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Fluorescence studies of the interaction of trichorzianine A IIIc with model membranes

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The biological activity and the chemical structure of the lipophilic peptides, trichorzianines, suggested that these substances could act on membrane permeability. The interaction of a major component of trichorzianines, trichorzianine A IIIc (TA IIIc), a 19-residue peptaibol containing tryptophanol as C-terminal amino-alcohol, with some synthetic phospholipid vesicles (egg phosphatidylcholine (egg PC), dipalmitoylphosphatidylcholine (DPPC) and sterol-containing egg PC) was studied by fluorescence spectroscopy. TA IIIc was found to bind to lipid vesicles either in liquid-crystalline or gel state. The accessibility to the aqueous phase of the embedded peptide was examined for various phospholipid compositions by fluorescence quenching experiments. We found that incorporation of TA IIIc in egg PC vesicles leads to reduced accessibility of the C-terminal tryptophanol to external quenchers, whereas when sterols are present in the bilayer, this accessibility is higher, consistent with a higher exposure of the chromophore to the aqueous phase. TA IIIc was shown to induce leakage of vesicular entrapped material. Incorporation of sterols in the bilayer seems to influence the position of the bound peptide within the bilayer but not its action on the membrane permeability.

Introduction

Trichorzianines are 19-amino-acid long linear peptides biosynthesized by *Trichoderma harzianum* which play an important role in the antagonistic properties exhibited by this fungus against some other microscopic fungi [1]. They are structurally related to peptaibols [2,3] of which alamethicin is widely studied and is known to alter the permeability of synthetic and biological membranes [4,5]. It was thus of interest to examine the interaction of trichorzianines with membranes and the subsequent changes that are induced.

Structural studies on a major component of trichorzianines, TA IIIc [3] reveal the following sequence:

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                1                   5                  10
TA IIIc: Ac-Aib-Ala-Ala-Aib-Aib-Gln-Aib-Aib-Aib-Ser-Leu-

                      15                  19
         Aib-Pro-Val-Aib-Ile-Gln-Gln-Trpol
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The 1–12 N-terminal part of TA IIIc mostly made up of hydrophobic residues with a high

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; SUV, small unilamellar vesicles; TA IIIc, trichorzianine A IIIc.

content of α-aminoisobutyric acid (Aib) is helically structured in organic solvent solutions. The C-terminal part contains more polar residues (Gln-17, Gln-18) and the amino-alcohol tryptophanol (Trpol-19).

We took advantage of the presence of the single fluorescent chromophore, tryptophanol, to study the interactions of TA IIIc with synthetic bilayer vesicles by fluorescence spectroscopy. The results will be discussed in relation to the depth of insertion of the peptide within the bilayer. We also report results on the peptide-induced membrane permeability by following the leakage of vesicle-entrapped carboxyfluorescein. The role of the chain-length was examined by using a peptide (NA VII) closely related to the 1–12 fragment of TA IIIc:

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                1                   5
NA VII: Ac-Aib-Ala-Ala-Aib-Iva-Gln-Aib-Aib-Aib-

          10
        Ser-Leu-Aib      (Iva = isovaline)
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Materials and Methods

TA IIIc was extracted from a spore culture of *Trichoderma harzianum* and purified according to the procedure described earlier [3]. Partial acid hydrolysis of trichorzianine TA VII led to a mixture of oligopeptides from which the fragment 1–12 (NA VII) was isolated by column chromatography: (1) Sephadex LH-20, MeOH; (2) SiO_2, CH_2Cl_2/MeOH (70 : 30, v/v). The peptides used were shown to be at least 95% pure by HPLC, Waters μBondapak C_{18}; EtOH/H_2O (65 : 35, v/v) (TA IIIc) or (50 : 50, v/v) (NA VII).

Tryptophan octyl ester, egg L-α-phosphatidylcholine (egg PC), type VII E, and dipalmitoyl-L-α-phosphatidylcholine (DPPC) were purchased from Sigma and used without further purification. Cholesterol (Sigma) was recrystallized from MeOH and ergosterol (Sigma) was purified by chromatography (SiO_2, hexane/ethyl acetate (70 : 30, v/v). Carboxyfluorescein (Eastman Kodak) was further purified according to Leserman and Barbet [6]. All other chemicals used for making buffers were of analytical grade.

Small unilamellar vesicles (SUV) were prepared by sonication to clarity (15 min) of 10^{-4} M solutions of either egg PC (0°C) or DPPC (60°C) in cacodylate buffer (pH 7.0) under nitrogen with a Branson sonifier Model B15 equipped with a microtip probe (duty cycle: 20%; power output: 30 W).

Carboxyfluorescein-encapsulated lipid vesicles were prepared according to Weinstein et al. [7]; the lipid vesicles were incubated in a 51 mM carboxyfluorescein solution (Hepes buffer, pH 7.4) for 15 h before sonication. Unencapsulated carboxyfluorescein was removed by filtering the vesicle solution through a Sephadex G-75 column (0.5 × 8 cm). Dilution factor was determined for each gel filtration.

Aliquots of methanolic solutions of peptides were added to SUV. The concentration of methanol in the final solution was kept below 0.5% by volume. About 10% of the added peptides were found to adsorb on the cell walls and this estimation was carried out by desorbing the peptides in methanol and measuring the concentration by fluorescence (TA IIIc) or absorption (NA VII) spectroscopy.

The concentrations of lipid vesicles were determined by measuring the radioactivity of [^{3}H]phosphatidylcholine which was used to label the phospholipid mother solutions.

Absorption measurements were done on an Uvikon spectrophotometer model 820 (Kontron) and fluorescence spectra were recorded on an Aminco SPF 500 spectrofluorimeter at 20°C.

Fluorescence quenching measurements were performed by adding KI to peptide/vesicles solutions. Results were analysed by using the Lehrer-modified Stern-Volmer relationship [8]: $F_0/\Delta F = 1/([Q]f_a\tau_0 k) + 1/f_a$ where F_0 is the fluorescence intensity in the absence of quencher, ΔF is the difference in fluorescence intensity in the absence and in the presence of quencher, [Q] is the concentration of quencher, τ_0, is the lifetime of the excited state, k is the rate constant for the deactivation of a fluorophore by a quencher, and f_a is the fraction of total fluorescence available to the quencher. K_{SV} is defined as equal to $\tau_0 k$.

Results and Discussion

Binding of tryptophan-containing peptides to phospholipid vesicles usually resulted in a shift of

the emission maximum (λ_{max}) to shorter wavelengths and to an enhancement of fluorescence intensity due to the transfer of the chromophore from a polar to a more hydrophobic medium. These spectral changes have been exploited for studies of lipid-peptide interaction [9–12]. TA IIIc in aqueous solution exhibited a λ_{max} at 360 nm, indicative of a highly polar environment of tryptophanol. When egg PC vesicles were added, this fluorescence maximum was shifted to shorter wavelengths till a final value of 340 nm ($\Delta\lambda = 20$ nm) was reached. Absorbance of the peptide at the excitation wavelength, 280 nm, remained constant in the presence of the vesicles. The relative fluorescence intensity F/F_0 (F and F_0 are the fluorescence intensities of the peptide in the presence and in the absence of vesicles, respectively) measured at 340 nm increased almost linearly as the lipid-to-peptide molar ratio (R_i) increased and then reached a plateau (Fig. 1A). The intercept of the linear part of the binding curve and the plateau allowed us to determine the parameter R_{iB} which represents the minimum number of phospholipid molecules required for the binding of one molecule of peptide. Tryptophan octyl ester was used as a simple model for a hydrophobic chain bearing a tryptophanyl polar head; the R_{iB} value measured for its interaction with egg PC is 20. In the presence of phosphatidylcholine vesicles (egg PC and DPPC), the number of phospholipids required for the binding of one TA IIIc molecule was higher (30 and 50, respectively). Thus, the gel state of phospholipids did not prevent the binding of TA IIIc. The only observed difference consisted in the higher R_{iB} value in the case of DPPC where acyl chains are highly organized at 20°C. This was accompanied by a smaller blue shift of the fluorescence maximum at a given R_i value for DPPC as compared to egg PC vesicles (Fig. 1B). In contrast, for egg PC vesicles containing sterols, the corresponding R_{iB} values were much lower (less than 10) and no λ_{max} shift was noted, indicating a greater exposure of the C-terminal residue into the aqueous phase.

The above results were confirmed by quenching experiments of tryptophanol fluorescence by KI. The study was made at a high R_i value (approx. 100) where a complete binding of TA IIIc to vesicles was assumed (Fig. 1). The modified Stern-Volmer plot (Fig. 2) allowed us to evaluate the Stern-Volmer constant, K_{SV}, and f_a, the fraction of fluorescence accessible to the quencher [8].

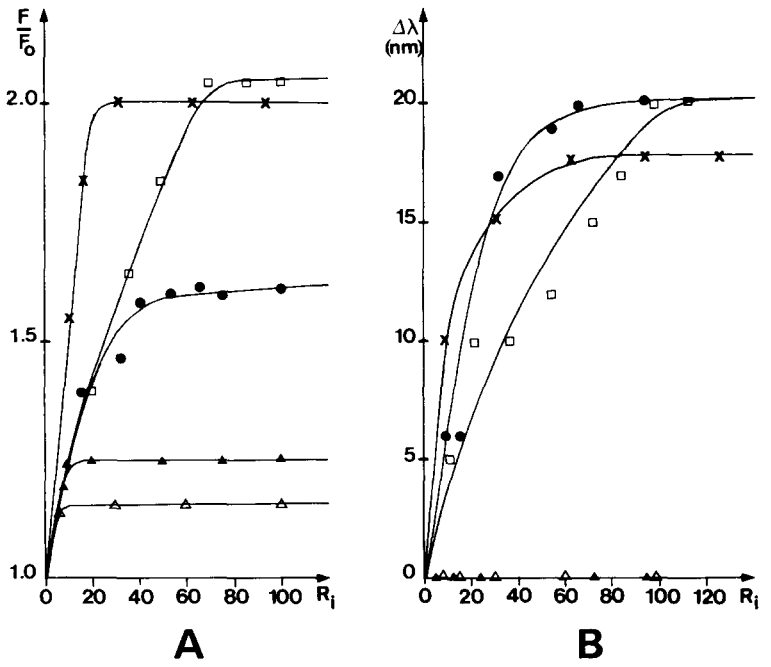

Fig. 1. (A) Relative fluorescence intensity (340 nm) and (B) $\Delta\lambda$ as a function of the lipid-to-peptide molar ratio, R_i, for the systems ($T = 20$°C): ●, TA IIIc, egg PC; □, TA IIIc, DPPC; ▲, TA IIIc, egg PC/ergosterol (70:30, mol/mol); △, TA IIIc, egg PC/cholesterol (70:30, mol/mol); and ×, tryptophan octyl ester/egg PC. TA IIIc = 0.35 μM; tryptophan octyl ester = 0.60 μM; $\lambda_{exc.}$ 280 nm.

The free peptide was characterized by a K_{SV} value of 16.6 M^{-1} and a full accessibility of the tryptophanol residue to water ($f_a = 1$). Once bound to egg PC vesicles, K_{SV} was found to increase (K_{SV} = 35.7 M^{-1}), while f_a fell to 0.4, showing embedment of an important fraction of the peptide within the bilayer. The increase of K_{SV} might be due to an increase in the tryptophanol fluorescence lifetime τ_0 as shown previously by Le Doan et al. [11] for tryptophan-containing peptides. In the presence of sterol-containing vesicles, K_{SV} values (14.5 M^{-1} for cholesterol and 11.4 M^{-1} for ergosterol) were found close to the K_{SV} value of the free peptide while the f_a values were higher (0.6) than that of pure egg PC vesicles. These results are consistent with an increase of surface exposure of the tryptophanol when sterols are incorporated into the membrane as shown by Borochov et al. [13] for membrane proteins in erythrocytes with different cholesterol contents.

The TA IIIc-membrane interaction is thus highly hydrophobic in character and not strongly affected by the physical state of the lipid vesicles. However, tryptophanol is less accessible to external fluorescence quencher in pure egg PC than in sterol-containing egg PC bilayers.

The influence of the peptide embedment on membrane permeability was assayed by following the peptide-induced release of the intravesicular content. In this series of experiments, the kinetics of leakage of entrapped carboxyfluorescein induced by TA IIIc on egg PC and egg PC-sterol vesicles were studied using the relief of quenching of carboxyfluorescein fluorescence upon dilution [7]. The method was also exploited to examine the ability of the 12-residue peptide, NA VII, to modify membrane permeability.

Fig. 3 illustrates typical time-course for the carboxyfluorescein fluorescence increase induced by TA IIIc on egg PC vesicles in the peptide concentration range: 0.06–0.3 μM. In the absence of peptide, the spontaneous leakage of entrapped carboxyfluorescein was less than 5% in 60 min. The carboxyfluorescein leakage was significantly but gradually increased upon addition of TA IIIc. The scattering peak intensity of the vesicle solution, indicative of the vesicle integrity, remained unchanged during the leakage process. It was re-

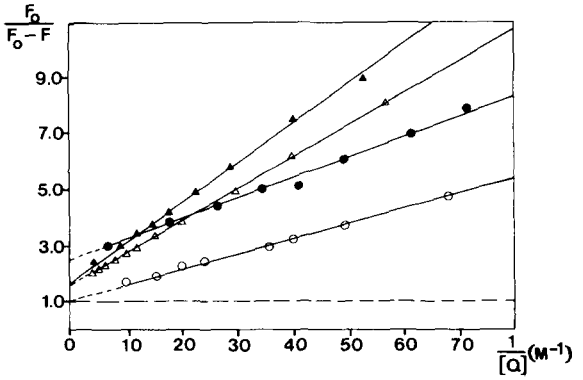

Fig. 2. Stern-Volmer plot of quenching of TA IIIc fluorescence by I^-. The experiments were carried out by adding small aliquots of a 5 M solution of KI to a 0.67 μM solution of TA IIIc (pH 7; 20°C), in the absence (○) or in the presence of vesicle: ●, egg PC; △, egg PC/cholesterol (70:30, mol/mol); ▲, egg PC/ergosterol (70:30, mol/mol). $R_i \approx 100$; $\lambda_{exc.} = 280$ nm; ------, KCl alone (on egg PC and sterol-egg PC vesicles containing TA IIIc). The medium contained 10^{-4} M $Na_2S_2O_3$ to prevent I_3^- formation.

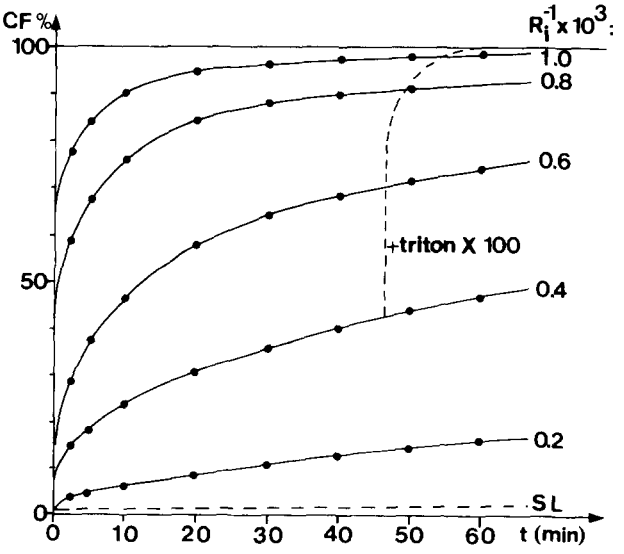

Fig. 3. TA IIIc-induced carboxyfluorescein leakage from egg PC vesicles. Time-course of carboxyfluorescein efflux for different ratios R_i^{-1} = [peptide]/[lipid] ([lipid] = 0.3 mM). The fluorescence of carboxyfluorescein was measured at 520 nm ($\lambda_{exc.}$ 488 nm). Percentage leaked carboxyfluorescein at time t was determined as % carboxyfluorescein = $(F_t - F_0)/(F_T - F_0) \times 100$, where F_0 is the fluorescence intensity of the original liposome suspension, F_t that at the time t and F_T that after addition of 20 μl of a 10% solution of Triton X-100. SL, spontaneous leakage.

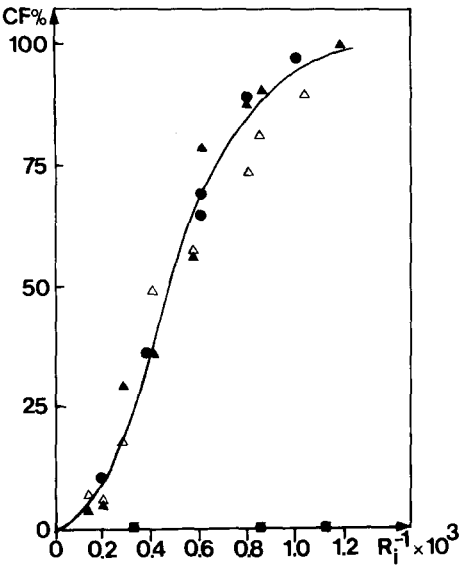

Fig. 4. TA IIIc-induced carboxyfluorescein leakage at $t = 30$ min from egg PC (●); egg PC/cholesterol (70:30, mol/mol) (△) and egg PC/ergosterol (70:30, mol/mol) (▲) vesicles as a function of R_i^{-1}. (■) NA VII-induced carboxyfluorescein leakage from egg PC vesicles (same conditions).

duced to approx. zero upon addition of Triton X-100, which solubilized the membrane. Thus, the observed leakage of carboxyfluorescein was not due to the lysis of the bilayer by TA IIIc. We examined the carboxyfluorescein leakage at 30 min as a function of R_i^{-1} ([peptide]/[lipid] ratio) and observed a rapid increase of escaped carboxyfluorescein in the range $R_i^{-1} = 2 \cdot 10^{-4}$ to $1 \cdot 10^{-3}$ (Fig. 4). The incorporation of sterols in the bilayer had no significant influence on the rate of TA IIIc-induced CF leakage (Fig. 4), indicating that no important change in the lipid-peptide association took place.

A number of amino acids greater than 12 appeared to be necessary for the activity, as we observed no significant leakage in the presence of the fragment NA VII in the same concentration range (Fig. 4). This effect of chain-length on the TA IIIc activity is in agreement with the structure-activity relationship previously described for the cation-translocating effect of alamethicin [14].

In conclusion, the results of this study show that the amphiphilic peptide, trichorzianine A IIIc, interacts with phospholipid bilayers either in gel or liquid-crystalline state and causes the leakage of vesicular entrapped material. Incorporation of sterols in the bilayer leads to a displacement of the embedded peptide in the membrane rather than to a change of the lipid-peptide association, as it does not affect the induced modifications of membrane permeability. It must be noted that TA IIIc induces the leakage of entrapped material starting from peptide/lipid ratio as low as 10^{-4}.

Further studies are now in progress in our laboratories in order to understand the nature of the membrane perturbation induced by the trichorzianines.

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