD and NMR data revealed that the linear hydrophobic trichorzins PA adopt an α-helical conformation [15]. This structure which has also been observed for alamethicin [38] and 19/20-residue analogues [11,29], includes a bend in the helix axis, involving the central proline residue located in the 10–14 region of the sequence. Such a bent helix is responsible for the ability of peptaibols to interact with lipid bilayers and to disturb their properties by forming either pores of poorly defined structure or channels, depending on the experimental conditions and peptide concentration. The primary target of the activity of these α-helical hydrophobic peptides should thus be the cell membrane. Hence, in order to elucidate the mechanism by which peptaibols and particularly the trichorzins PA are toxic for mollicutes, we have analysed and compared the effects of alamethicin and the trichorzin PA V on the transmembrane electrical potential ($\Delta\Psi$) in *A. laidlawii* and *S. melliferum*, chosen as representative targets. Alamethicin F50 and trichorzin PA V decreased the membrane potentials of both mollicutes and, as expected from the results of growth inhibition tests and spiroplasma cell deformation experiments, alamethicin proved slightly more efficient than trichorzin PA V (Figs. 5 and 6).

Since the depolarization of the plasma membrane of mollicutes strongly suggests perturbations of the transmembrane ionic balance, liposomes have been used to further analyse the membranotropic behaviour of the trichorzins PA and their ionophoric properties. Firstly, probing the interaction of the trichorzins PA with SUVs by fluorescence spectroscopy has demonstrated their insertion within the lipid bilayer, which is a prerequisite for the permeabilisation of the membrane. Secondly, we have shown membrane permeabilisation by trichorzins PA in carboxyfluorescein leakage and Na^+ exchange experiments (Figs. 7 and 8). The latter data show that trichorzin PA VI is able to permeabilize LUVs to Na^+ and Cl^- ions, indicating the absence of selectivity for anions or cations. This is consistent with the effect of trichorzin PA V on the membrane potential of mollicutes, since such nonspecific ionophores are expected to enhance the leakage of protons and other ions through the plasma membrane. For each peptide, the same concentration range (0.2×10^{-6}–10^{-6} M) was effective on both liposomes (LUV and SUV) and mollicutes.

We have recently shown that the trichorzins PA are also able to induce voltage-dependent channels in

planar bilayers at concentrations ranging from 3×10^{-9} and 3×10^{-8} M and that the conducting aggregates are better stabilized by the C-terminal Trpol [16]. In contrast, such a role of the trichorzins PA C-terminal amino alcohol has not been observed here, neither for the ionophoric activity on liposomes nor for the antimycoplasmic activity.

Mollicutes are much more sensitive to amphipathic peptides of microbial origin (bacterial or fungal) than to animal defence peptides [8], which is supported by the potent antibiotic activity of the fungal peptaibols on these bacteria. Actually, as obligate parasites of animals or plants, mollicutes have presumably evolved mechanisms to evade peptide defence systems of their hosts and they have in principle not undergone the selective pressure of peptaibols during their evolution, since these antibiotics are synthesized by unicellular fungi which cannot be contaminated by mollicutes. The sensitivity of mollicutes to melittin could be explained in the same way, since this toxic component of bee venom is not used as a means of protection against microbes, but against animals.

In this work, we have also observed that a serum component, possibly lipoproteins, has a protective effect against the peptides. This explains why the MICs determined by growth inhibition tests in serum-containing culture media were higher than the spiroplasma minimal deforming concentrations (MDCs) determined in serum-free buffer. We have also verified that the minimal lethal concentrations (MLCs) determined in the absence of serum where 10-fold lower than those obtained in the presence of serum. Further investigation will be necessary to know which specific serum component is actually responsible for this effect.

In conclusion, this work shows that the primary target of the toxic activity exhibited by the trichorzins PA is the cell membrane and that these peptides are able to kill mollicutes owing to their strong, non-specific ionophoric activity. The antibacterial activity seems neither hampered nor favoured by the presence of cholesterol in the plasma membrane of mollicutes, whereas the lipoprotein spiralin does not seem to protect spiroplasma cells against these peptides. Experiments are now in progress to specify the nature of the membrane defects induced by the trichorzins PA and responsible for the death of mollicutes.

Acknowledgements

This work was supported by the 'Fondation Langlois' (Rennes), the 'Groupement de Recherches' GDR CNRS 790, the 'Ministère de l'Enseignement Supérieur et de la Recherche' (ACC 'Physico-Chimie des Membranes Biologiques'), and the NMR Discussion Group (Beckenham, UK). We thank Professor Théophile Bernard and the members of the GDR CNRS No. 790 for many stimulating and fruitful discussions, and Marie-Madeleine Guéguen and Christophe Goulard for skilfull technical assistance.

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