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Interaction of trichorzianines A and B with model membranes and with the amoeba *Dictyostelium*

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Trichorzianines A (TA) and B (TB) are microheterogeneous mixtures of antibiotic nonadecapeptides of the peptaibol class which interact with lipidic membranes and modify their permeability properties. The TB differ from the TA by replacement of the Gln-18 by a Glu, giving rise to a C-terminal negative charge at neutral pH. The role of this charge on the trichorzianine–lipid interaction was investigated with model membranes by fluorescence spectroscopy and the results were correlated with the biological activity toward the amoeba *Dictyostelium discoideum*. The interaction of the acidic trichorzianine TB IIIc (Glu-18) with phospholipid bilayers and the subsequent induced permeability were weaker than that exhibited by the uncharged TA IIIc (Gln-18) and MeTB IIIc (TB IIIc monomethyl ester). The unfavourable effect of the negative charge in TB IIIc was strongly enhanced by incorporation of cholesterol in the bilayer. Similarly, TA IIIc as well as MeTB IIIc induced growth inhibition and lysis of the amoeba *Dictyostelium* at four times lower concentrations than TB IIIc. The results suggested that the interaction of trichorzianines with the phospholipid bilayer and the subsequent modifications of permeability were involved in the inhibitory properties and cell lysis induced by trichorzianines toward *Dictyostelium*.

Introduction

Trichorzianines are nonadecapeptides of the peptaibol class isolated from the fungus *Trichoderma harzianum* [1,2,3]. They are separated by chromatography into two main groups, the neutral trichorzianines A (TA) [3], and the acidic trichorzianines B (TB) [4], that are negatively charged at physiological pH. The two groups consist in microheterogeneous mixtures resulting from minor replacements of residues in the sequence. The distinction between the TA and TB concerns the 18-position which is occupied by a glutamine in TA and by a glutamic acid in TB. The C-terminal amino alcohol is either phenylalaninol or tryptophanol (Trpol). From each microheterogeneous group, two major components containing Trpol (TA IIIc and TB IIIc) were isolated by HPLC and their sequence determined.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	**18**	19
TA IIIc:	Ac	Aib	Ala	Ala	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	**Gln**	Trpol
TB IIIc:	Ac	Aib	Ala	Ala	Aib	Aib	Gln	Aib	Aib	Aib	Ser	Leu	Aib	Pro	Val	Aib	Ile	**Glu**	Trpol

As shown in a previous paper, TA IIIc binds to phospholipid bilayers and induces leakage of vesicular-entrapped material [5]. This interaction is mainly governed by hydrophobic effects. Incorporation of sterols (cholesterol or ergosterol) in the bilayer modifies the peptide embedment within the bilayer but does not affect the induced modifications of membrane permeability.

Abbreviations: CF, carboxyfluorescein; egg PC, egg L-α-phosphatidylcholine; HPLC, high performance liquid chromatography; Mes, 4-morpholineethanesulfonic acid; MeTB, monomethylester of TB; R_i, [lipid]/[peptide]; TLC, thin-layer chromatography; Trpol, tryptophanol; TA, trichorzianines A; TB, trichorzianines B; SUV, small unilamellar vesicles.

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As already reported for the peptaibol alamethicin, [6] and for melittin [7], trichorzianines induce voltage-dependent and multi-state ionic channels in planar bilayers [8], and a different behaviour of the homologous neutral TA IIIc and charged TB IIIc peptides was observed.

We present, in this paper, a comparative study of the interaction of TA IIIc and TB IIIc with model membranes and their biological effects toward the amoeba *Dictyostelium discoideum*.

In the first part, we investigated on model membranes the effects of the presence of a Glu-18 negative charge in the C-terminal extremity of trichorzianines and of incorporation of cholesterol within the bilayer. We took advantage of the presence of a single fluorophore (Trpol) in TA IIIc, TB IIIc and MeTB IIIc (the uncharged monomethyl ester of TB IIIc) to study their interaction with bilayers by fluorescence spectroscopy. The subsequent modifications of membrane permeability were further examined. The results show the critical role of the Glu-18 negative charge in the interaction of trichorzianines with phospholipid bilayers.

In the second part, the toxic effects of trichorzianines of the three peptide groups (TA, TB, and MeTB) were measured on an eucaryotic cell, the amoeba *Dictyostelium discoideum* [9,10]. The inhibitory properties of the trichorzianines on the growth of *Dictyostelium* as well as their direct lytic potencies were compared to their ability to interact with model membranes.

Materials and Methods

TA IIIc and TB IIIc were isolated from the spores of *Trichoderma harzianum* and purified as already described [2,4]. The methyl ester derivatives of TB and TB IIIc (MeTB and MeTB IIIc) were obtained by methylation of the carboxylic function of TB and TB IIIc with diazomethane (20 °C, 30 min). TLC (KC_{18} Whatman, $MeOH/H_2O$, 90:10): R_f TA and TA IIIc (0.25), TB and TB IIIc (0.58), MeTB and MeTB IIIc (0.25). Egg PC type VII E was purchased from Sigma and used without purification. Cholesterol (Sigma) was recrystallized from MeOH. CF (Eastman Kodak) was purified according to a published procedure [11]. The concentration of the peptide solutions was determined on an Uvicon spectrometer model 820 using ε (281 nm) = 4800 $M^{-1} \cdot cm^{-1}$. Fluorescence spectra were recorded at 20 °C on a Spex Fluorolog Model 1681 and given with correction of the emission photomultiplier.

Incorporation experiments. SUV were prepared by sonication to clarity of $4 \cdot 10^{-4}$ M solutions of egg PC (or egg PC/cholesterol) in 0.1 M NaCl, 10 mM cacodylate buffer (pH 7.0) at 0 °C, under nitrogen (15 to 20 min, duty cycle 20%; power output 30 W, Branson sonifier Model B 15, equipped with a microtip probe). The binding of the peptides to SUV was followed by monitoring the fluorescence spectrum modifications of the Trpol residue upon addition of lipid vesicles.

In typical fluorescence measurements, aliquots of a vesicle suspension were added to a $6 \cdot 10^{-7}$ M peptide solution in cacodylate buffer (5 μl of a $1.8 \cdot 10^{-4}$ methanolic peptide solution in 1400 μl buffer in a 1×1 cm quartz cell); the fluorescence emission spectra were recorded after an incubation period of 10 min at 20 °C under magnetic stirring. The system was at equilibrium after such a period as λ_{max} and fluorescence intensity remained constant. Emission spectra of the peptide-vesicle solutions were systematically corrected by subtracting that of corresponding solutions containing the same concentration of vesicles. The amount of vesicles added to the constant concentration peptide solution was expressed by the lipid to peptide molar ratio R_i. The amount of peptide adsorbed on the cell walls (decreasing from 20% to 5% as the phospholipid concentration increased from 5 μM to 140 μM) was determined by dissolution of the peptide with methanol (1400 μl) and measuring its concentration by fluorescence spectroscopy. All R_i values were thus corrected, taking into account the amount of adsorbed peptide. Typical fluorescence measurements conditions were: λ_{exc} 282 nm, band pass 1.8 nm, λ_{em} 300 to 450 nm, band pass 3.6 nm. Integrity of the vesicles was followed by measuring the scattering peak intensity at 282 nm before and after addition of vesicle suspensions to peptide solutions. The relative affinities of the peptides for the vesicles were given by characteristic R_i values graphically determined (dotted lines) as shown in Fig. 1C.

Membrane permeability measurements. Leakage from vesicles was measured, using the CF-entrapped vesicle technique [12]. The preparation of the CF-encapsulated vesicles consisted in weighing the appropriate amount of lipids either egg PC, or egg PC-cholesterol (4:1, 3:1 and 7:3 mol/mol). The lipids were dried under vacuum and then allowed to hydrate at room temperature, in a 51 mM CF solution in 0.1 M NaCl, 10 mM cacodylate buffer (pH 7.0) for 15 h. After sonication, the lipid solution (usually 9 mM) was separated from unencapsulated CF by filtration through a Sephadex G-75 column (0.5×15 cm for 1 ml solution) in the above buffer. Dilution factor was determined for each gel filtration and was usually about 3. A 200 μl aliquot of the vesicle solution was added in a 1×1 cm quartz cuvette to 1200 μl of cacodylate buffer. The phopholipid concentration was kept constant and increasing peptide/lipid molar ratios (R_i^{-1}) were obtained by adding aliquots of methanolic solutions of peptides, the methanol concentration being kept below 0.5% by volume. After rapid and vigorous magnetic stirring, the time-course of fluorescence change corresponding to CF efflux was recorded at 520 nm (0.72 nm band pass) with λ_{exc} 488 nm (0.72 nm band pass).

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 in the presence of peptide and F_T is the total fluorescence determined by disrupting the vesicles by addition of 20 µl of a 10% solution of Triton X-100. The integrity of vesicles during the CF leakage process was followed by measuring the scattering peak intensity at 488 nm before and after addition of peptides to vesicles.

Dictyostelium discoideum culture conditions. The amoeba *Dictyostelium discoideum*, strain AX2 (ATCC 24397) was grown axenically at 22 ± 1°C in a peptone-yeast extract medium supplemented with 18 g/l maltose [13]. Growth was monitored by counting the cells in a Coulter counter. The lower threshold was set for particles with a diameter higher than 5 µm. Amoebae in their exponential phase of growth ((4–8) · 10^6 cells/ml) were harvested by centrifugation at 800 × *g* for 4 min in a Jouan GR 4.11 centrifuge at 4°C. Cells were washed with ice-cold 20 mM Mes-Na buffer (pH 6.3) and the packed cell pellet was stored at 0°C before experiments.

Lysis assay of Dictyostelium amoebae with trichorzianines. Lysis assay was conducted as described for the lytic protein phallolysin [14]. Amoebae were suspended in 20 mM Mes-Na (pH 6.3) to give cell concentrations between 2 · 10^5/ml to 4 · 10^6/ml and incubated at 22°C in the presence of increasing concentrations of trichorzianines (see figure legends for specific conditions). The final volume was 0.5 ml and methanol concentration was adjusted to 1% (v/v) in all samples. It was checked that methanol itself at this concentration had no lytic effect on the cells and that no swelling occurred in 20 mM Mes-Na buffer in the absence of trichorzianines. Incubation was started by addition of amoebae, and samples were slowly shaken to avoid cell settling. After 15 min, incubation was stopped by addition to the samples of 10 ml of 5-fold diluted Isoton II (Coulter) containing 0.1% (v/v) formaldehyde. The number of remaining cells was immediately measured with a Coulter counter. Microscopic observations during the incubation period indicated that swelling of the cells occurred before lysis.

Results

Fluorometric measurements of the interaction of TA IIIc, TB IIIc, MeTB IIIc with lipid bilayers

The observed fluorescence maxima (corrected spectra) of TA IIIc, TB IIIc and MeTB IIIc in aqueous solution were 347 nm *. In a previous study [5], we observed that addition of egg PC vesicles (SUV) to a TA IIIc solution resulted in a shift of the Trpol fluorescence maximum to shorter wavelengths and in a marked enhancement of the relative fluorescence intensity F/F_0. The shift of 12 nm to shorter wavelengths and the increase in fluorescence intensity were both characteristic of the transfer of the Trpol fluorophore from a polar to a more hydrophobic environment. This was interpreted as a measure of the accessibility of the C-terminal Trpol to the lipids and thus as the result of the embedment of the peptide within the egg PC bilayer, the N-terminal part being more hydrophobic.

When the interaction experiment was carried out with TB IIIc (Fig. 1A), the λ_{max} shift and the relative fluorescence intensity enhancement were still observed but to a lesser extent. The plots of the λ_{max} shifts (Fig. 1C) and of the relative fluorescence intensity (Fig. 1D) allowed the comparison of the different affinities of the three trichorzianines for the lipidic phase. The increase in the $\Delta\lambda_{max}$ and F/F_0 values reached a plateau at higher R_i values for TB IIIc than for TA IIIc or MeTB IIIc. A R_i value characteristic of the lipid–peptide interaction could be derived from the intercept between the linear part of the curve and the plateau. For example, these R_i values, graphically determined (Fig. 1C) were 45 and 113 for TA IIIc and TB IIIc, respectively, indicating that the lipid–peptide interaction was weaker in the case of TB IIIc as compared to TA IIIc. This difference might be explained by the presence of a negative charge in TB IIIc arising from the ionization of the carboxylic acid function of the Glu-18 at neutral pH. By contrast, the accessibility of the Trpol residue of the methyl ester derivative MeTB IIIc, when incorporated into the bilayer, appeared to be greater not only than that of TB IIIc but also than that of TA IIIc, as the final value of $\Delta\lambda_{max}$ was 15 nm and the characteristic R_i value was 28 (Figs. 1B–1D). This could be due to the absence of a negatively charged carboxylate and the presence of a carboxymethyl group which possesses a more hydrophobic character than the carboxamide one in TA IIIc. These results indicated a more hydrophobic environment of the C-terminal Trpol in the order MeTB IIIc > TA IIIc > TB IIIc upon phospholipid vesicles addition, due very likely to a better incorporation of the peptide into the lipid matrix.

When egg PC vesicles containing cholesterol (30%) were used, no λ_{max} shift was observed in the emission spectra of TA IIIc and TB IIIc while a λ_{max} shift was still observed for MeTB IIIc (Figs. 1C and 1D). In this latter case, the $\Delta\lambda_{max}$ (7 nm) and F/F_0 values were much lower than those determined in the absence of sterol. These results suggested a greater exposure of the C-terminal residue to the aqueous phase when

* The observed lower value as compared to that of 360 nm previously published for TA IIIc [5], results from the application of a photomultiplier correction, whereas the previously published data had not been so corrected. The same value of 347 nm was observed on our instrument for an aqueous solution of *N*-acetyltryptophanamide used as fluorescence standard.

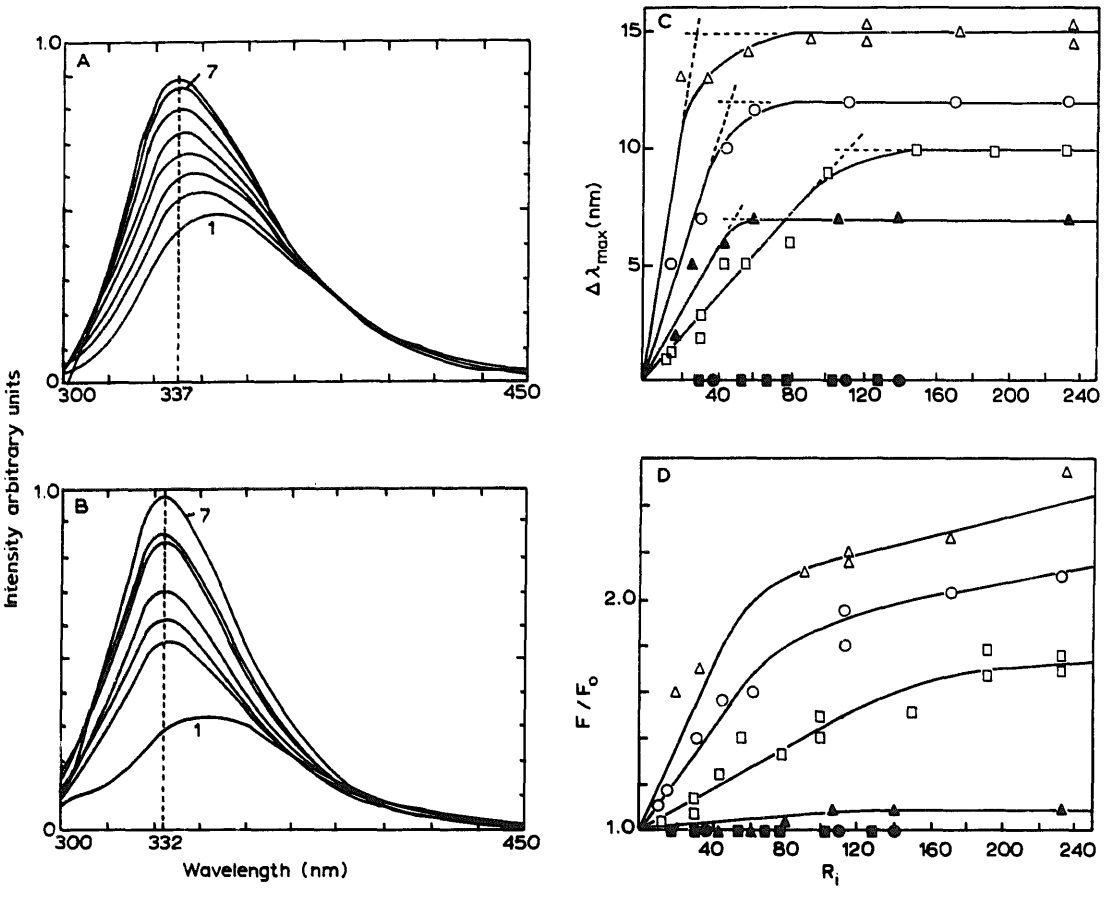

Fig. 1. Binding of trichorzianines to egg PC and egg PC/cholesterol (70:30, mol/mol) vesicles. Fluorescence emission spectra of TB IIIc (A) and MeTB IIIc (B) for increasing [lipid]/[peptide] molar ratios R_i, in the presence of increasing amounts of egg PC vesicles from $R_i = 0$ (curve 1) to $R_i = 230$ (curve 7). Fluorescence maxima shift (C) and relative fluorescence intensity F/F_0 (D) measured at the final wavelength value (MeTB IIIc: 332 nm, TA IIIc: 335 nm, TB IIIc: 337 nm) as a function of R_i for egg PC vesicles: (○) TA IIIc, (□) TB IIIc, (△) MeTB IIIc and egg PC/cholesterol (70:30, mol/mol) vesicles: (●) TA IIIc, (■) TB IIIc, (▲) MeTB IIIc. $T = 20\,°C$; cacodylate buffer (0.1 M NaCl); pH = 7; [peptide] = $0.6 \cdot 10^{-6}$ M; $\lambda_{exc} = 282$ nm.

cholesterol was incorporated into the bilayer, in agreement with the previous fluorescence quenching experiments [5].

To get deeper insight into the requirements for the trichorzianine–lipid interaction, permeability measurements were undertaken.

Trichorzianine-induced leakage of vesicular-entrapped CF

Trichorzianine-induced efflux of entrapped CF from egg PC vesicles was monitored by following the fluorescence increase, as a result of CF release from the liposomes and dilution in the external medium. The permeability experiments were performed with peptide/lipid molar ratios corresponding to a complete incorporation of the peptides within the bilayer ($R_i^{-1} = (0.2\text{–}1) \cdot 10^3$).

Addition of TA IIIc or MeTB IIIc in a concentration range of 0.3–0.4 μM to CF-entrapped vesicles induced a progressive leakage of more than 50% of the entrapped material in 20 min (Figs. 2 and 3). By contrast, addition of TB IIIc in the same concentration range led to a lower peptide-induced release of the dye (Fig. 3).

The scattering peak intensity (488 nm) remained unchanged during the leakage process, whereas it was reduced to zero when the detergent Triton X-100 was added. This indicated that the release of the intravesicular content occurred without lysis of the lipid vesicles by the trichorzianines. As the kinetic profiles of CF efflux were of the same type when varying the peptide/lipid molar ratios (Fig. 2), the observed percentage CF released at time 20 min after addition of trichorzianine was taken as a measure of the membrane permeability.

Results presented in Fig. 3 allowed us to compare the ability of the three peptides to induce permeability at neutral pH: the striking feature in these results was the weak effect of the negatively charged peptide TB IIIc. The TB IIIc potency was even more drastically reduced when cholesterol was present in the bilayer and was sharply dependent upon the sterol content (Fig. 3). The TB IIIc-induced membrane permeability of egg PC vesicles was almost unaffected between 0 and 20% cholesterol, but decreased rapidly between 20 and 30% cholesterol, indicating a threshold effect of the bilayer cholesterol content on the membrane permeability mod-

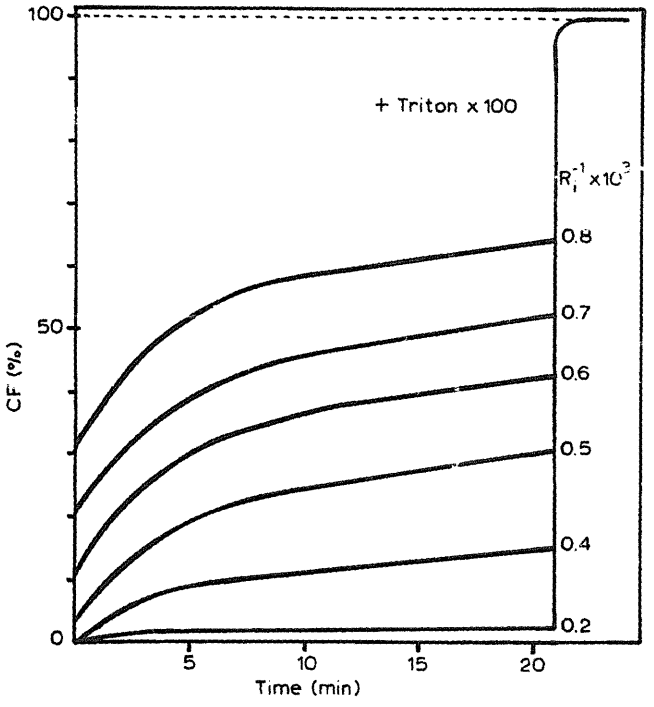

Fig. 2. MeTB IIIc-induced CF leakage from egg PC/cholesterol (70:30, mol/mol) vesicles. Time-course of CF efflux for different ratios R_i^{-1} = [peptide]/[lipid] $T = 20\,^{\circ}C$, cacodylate buffer (0.1 M NaCl), pH = 7, [lipid] = $0.45 \cdot 10^{-3}$ M, $\lambda_{exc} = 488$ nm, $\lambda_{em} = 520$ nm. Curves are re-drawn from continuous recording of the CF fluorescence increase and normalized to 100% corresponding to complete lysis of vesicles in the presence of Triton X-100. The percentages of leaked CF at time t are determined as described under Materials and Methods.

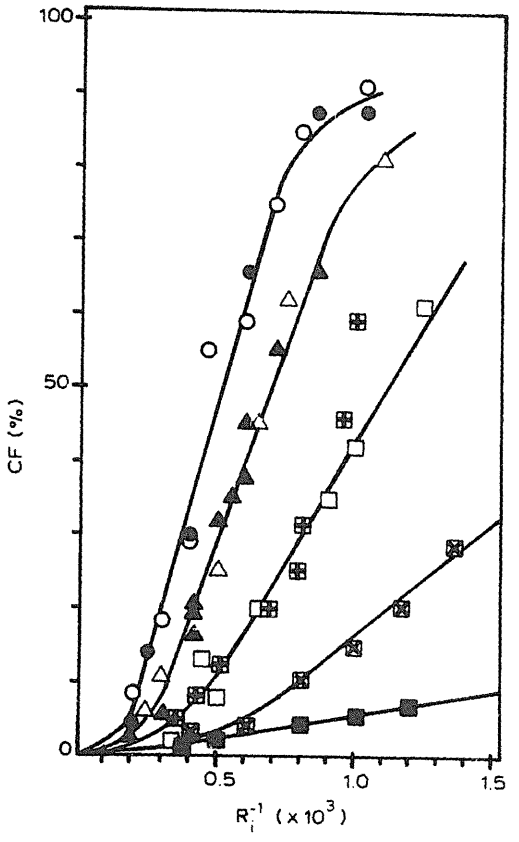

Fig. 3. Trichorzianine-induced CF leakage at $t = 20$ min for different ratios R_i^{-1} = [peptide]/[lipid] from egg PC vesicles: (○) TA IIIc, (△) MeTB IIIc and (□) TB IIIc; and from egg PC/cholesterol (70:30, mol/mol) vesicles: (●) TA IIIc, (▲) MeTB IIIc and (■) TB IIIc. For TB IIIc various egg PC/cholesterol contents were tested: (⊞) 80:20, mol/mol; (⊠) 75:25, mol/mol.

ifications induced by the charged peptides. Here again, the behaviour of TB IIIc greatly differed from that of the two uncharged peptides TA IIIc and MeTB IIIC as their membrane properties were not significantly affected by the presence of 30% cholesterol in the bilayer.

The relative ability of the trichorzianines to induce CF release was thus in the order TA IIIc ≈ MeTB IIIc > TB IIIc, which nearly followed the same order of decreased affinity for the lipid bilayer as derived from fluorescence titration curves. The presence of a negative charge at the penultimate residue in the sequence appeared thus to be a critical parameter in the trichorzianine–bilayer interaction and the peptide-induced membrane permeability, especially in the presence of a high content of cholesterol in the membrane.

The induced permeability experiments indicated that the interaction of TA IIIc with egg PC bilayers containing 30% cholesterol occurred even without observing modifications on the Trpol fluorescence, as a CF leakage was noticed. Correlation of the results derived from the Trpol fluorescence and the permeability measurements suggested that both incorporation of cholesterol in the bilayer and introduction of a negative charge at the C-terminal end of the peptide decreased the peptide embedment within the membrane and consequently their action on the membrane permeability.

Trichorzianines as inhibitors of the growth of Dictyostelium

Various amounts, up to 10 μM of trichorzianine A and 25 μM of trichorzianine B were tested for their effect on the growth of *Dictyostelium* amoebae in axenic medium and representative curves are shown in Fig. 4. In control experiments, amoebae grew with a doubling time of 9–10 h and reached a maximal density of $2 \cdot 10^7$ cells/ml. A sharp threshold level was observed for trichorzianines activity toward *Dictyostelium*. A marked inhibitory effect was noticed in the presence of 7.5 μM TA as shown first by a delay of about 20 h before growth started. The increase in doubling time during the exponential phase of growth was moderate (15 h as compared to 10 h in the control experiment) but the maximal cell yield was drastically reduced (7–8-fold) and reached only a value of $3 \cdot 10^6$ cells/ml. When the culture medium contained 10 μM TA, lysis of the cells used as inoculum occurred without any growth.

group in MeTB IIIc results in an increased efficiency of the peptide incorporation, but has no significant effect on the induced permeability.

An unfavourable role of the C-terminal negative charge is also observed toward the activity against *Dictyostelium*, as both the growth inhibition and the lysis of the amoebae are induced by lower concentrations (4–5-fold) of the neutral peptides than of the acidic one.

The biological effects toward *Dictyostelium* seem thus to be related to the membrane property modifications; therefore, the interaction of the peptides with the cell membrane lipids can be involved in the biological properties exhibited by trichorzianines.

Trichorzianine–liposome interaction

Our results demonstrate that the interaction of trichorzianines with phospholipid bilayers and the modifications of membrane permeability occur even in the absence of a transmembrane potential gradient, in agreement with recent studies on alamethicin [19,20]. The permeability modifications may be explained by disruptions in the bilayer structure leading to pores or discontinuities in the liposomes allowing free passage of the entrapped material. Leakage may also occur during fusion of the liposomes as already postulated for alamethicin [21] and melittin [22].

Although alamethicin and melittin seem to be also mainly helical in the presence of phospholipids [18,23,24], their precise conformation, orientation, and aggregation are yet open for speculation. Two types of models, still controversial are proposed to explain the interaction of alamethicin or melittin with the membranes: the pore formation which results from aggregation of helices spanning the bilayer [6,25] or the incorporation of the helices near the bilayer surface in a wedge-like structure [22,26]. Orientation of the helices within the bilayer would also depend on the physical state and hydration of the lipids [24].

The trichorzianine activity is favored by the absence of the Glu-18 negative charge, the presence of which may be thus assumed to perturb the lipid–peptide interaction. The presence of the negative charge may be critical for the deepness of the peptide embedment, or the orientation of the helices with respect to the phospholipid chains and for the aggregation state of the helices. Neutral alamethicin was shown recently to exist in an aggregated form when incorporated into a bilayer [20,27].

The unfavourable effect of the negative charge is strongly enhanced by the incorporation of 30% cholesterol in the bilayer. The presence of cholesterol in a bilayer usually results first in a compaction of the lipid chains [28] which renders the chain rigidity comparable to that of gel phase. Moreover, it induces a pushing out effect on the embedded molecules [29] resulting in an increase of exposure of their external part to the aqueous phase. Previous studies in our laboratory showed that the gel phase did not prevent the binding of TA IIIc, but that the accessibility of the Trpol fluorophore to aqueous soluble quenchers was significantly increased in the presence of cholesterol [5]. Therefore, the principal effect of cholesterol on the trichorzianine–bilayer interaction comes rather from a modification of the peptide embedment within the bilayer. When the peptide is efficiently buried into the bilayer as observed with neutral TA IIIc or MeTB IIIc, the induced permeability is not affected by the pushing-out effect. However, the presence of the Glu-18 negative charge in TB IIIc may prevent a maximal hydrophobic interaction of the peptide with the acyl chains of the lipids, resulting in a reduced membrane permeability. The threshold effect on the permeability modifications at 20% cholesterol, remarkably coincides with the disappearance of pure phospholipid domains occurring at such a sterol ratio [28].

Trichorzianine–Dictyostelium interaction

Few studies have been reported with respect to the sensitivity of *Dictyostelium* toward antibiotic peptides. Growth and differentiation were shown to be inhibited by the peptides polymixine and colimycine [30] which belong to a quite different class from the peptaibols.

The plasma membrane of *Dictyostelium* is made up mostly of phosphatidylcholine and phosphatidylethanolamine, and contains 40% sterols [31]. The differences noticed between the activity of TA IIIc and TB IIIc on model membranes and on *Dictyostelium* follow a similar trend as the neutral peptides are much more efficient on both systems. Although it appears evident that trichorzianines induce in fact complex effects on amoeba cells, a tentative explanation of their action on *Dictyostelium* may take into account the conclusions derived from the experiments conducted on model membranes. By formation of disruptions in the plasma membrane, the trichorzianines would act by increasing the membrane permeability, ending finally with cell lysis.

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