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Interaction of the 14-residue peptaibols, harzianins HC, with lipid bilayers: permeability modifications and conductance properties

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Abstract

Harzianins HC are a series of 14-residue peptaibols containing three Aib-Pro motives separated by sequences of two usual amino acids (Aib-Pro-Xaa-Xaa)*n*. They are organized in a subtype of the 3_{10}-helix, which results in an approximate length of about 27–30 Å for the helical rods, allowing them to span a bilayer. Permeabilization of small unilamellar vesicles composed of zwitterionic lipids (egg phosphatidylcholine/cholesterol 7/3 and 8/2) by harzianins HC was observed, as well as voltage-gated macroscopic conductance and single-channel formation in planar lipid bilayers (DOPE/POPC 7/3). The permeabilization process was shown to increase with increasing the helix global hydrophobicity. The ion channel-forming properties appeared rather favoured by an increase in the peptide amphipathicity. The set of conductance levels increasing in geometrical progression, reflecting the sequential uptake and release of monomers which is characteristic of the barrel-stave model for ion-channels described for alamethicin was not observed. The passage of ions through the bilayer would rather be the result of a set of aggregates with fixed numbers of monomers formed in the bilayer. The permeability process and the voltage-gated properties could thus result from different mechanisms showing that harzianins HC can permeabilize membranes via bilayer destabilization or channels, depending on the membrane system, composition and application of voltage.

Keywords: Lipid bilayer; Ion channel; Small unilamellar vesicle; Membrane permeability; Harzianins HC; Peptaibol

Abbreviations: CF, 5,6-dicarboxyfluorescein; Chol, cholesterol; DOPE, 1,2-dioleoylphosphatidylethanolamine; ePC, egg phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MLV, multilamellar vesicles; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SUV, small unilamellar vesicles; The standard one-letter code has been used for *L*-amino acids: U = Aib (α-aminoisobutyric acid), J = *D*-Iva (*D*-isovaline) and Lol = *L*-Leuol (*L*-leucinol).

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1. Introduction

A large number of antimicrobial peptides isolated from animal cells (mammalian defensins [1], magainins [2]), from insects (cecropins [3], melittin [4]), as well as from microorganisms (alamethicin [5] and related peptaibols [6–9], gramicidins [10,11], lantibiotics [12]), are known to interact with phospholipid bilayers, causing membrane perturbation. Some of these peptides have antibiotic activity or are involved in immune response, but the activities are not resolved at the molecular level. Among them, peptaibols have been much investigated because they form ionic channels and provide ideal model systems for voltage-gated phenomena [13,14].

Peptaibols form a wide class of 7- to 20-residue linear hydrophobic peptides containing a high proportion of the α,α-dialkylated amino acid, α-aminoisobutyric acid (Aib), an acetylated (or acylated) N-terminal residue and a C-terminal amino alcohol. They can be classified into three subclasses: (1) the long-sequence peptaibols [5–9,15] including the well-known alamethicin; they contain 18 to 20 residues with a centrally located proline and glutamine residues near both ends; (2) the short-sequence peptaibols with 11 to 16 residues and several Aib-Pro motives [16,17]; most of them contain either Ac-Aib-Asn- or Ac-Aib-Gln- as N-terminus; (3) the lipopeptaibols with 7 or 11 residues, an N-terminal amino acid acylated by a C8 or C10 fatty acid and a high content in glycine [18,19].

The presence of Aib residues in peptaibol sequences restricts the possible conformations, so that α- and 3_{10}-helical structures are favoured [20]. Thus, peptaibols form weakly amphipathic or hydrophobic helices, either of the α-type [21,22] or 3_{10}-type [16,21,23]. Such structures are involved in the action mechanism of these peptides. The process of binding of alamethicin to lipids, and the transition from the non-conducting state to the conducting state are still debated and several models have been proposed to account for the voltage-dependence of alamethicin and long-sequence analogues (for reviews see [14,24]). Nevertheless, it is commonly assumed that the alamethicin channel in its conducting state involves an aggregate of several transbilayer helical monomers and that transitions between sub-conductance states result from uptake and release of monomers. As currently envisioned in such a model, a central pore is surrounded by trans-bilayer α-helices which must contain at least 18–20 residues in order to span the hydrocarbon region of the bilayer of an average 30 Å thickness. However, in agreement with the mattress model of lipid/peptide interaction [25], a degree of peptide/bilayer mismatch is tolerated, implying local distortions of the bilayer which could modulate channel kinetics.

Recently, we have isolated and sequenced harzianins HC (Fig. 1), a series of original short-sequence peptaibols with 14 residues [17]. Preliminary conformational data for these peptides in methanol solution has suggested that they would be organized into a subtype of the 3_{10}-helix, as a result of the peculiar sequence including three Aib-Pro motives separated by sequences of two usual amino acids (Aib-Pro-Xaa-Xaa)*n* [17]. If maintained in the bilayer environment, such a secondary structure would result in a helix length of about 27–30 Å. Taking into account a possible distortion of the bilayer, such short-sequence helical Aib-containing peptides could thus be expected to be membrane active, in spite of the lower number of residues.

In the present paper, we describe the membrane-perturbing properties of harzianins HC. Their permeability modifications were measured in the absence of transbilayer voltage, by using small unilamellar vesicles as model membranes. A number of sequences which are the most relevant for single residue substi-

		1	***2***	*3*	*4*	*5*	***6***	***7***	*8*	*9*	***10***	*11*	*12*	*13*	*14*
HC I	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ser**	**Val**	Aib	Pro	**Aib**	Leu	Aib	Pro	Leuol
HC III	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ser**	**Val**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol
HC VI	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ala**	**Val**	Aib	Pro	**Aib**	Leu	Aib	Pro	Leuol
HC VIII	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ala**	**Val**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol
HC IX	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ala**	**Ile**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol
HC X	:	Ac	Aib	**Gln**	Leu	Aib	Pro	**Ala**	**Val**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol
HC XII	:	Ac	Aib	**Asn**	Leu	Aib	Pro	**Ser**	**Ile**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol
HC XV	:	Ac	Aib	**Gln**	Leu	Aib	Pro	**Ala**	**Ile**	Aib	Pro	**Iva**	Leu	Aib	Pro	Leuol

Fig. 1. Sequences of eight harzianins HC isolated from *Trichoderma harzianum.*

tutions were selected in order to characterize in macroscopic and single-channel experiments their pore-forming properties in planar lipid bilayers. Thus, four harzianins HC were selected which may account for the role of a longer amino acid side chain at position 2 which can modify the stabilization of peptide aggregates by hydrogen bonds (Asn2/Gln2), the role of a polar residue at position 6 (Ala6/Ser6), and the influence of a longer and more hydrophobic side chain at position 7 (Leu7/Val7). The results are discussed as regards the tridimensional structure, hydrophobicity and amphipathicity of harzianins HC.

2. Materials and methods

2.1. Harzianins HC isolation

Harzianins HC were obtained by fermentation of *Trichoderma harzianum* strain M-903603 and isolated by different chromatography steps followed by semi-preparative reversed-phase HPLC (C18 Spherisorb ODS2 (AIT France), 5 μm, 7.5 × 300 mm, MeOH/H_2O: 82.5/17.5, flow rate 2 ml/min) carried out with a Waters liquid chromatograph (6000A and M45 pumps, 680 automated solvent programmer, WISP 717 plus automatic injector, 481 UV-vis. detector) equipped with a Gilson 201 fraction collector, as described [9]. The retention times given in Table 1 were determined in such conditions, with $R_{t0} = 4$ min. The peptides were shown to be at least 98% pure by analytical HPLC (Spherisorb ODS2, 5 μm, 3.5 × 250 mm, MeOH/H_2O: 81/19, flow rate 1 ml/min).

Table 1
Relative hydrophobicity (HPLC retention times (R_t) on a C18 ODS2 reversed phase), and [lipid]/[peptide] ratios allowing 50% leakage in 20 min of the entrapped CF (R_{i50}) for harzianins HC

Peptide	R_t (min)	R_{i50} [a]	R_{i50} [b]
HC XV (Q2, A6, I7, J10)	67	250	
HC X (Q2, A6, **V7**, J10)	56	111	167
HC IX (**N2**, A6, I7, J10)	58	83	
HC VIII (**N2**, A6, **V7**, J10)	49	45	
HC XII (**N2**, **S6**, I7, J10)	38	36	
HC VI (**N2**, A6, **V7**, **U10**)	43	25	
HC III (**N2**, **S6**, **V7**, J10)	31	19	33
HC I (**N2**, **S6**, **V7**, **U10**)	29	8	

[a] ePC/Chol: 7/3.
[b] ePC/Chol: 8/2.

2.2. Preparation of lipid vesicles and permeability modification measurements

Egg phosphatidylcholine (ePC) type V E and cholesterol (Chol) were purchased from Sigma; ePC was used without further purification and Chol was recrystallized from methanol. Stock solutions in $CHCl_3$ were kept at −40°C. Carboxyfluorescein (CF) from Eastman Kodak was separated from hydrophobic contaminants and recrystallized from ethanol as previously described [26].

The peptide-induced release of intravesicular content was monitored by the CF-entrapped vesicle technique [27], that uses the property of quenching relief upon dilution of the encapsulated fluorescent probe. CF-entrapped small unilamellar vesicles (SUV) were prepared, as previously described [26,28,29]. The appropriate amounts of lipids (egg PC/cholesterol: 7/3 or 8/2) were severely dried under vacuum and then allowed to hydrate at room temperature in a 56 mM CF solution in Hepes buffer (0.1 M NaCl, 5 mM Hepes, pH 7.4) for 15 h. The multilamellar vesicles (MLV) thus obtained were sonicated (40 min for 4 ml of MLVs, Heat Systems Ultrasonics model W-225R, equipped with a microtip probe; duty cycle 20%; power output 40 W) and then separated from unencapsulated CF by gel filtration through a Sephadex G75 column (1 cm × 15 cm for 4 ml of liposomes), with Hepes buffer as eluent. The dilution factor was calculated, allowing to determine the lipid concentration of the SUVs.

Liposome permeabilization was measured by fluorescence spectroscopy at 20°C on an Aminco SP F500 spectrofluorometer. A final lipid concentration of 0.6 mM in the fluorescence quartz cuvette was typically used. Leakage kinetics were monitored for different $R_i^{-1} = [\text{peptide}]/[\text{lipid}]$ molar ratios obtained by adding aliquots of methanolic solutions of peptides (methanol concentration kept below 0.5% by volume). The time course of fluorescence change corresponding to CF efflux was recorded ($\lambda_{exc} = 488$ nm, 1 nm band pass, $\lambda_{em} = 520$ nm, 1 nm band pass) after rapid and vigourous stirring. Percentage of released CF at time t was determined as $\%CF = (F_t - F_0)/(F_T - F_0) \times 100$, where F_0 is the fluorescence

intensity of the vesicle suspension in the absence of peptide, F_t is the fluorescence intensity measured at time $t = 20$ min in the presence of peptide, F_T is the total fluorescence intensity determined by disrupting the vesicles by addition of 50 μl of a 10% solution of Triton X-100.

2.3. Peptide reconstitution into planar lipid bilayers

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and stored at -74°C. For macroscopic conductance experiments, virtually solvent-free lipid bilayers were formed over a 125 μm hole in a 25 μm thick PTFE (Goodfellow, Cambridge, UK) septum sandwiched between two half-glass cells. Lipid films were spread on top of electrolyte solutions (1 M KCl, 10 mM Hepes, pH 7.4) in both compartments and bilayer formation was most often achieved by lowering then raising the level in one or both sides [30] and was monitored by capacitance. The lipid solution at 5–10 mg/ml in hexane (Fluka, spectroscopic grade) was a neutral mixture: 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC)/1,2-dioleoylphosphatidylethanolamine (DOPE), 7/3 (w/w). Peptides (from a 10^{-5} M stock solution in methanol) were added with stirring to the *cis*- or positive side, according to the usual conventions; the bulk methanol concentration did not exceed 1%. Transmembrane currents were fed to a Keithley amplifier (model 427, Cleveland, OH) virtually grounded to the *trans* Ag/AgCl electrode. Current-voltage curves were recorded with an X-Y plotter.

In single-channel experiments, lipid bilayers were formed at the tip of fire-polished patch-clamp pipettes [31] pulled in two steps (model PP-83, Narishige, Tokyo, Japan) from Vitrex borosilicate tubes (Modulohm, Herlev, DK). The outer tip diameter was about 1 μm and its resistance in the standard solution used for conductance measurements described above was of the order of 5 MΩ. The same neutral lipids as in macroscopic conductance configuration but at 0.5–2 mg/ml in hexane, were allowed to evaporate on top of 2 ml of the electrolyte solution in a glass beaker (spreading area: 3 cm^2). To form bilayers, either the pipette was withdrawn and then slowly dipped again through a hydraulic micromanipulator, or a lipid droplet was applied to the pipette shank. All planar bilayer experiments were performed at room temperature. A programmable waveform generator (SMP 310 model) and a patch-clamp amplifier (RK-300), both from Bio-Logic (Claix, France), were used to record single-channel currents. The latter were fed to an 8-pole low-pass filter and stored on a digital tape recorder (respectively, AF 180 and DTR 1200 models from Bio-Logic). Numeric signals were subsequently analyzed through Satori v. 3.01 software from Intracel Ltd (Royston, UK).

3. Results

3.1. Permeability measurements

The liposome methodology offers a wide range of experiments that allow studies of peptide/lipid interactions. The subsequent changes in the liposome permeability properties were measured as an indirect probe of the interaction of harzianins with lipid bilayers. Peptide-induced release from lipid vesicles of a fluorescent probe, carboxyfluorescein (CF), entrapped at a self-quenched concentration [27], was monitored for the different harzianins HC and for different peptide to lipid R_i^{-1} ratios ranging between 10^{-3} and 10^{-1}. Small unilamellar vesicles (SUV) made of a mixture of the zwitterionic phospholipid, egg phosphatidylcholine (ePC), and cholesterol (Chol) in the proportion 7/3 were used as model membranes. Such a lipid mixture, previously used to analyse membrane properties of other peptaibols [26,28,29] results in negligible spontaneous leakage over a period of several hours.

The kinetics of the leakage process induced by the different harzianins HC were monitored over 20 min with varying peptide concentrations ranging between 1–100 μM, while keeping constant the lipid concentration (600 μM). Integrity of the vesicles during the leakage process was shown by following the scattering peak intensity at 488 nm, which remained unchanged upon addition of harzianins and all over the kinetics, whereas it was completely abolished when the detergent Triton X-100 was added. In order to compare the potency of the different harzianins HC, the percentage of released CF at time $t = 20$ min was plotted as a function of the [peptide]/[lipid] ratios R_i^{-1} (Fig. 2). The [lipid]/[peptide] ratios allowing 50% leakage of the entrapped probe (R_{i50}) were

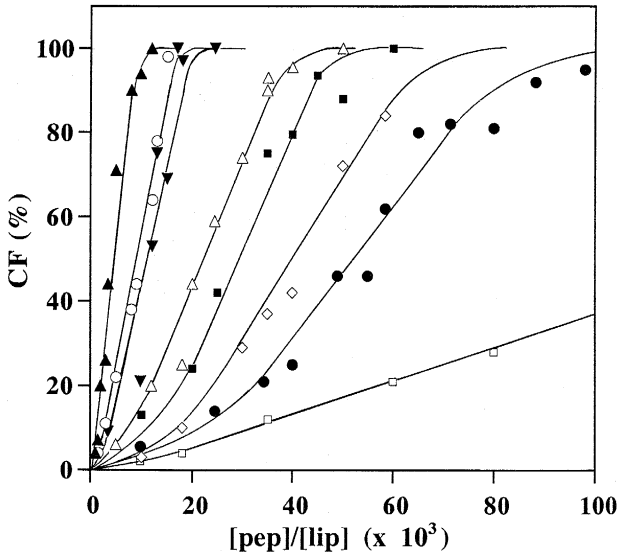

Fig. 2. Percentage of escaped CF from ePC/Chol: 7/3 SUV for different R_i^{-1} ratios = [peptide]/[lipid]; [lipid] = 0.6 mM; □ HC I, ● HC III, ◇ HC VI, ■ HC XII, △ HC VIII, ▼ HC IX, ○ HC X, ▲ HC XV.

taken as a measure of the peptide efficiency (Table 1), which could be related to the membrane affinity of harzianins HC.

Harzianin HC XV, which is the most hydrophobic peptide of the HC series, as reflected by the retention times (Table 1), exhibited the highest efficiency. The membrane activity was decreased by approximately a factor 30 when examining the leakage induced by HC I, which had the less hydrophobic sequence from substitutions of Gln, Ala, Ile and Iva at positions 2, 6, 7 and 10 by the less hydrophobic residues Asn, Ser, Val and Aib, respectively. In order to describe precisely the role of each of these residues in the sequence, the effects of single or double amino acid substitutions on the permeabilization process were examined. The HC XV efficiency was decreased by factors 3 and 2, when substitutions at positions 2 (Gln2/Asn2) and 7 (Ile7/Val7) were considered by using HC IX and HC X, respectively. Such consequences of a single amino acid substitution in the harzianins HC sequence were also observed by comparing the activity of harzianin HC VIII to those of HC X (Asn2/Gln2), HC III (Ala6/Ser6), HC IX (Val7/Ile7) and HC VI (Iva10/Aib10) (Table 1). Double or triple substitutions in regard to HC XV at positions 2 and 6 (HC XII), 2 and 7 (HC VIII), 2, 6,

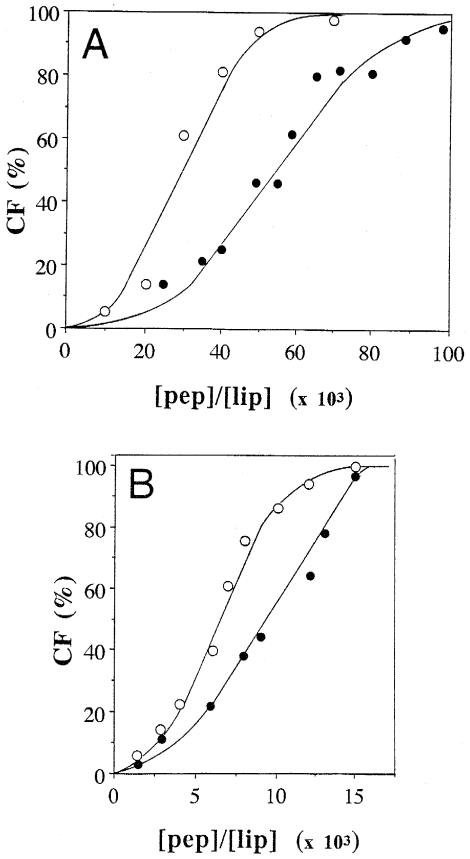

Fig. 3. Effect of the cholesterol amount in the bilayer on the permeabilization of ePC/Chol SUV by harzianins HC III and HC X; A: HC III; B: HC X; ● ePC/Chol: 7/3; ○ ePC/Chol: 8/2.

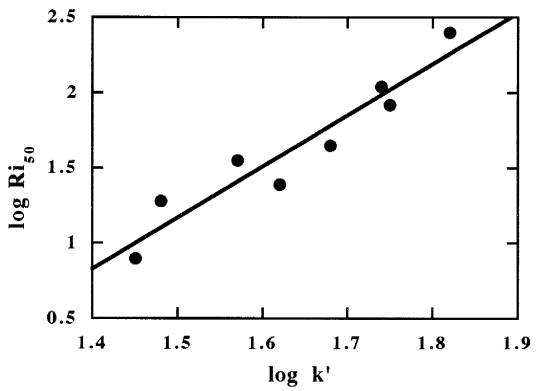

Fig. 4. Relationship between the log (R_{i50}) and log k' taken as a measure of the relative hydrophobicity of harzianins HC (k' values were determined using the retention times measured on a C18 ODS2 column 7.5×300 mm, from the relationship $k' = t - t_0 / t_0$).

7 (HC III) and 2, 7, 10 (HC VI) also resulted in a decrease of the membrane activity by a factor ranging between 2 and 3 for each position.

These results agree with the membrane affinity values of individual amino acids [32], which also exhibit a strong correlation with individual residue hydrophobicity determined from different authors [14,33–35]. Furthermore, we measured the relative hydrophobicity of harzianins HC by using the capacity factors k' obtained by HPLC on a reversed phase column [36,37]. A linear relationship with a good correlation coefficient (0.95) was observed between log (R_{i50}) and log k' (Fig. 4). The permeabilization process by harzianins HC appeared thus to be strongly dependent on the peptide global hydrophobicity.

Previous results in our laboratory on the lipid/peptide interaction of 19-residue α-helical peptaibols bearing a C-terminal negative charge at physiological pH [26,28] have shown that, when decreasing the cholesterol amount in the bilayer from 30% to 20%, the liposome permeabilization induced by such a charged peptide gradually increases. This variation in cholesterol content is without effect on the spontaneous leakage. The induced permeabilization then remains constant in the range 20–0% cholesterol; it has been noticed that the threshold effect at 20% cholesterol corresponds to the disappearance of pure lipid domains. By contrast, the activity of the neutral peptide analogue remains insensible to the membrane cholesterol content over the range 30–0%. However, tryptophan fluorescence quenching experiments have proved that the peptide was lesser embedded in the membrane in the presence of 30% cholesterol. As cholesterol usually exerts a pushing out effect of the embedded molecules [38], the results have stressed the role of the length and embedding of the hydrophobic helices on the permeabilization process by long-sequence peptaibols

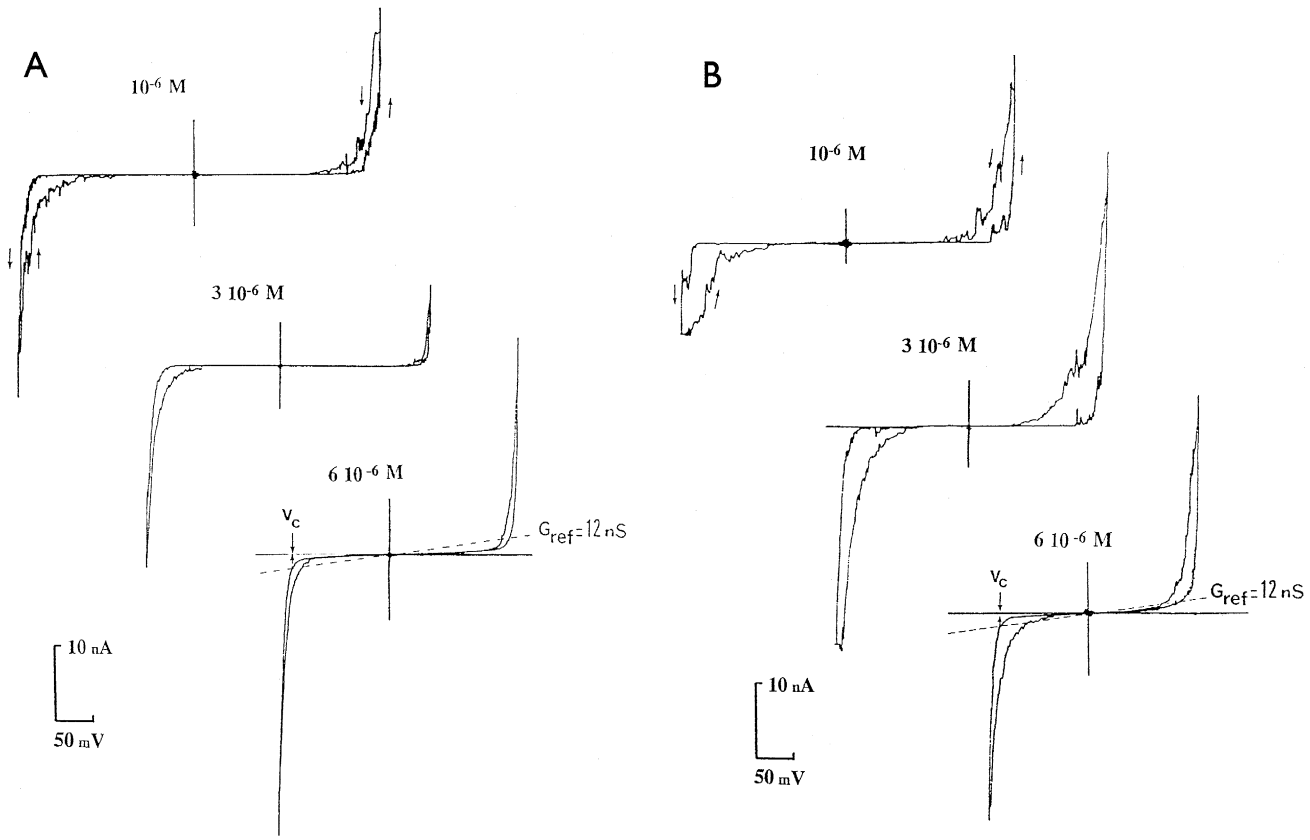

Fig. 5. Current–voltage relationship for planar bilayers (POPC/DOPE: 7/3) treated with increasing concentrations 10^{-6}, 3×10^{-6} and 6×10^{-6} M of A: HC III and B: HC VIII; 1 M KCl both sides, room temperature; characteristic voltages V_c are defined as crossings of the exponential branches with the reference conductance shown by the broken lines in A and B.

[26,28]. In order to give an insight into the influence of the peptide embedding on the harzianin HC-induced permeabilization of liposomes, the effect of decreasing the cholesterol amount in the bilayer was examined. Harzianins HC III and HC X were chosen as examples of neutral short-sequence peptaibols with weak and strong activity. The activity of both peptides was increased by approximately a factor 1.6 when the cholesterol amount was decreased from 30% to 20% (Fig. 3), indicating the influence of the matching between peptide hydrophobic helix length and bilayer thickness.

3.2. Ion channel formation in planar lipid bilayers

3.2.1. Macroscopic conductance

Large bilayers allow the incorporation of typically hundreds to thousands of channels after peptide partitioning from bath solution and the recording of current-voltage (I–V) curves. Macroscopic conductance measurements with alamethicin [39], trichorzianins [40] and analogues [29] have been previously performed in planar bilayers, providing information on the simultaneous activity of many channels. The same technique was used to probe the channel-forming ability of the short-sequence peptaibols, harzianins HC III, VIII-X and its eventual modulation by the side chain hydrophobicity.

Methanolic solutions of peptides were added to the *cis*-side and after allowing 15–20 min for equilibrium to be reached, the bilayer was submitted to a voltage triangular waveform (3–6 mV/s). Several runs insured that a steady-state was achieved and the resulting I/V curves were recorded (Fig. 5). As previously noticed for alamethicin and analogues but quite unexpectedly for harzianins HC, a steep development of membrane current above a voltage threshold was observed, despite the low number of residues of these peptides. The I/V curves were quasi-symmetrical, contrasting with the alamethicin and zervamicin curves obtained in similar experimental conditions [14]. This most likely resulted from a fast equalisation of the peptide concentration on both *cis* and *trans* sides of the bilayer with the usual forward gating still applying, the peptide N-terminus or positive end of the helix dipole crossing the bilayer. This was favoured by the structure of harzianins which are relatively short and electrically neutral with segregation of the hydrophilic residues in the N-terminal half. Indeed, as shown previously with alamethicin, blocking the negative C-terminal charge of Glu18 and slightly shortening the peptide results in symmetrical I/V curves [13].

Both the high voltage-sensitivity and concentration-dependence of threshold (Fig. 6) were retrieved with the four harzianins, as analyzed in Table 2. The concentration-dependence V_a, i.e., the sensitivity of the macroscopic conductance to changes in aqueous peptide concentration, appeared of the same magnitude for the four harzianins HC, however, slightly decreasing from HC X to HC III with the hydrophobicity of the peptide. The voltage-dependence V_e and the apparent mean number of monomers building up the conducting aggregate N_{app}, derived through the analysis already described for alamethicin [13], were similar for the four examined harzianins HC and about the same as found for analogues of alamethicin [29,40]. In spite of this, these parameters did not seem much modulated by harzianin HC amino acid sequence (Table 2); the slightly lower V_e shown by HC III, the least efficient in terms of concentration-dependence, could be correlated to the presence of the more hydrophilic Ser at position 6, suggesting a greater voltage-sensitivity. However, when plotting for different micromolar concentrations of HC, the

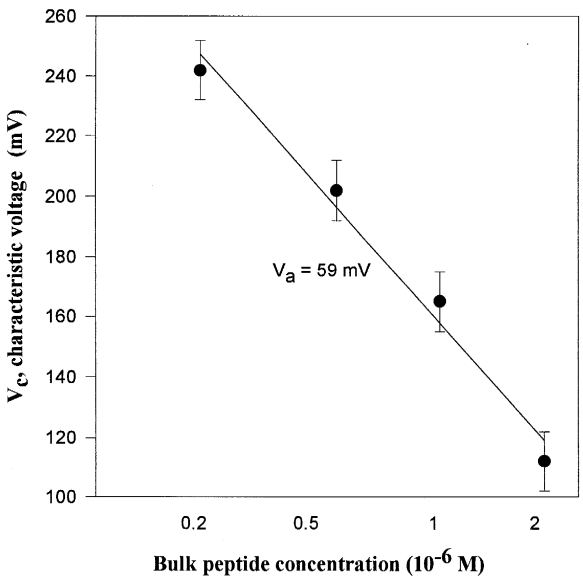

Fig. 6. Characteristic voltage V_c as a function of the HC IX concentration.

logarithmic value of the membrane conductance at 100–130 mV versus the relative hydrophobicity (k') (not shown), as previously done with the 20-residue trichosporins Ts-Bs [41], a non-linear relationship appeared. Thus, the voltage-dependent and liposome permeabilization processes did not appear to strictly follow the same rule as regards the peptide hydrophobicity.

3.2.2. Single-channel conductances

The single channel properties of the four selected harzianins HC were examined in planar lipid bilayers formed at the tip of patch-clamp pipette by using the 'tip-dip' method. The much-reduced membrane area and peptide aqueous concentration allowed the resolution of single-channel events. Bursts of channel activity were observed for harzianins HC. The traces shown in Fig. 6 are selected but typical examples of single-channel activity displayed by the peptides at similar concentrations and applied voltages. The trace for HC III exhibited well-defined levels of current reflecting the occurrence in the membrane of several discrete conductive entities. Three different and roughly equally spaced current levels could be seen (Fig. 7A) and the associated amplitude histogram (Fig. 8A) allowed to estimate the conductance levels

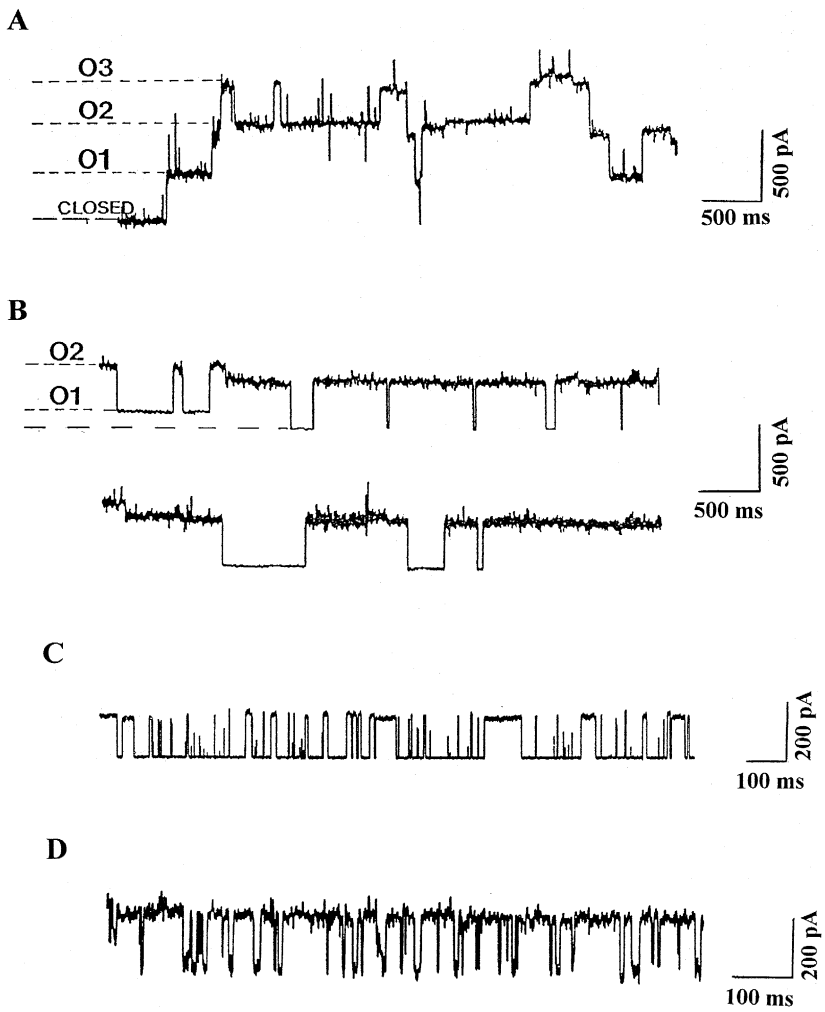

Fig. 7. Single channel current fluctuations induced in POPC/DOPE: 7/3 'tip-dip' bilayers by A: HC III at a peptide concentration of 5×10^{-8} M and an applied voltage of +100 mV; B: HC VIII, 2.5×10^{-8} M and +130 mV; C: HC IX, 2×10^{-8} M and +140 mV; D: HC X, 2×10^{-8} M and +140 mV. In all instances, 1 M KCl, room temperature; openings are upwards deflections.

as multiples of about 3300 pS. In addition, a smaller level labeled O1 at 700 pS was more rarely observed. As shown on the amplitude histogram, HC VIII exhibited a sequence of conductance levels (Fig. 8B) at 700, 2500 and 4600 pS, but contrasting with alamethicin single-channel pattern, smaller levels often occurred on top of the higher conductance level. Single-channel events exhibited by HC IX and HC X were of much shorter duration and smaller amplitude (Fig. 7C, 7D). They both displayed a single level of 1200–1400 pS, with a greater probability of the open-state in the case of HC X. Although all harzianins HC exhibited in macroscopic conductance experiments the same number of monomers implied in the most probable conducting state ($N_{app} = 8$), differences in the single-channel traces were observed. Such an apparent discrepancy reflected the occurrence of less probable conductance states arising from aggregates of different numbers of monomers. The activity of these aggregates was revealed in the single-channel experiments, thus preserving the validity of the analysis of the I/V data.

It is worth noting that the regular progression of conductance levels seen with alamethicin and analogues [29,40] or to some extent with zervamicin [42] was not observed with harzianins HC, suggesting a different mode of action in the bilayer.

Table 2
Macroscopic conductance analysis for harzianins HC III, and HC VIII-X

Peptide	C (M)	$V_{c\,(mV)}$	$V_{a\,(mV)}$	$V_{e\,(mV)}$	N_{app}
HC III	10^{-6}	228			
	$3\cdot10^{-6}$	175	47.5	6	8
	$6\cdot10^{-6}$	140			
HC VIII	10^{-6}	225			
	$3\cdot10^{-6}$	162	53	7.5	7
	$6\cdot10^{-6}$	125			
HC IX	$2\cdot10^{-7}$	242			
	10^{-6}	155	59	7.5	8
	$2\cdot10^{-6}$	112			
HC X	$3\cdot10^{-6}$	203			
	$4.5\cdot10^{-6}$	177			
	$6\cdot10^{-6}$	158	65	7.8	8
	$7.5\cdot10^{-6}$	147			

V_c is the characteristic voltage defined as in Fig. 5; the voltage-dependence V_e is the increment producing an *e*-fold conductance change; the concentration-dependence V_a is the voltage shift resulting from an *e*-fold concentration change; the apparent mean number of monomers involved in conducting aggregate is determined as $N_{app} = V_a/V_e$ according to Ref. [13]. Values of V_c, V_a and V_e are given within ± 7 to 9 mV, ± 8 to 10 mV and ± 0.5 mV, respectively; resulting N_{app} rounded values are within ± 1.

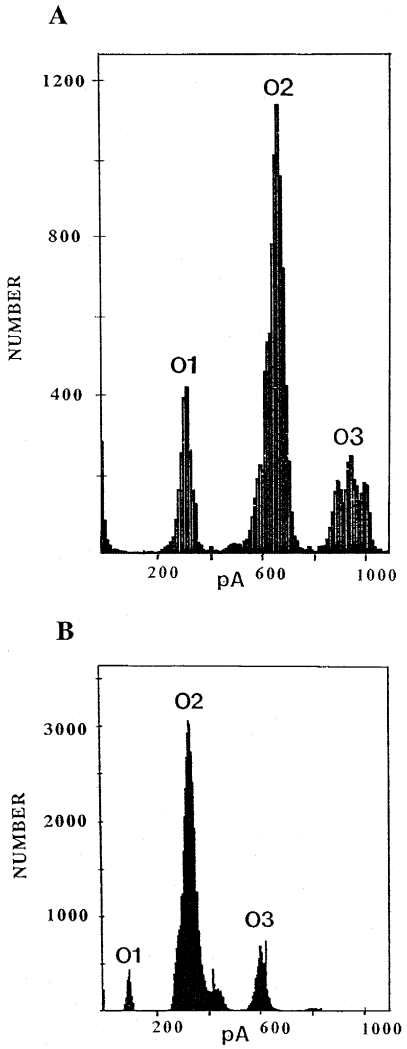

Fig. 8. Amplitude histograms (A: HC III, B: HC VIII) associated to single channel current fluctuations induced in the conditions of Fig. 7 (in ordinates, number of events; the closed states correspond to 0 pA).

4. Discussion

Harzianins HC are 14-residue hydrophobic peptides organized in a 3_{10}-helix subtype [17]. They are stabilized by $4 \rightarrow 1$ hydrogen bonds, making successive β-turns twisted into an approximate 3_{10}-helix.

Taking into account a number of 3.2 residues per helix turn assumed for such an helix type [23,43], the resulting helical rods with approx. 27–30 Å long appear mostly hydrophobic with a slight amphipathicity resulting from the sequestration in the same helix sector of Asn2 (Gln2), Pro5 and Pro9. The anomalously high hydrophilic character of proline [44] and its lower membrane affinity compared to Asn, Gln and Ser [32] allow it to contribute to the hydrophilic sector of the helix. Especially in the case of HC III which contains a Ser residue at position 6, the hydrophilic sector is strengthened. The plot of the mean hydrophobic moment $<\mu_H>$ versus the mean hydrophobic index $<H>$ calculated according to Eisenberg et al. [33] and using the appropriate periodicity for a β-turn ribbon is shown in Fig. 9. All examined harzianins fall in the domain of multimeric transmembrane segments, compatible with their pore-forming properties. The Ser6-containing harzianin HC III, with slightly lower hydrophobicity and increased hydrophobic moment appears somewhat detached from a cluster made of the other peptides. For comparison, alamethicin and its N-terminal 1-14 fragment are still lying in the same domain of the plot, though located away from the harzianin HC cluster in agreement with the different pore-forming properties observed, especially at the single-channel level.

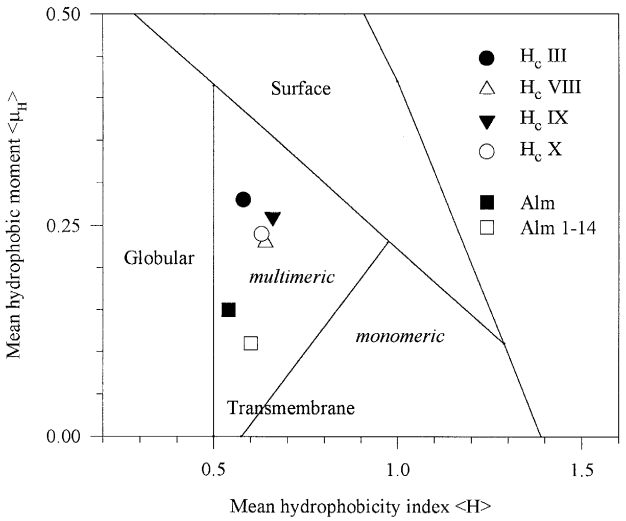

Fig. 9. Hydrophobic moment plot (mean value per residue) for harzianins HC III and HC VIII-X assayed for their pore-forming properties and compared with alamethicin (Alm) and its N-terminal 1-14 fragment (Alm 1-14). The normalized consensus hydrophobicity scale [33] was used together with periodicities of 3.2 and 3.6 residues per turn associated to 112.5° and 100° values for the angle between successive residues in the helical wheel, for harzianins HC and alamethicin, respectively. Calculated values of $<\mu_H>$ and $<H>$: HC III = 0.28, 0.58; HC VIII = 0.23, 0.64; HC IX = 0.26, 0.66; HC X = 0.24, 0.63; Alm = 0.15, 0.54; Alm1-14 = 0.11, 0.60.

In the present work, it was shown that harzianins HC increase the permeability of ePC/Chol bilayers in the absence of voltage in the concentration range 2 μM–20 μM and form voltage-dependent channels for concentrations starting from 10^{-2} μM. Whatever position in the sequence, hydrophobicity of the residue lateral chains clearly appeared as the predominant factor involved in the voltage-independent liposome permeabilization process, indicating that the harzianin HC/membrane interaction was highly hydrophobic in character, as also shown previously for long-sequence peptaibols [26,28]. The interaction with membrane bilayers and the membrane-perturbing effects were affected by the amount of cholesterol in the phospholipid bilayer. As cholesterol has been shown to exert a pushing out effect of the embedded molecules [38] and particularly of long-sequence peptaibols [26,28], the present results could be interpreted in terms of a lesser embedding of the hydrophobic harzianin HC helices in the bilayer with higher cholesterol content, thus decreasing the perturbing effect on the liposome bilayer. These results point to the role of the mismatch of the helix length and hydrophobic thickness of lipids in the interaction of harzianins HC with membranes and subsequent permeabilization.

Although the sensitivity of macroscopic conductance to changes in peptide concentration (V_a) increased with the peptide hydrophobicity, the other macroscopic and single channel data did not show the same dependence. The sequence of efficiency observed in liposome permeabilization assays, hydrophobicity and membrane activity being correlated, did not hold any more. Among the examined harzianins HC, the most efficient analogue is HC III, both at the macroscopic conductance level (higher concentration- and voltage-dependence, Table 2) and at the single-channel level (number of active channels and their lifetimes, Fig. 7A). This could reflect the involvement in the pore lumen of the hydrophilic Ser6, responsible for an increase of the helix amphipathicity. With synthetic analogues of alamethicin adopting

an α-helical conformation, we have recently shown that an Ala7/Ser 7 substitution restores an alamethicin-like pattern of channel activity [45]. The special behaviour of HC III as regards the pore-forming properties is also singled out when examining its location in the hydrophobic moment plot (Fig. 9), as discussed above.

From macroscopic and single channel experiments, it appeared that oligomerization of harzianin monomers takes place, but contrasting with alamethicin [46] and natural or synthetic analogues [28,37,47], harzianins HC failed to show the typical pattern of a set of conductance levels increasing in geometrical progression. The dynamic barrel-stave model with pores of varying diameter, reflecting the sequential uptake and release of monomers did not seem to apply in this case. The observed conductance levels could be the result of a set of aggregates, each with fixed numbers of monomers formed in the bilayer.

Taking into account the helical structure of harzianins HC, it is possible to speculate about a hypothetical organization of such channels; the helix aggregates could be accommodated in the bilayer provided that local distortions of the bilayer could help the monomers to span the membrane. However, if the mismatch between the bilayer thickness and the helix length is limited in the case of the 14-residue harzianins HC, it appears more stringent when considering the voltage-dependent conductance induced by the 11-residue-related trichorozins [48]. Such peptides share the same structural characteristics as harzianins HC (i.e., the Ac-Aib-Asn/Gln and Aib-Pro-Xaa-Xaa sequence patterns) and would presumably act by a similar mechanism implying local distortions of the bilayer to allow the short helices to adopt a transmembrane orientation [14,25]. However, other hypotheses such as elongation of the helix upon the influence of intrinsic molecular interactions or head-to-tail dimerization of peptide helices to form the functional channel could be suggested too [14].

In conclusion, the permeability process appears mainly governed by the peptide hydrophobicity, while the voltage-dependent properties seem more dependent upon the helix amphipathicity. Assuming that the modifications of membrane permeability by helical peptides, upon voltage application or not, may generally imply different interaction modes depending upon the peptide orientation towards the bilayer surface [14,49], which can be parallel or oblique ('carpet-like' mechanism [50] and 'wedge-effect') or perpendicular (pore formation), the liposome permeabilization and voltage-gated ion channel activity exhibited by harzianins HC could presumably occur through two distinct mechanisms. Local disorder of phospholipids adjacent to harzianins HC or perturbation of the integrity of the bilayer, affecting the spontaneous monolayer curvature [51] could be involved for the permeabilization process, while pores made up of helical clusters of limited size could result in the voltage-dependent properties.

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