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LIPID-PROTEIN INTERACTIONS

A COMPARATIVE STUDY OF THE BINDING OF CARDIOTOXINS AND NEUROTOXINS TO PHOSPHOLIPID VESICLES

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Summary

It has recently been shown that cardiotoxin II from *Naja mossambica mossambica* specifically interacts with negatively charged phospholipids (Dufourcq, J. and Faucon, J.F. (1978) Biochemistry 17, 1170—1176). In order to investigate whether or not short neurotoxins give rise to similar interactions, four techniques have been used, namely intrinsic fluorescence, fluorescence polarization of 1,6-diphenylhexatriene, turbidity measurements and release of 6-carboxyfluorescein trapped inside single shelled vesicles.

Neurotoxin III from *Naja mossambica mossambica* and neurotoxin I from the venom of the scorpion *Androctonus australis* Hector, specifically interact with negatively charged phospholipids leading to changes in tryptophan fluorescence and to a decrease of the fluidity of the bilayer. Cardiotoxin II from the same snake venom gives similar results. On the other hand, it seems that either a very weak or no interaction at all occurs in the case of neurotoxin I from the same *Naja* venom.

There are important differences in the behaviour of cardiotoxin and neurotoxins: (i) neurotoxins lead to only weak release of 6-carboxyfluorescein from lipid vesicles, whereas cardiotoxin II induces fast and quantitative escape of the dye and then a general breakdown of the vesicular structure; (ii) binding of neurotoxins can be easily reversed by 100—200 mM NaCl or less than 1 mM Ca^{2+} and so it is essentially electrostatic, whereas binding of cardiotoxin II seems to involve some hydrophobic contribution.

The short neurotoxins and cardiotoxins from snake venom having a great

homology in sequence, their differences on binding properties are discussed in terms of changes in a particular area of the sequence.

Snake neurotoxins and cardiotoxins, in spite of their great homology in sequences, act in a very different way towards membranes. Neurotoxins are highly specific for only a few cell targets, whereas cardiotoxins are described as direct lytic factors or cytotoxins, due to their ability to disrupt many kinds of membrane. It has recently been shown that strongly basic cardiotoxins interact selectively with negatively charged phospholipids [1,2]. The formation of such lipid-protein complexes can account for most of the known features of cardiotoxin action on natural membranes. Since neurotoxins are also basic proteins, one may wonder if they lead to the same type of interaction with phospholipids.

In the present work, an attempt is made to analyse this possibility. Neurotoxins I and III and cardiotoxin II from the venom of the *Elapidae* snake *Naja mossambica mossambica* were compared for their binding to lipids. A scorpion neurotoxin, toxin I, from the venom of *Androctonus australis* Hector, which is also a small molecular weight basic protein, was investigated.

Detection of lipid-protein interactions has been carried out by four independent methods which have already been proved useful: intrinsic fluorescence of the proteins [1—5]; turbidity measurements [6]; fluorescence polarization of a probe embedded in the lipid bilayer [7]; and release of a dye trapped in single shelled vesicles.

Material and Methods

Phospholipids were purified in the laboratory, phosphatidylcholine from hen egg yolks [8], phosphatidylserine from bovine brain [9] and phosphatidylinositol from baker's yeast [10]. Phosphatidylserine and phosphatidylinositol have also been purchased from Lipid Products (U.K.).

The selected phospholipids were suspended in 20 mM Tris-acetate buffer, pH 7.5/1 mM EDTA, and sonicated for about 10 min at 4° C (egg phosphatidylcholine and phosphatidylinositol) or 20° C (phosphatidylserine) under nitrogen. As was previously shown [11,12], it resulted mainly in single shell vesicles which were used directly.

Leakage experiments were done with 6-carboxyfluorescein from Eastman Kodak, purified according to Weinstein et al. [13]. Lipids were suspended in a buffer containing 200 mM 6-carboxyfluorescein, then sonicated and filtered on a Sephadex G25 column to eliminate the untrapped dye [13]. Vesicles were then kept at 4° C in order to minimize their leakiness; they could be used in these conditions several days later.

Fluorescence spectra and light scattering measurements were performed on a FICA 55 MK II differential spectrofluorometer which automatically recorded corrected excitation and emission spectra. Unless otherwise mentioned, all the spectra were obtained in the following conditions: excitation wavelength 280 nm, excitation and emission slits 7.5 nm, temperature 25° C. Fluorescence polarization measurements were carried out on an apparatus built in the laboratory.

The hydrophobic probe, 1,6-diphenyl-1,3, 5-hexatriene, obtained from Aldrich Chem. Co., was introduced in the lipid bilayer at a molar ratio always less than 1%, either by colyophilization with lipid or by addition of some μl of a tetrahydrofuran solution to preformed vesicles [14].

Scorpion neurotoxin I of *Androctonus australis* Hector and snake neurotoxins I and III of *Naja mossambica mossambica* were purified as previously described [15,16]. Cardiotoxin II from the same snake venom corresponds to cytotoxins v_2^{II} of Louw [17]; it was purified as described by Jover [18].

Results

Intrinsic fluorescence

Intrinsic fluorescence has first been used to detect interaction with lipids. The fluorescence spectra of these toxins are dominated by their tryptophan emission, one or two residues, although they possess several tyrosines. The maxima centered around 350 nm indicate that all tryptophan residues are exposed to water.

Upon addition of phosphatidylserine vesicles, no significant change is observed on the maximum wavelength; however, it results in various effects on the fluorescence intensity, depending on the toxin used (Fig. 1). With scorpion neurotoxin I, a 25% decrease is observed when lipids are added. No change is detected for snake neurotoxin I, but neurotoxin III leads to an increase of fluorescence intensity of 15%. In all cases, these effects are much more weak than those previously obtained for cardiotoxin II, in which case a 15 nm blue shift is paralleled by a 230% increase in fluorescence intensity [1].

Another negatively charged lipid, phosphatidylinositol, gives with snake neurotoxin III results very similar to those obtained with phosphatidylserine (Fig. 1). On the other hand, when a zwitterionic phospholipid, such as egg

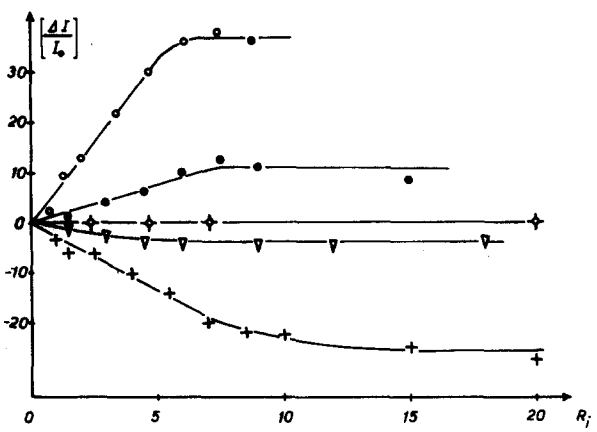

Fig. 1. Relative changes in the fluorescence intensity at 350 nm of the different neurotoxins upon addition of phospholipid. 20 mM Tris-acetate buffer/1 mM EDTA, pH = 7.5. R_i is the lipid to protein molar ratio. Binding to phosphatidylserine vesicles: ▽———▽, snake neurotoxin I (5 μM); ●———●, snake neurotoxin III (1 μM); + ——— +, scorpion neurotoxin I (5 μM). Binding to egg phosphatidylcholine vesicles (—◇—) and phosphatidylinositol (○———○) of snake neurotoxin III (1 μM).

phosphatidylcholine, is allowed to interact with toxins no effect at all is detected despite the large lipid to protein molar ratio used (Fig. 1).

One has to notice that increasing the ionic strength of the medium up to 100—200 mM NaCl or 1 mM Ca^{2+} after formation of the complexes with phosphatidylserine leads to the recovery of the signal of free protein.

Light scattering

Turbidity changes have been followed during the fluorescence experiments by measuring the intensity of the incident light scattered at 90°. The relative changes induced by toxin additions to phosphatidylserine vesicles are plotted versus the protein to lipid molar ratio $10^2/R_i$ in Fig. 2. A drastic increase is observed in the case of cardiotoxin II and neurotoxin III; only very small variations are detected with both snake and scorpion neurotoxins I. Moreover, on increasing ionic strength for neurotoxin III, the turbidity decreases down to the value of vesicles alone; the mid-point is 60—80 mM NaCl or less than 1 mM Ca^{2+}.

Release of a dye trapped inside the vesicles

As it has been shown by Weinstein et al. [13], the fluorescence of 6-carboxyfluorescein trapped at high concentration inside the vesicles is severely quenched by interactions between fluorophore molecules. When 6-carboxyfluorescein is released either by passive permeation or by the disruption of the vesicles, the dye dilutes into the entire solution volume and its fluorescence is no longer quenched. The fluorescence intensity then increases proportionally to the amount of dye released up to about 20 times its initial value.

The leakage of 6-carboxyfluorescein induced by the toxins is generally not very reproducible, and so it is difficult to deduce quantitative information from

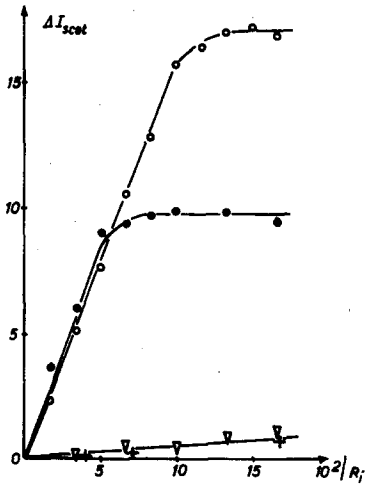

Fig. 2. Turbidity increase induced by the different toxins (arbitrary units). The toxins are added to vesicles [PS] = 15 μM; 20 mM Tris-acetate/1 mM EDTA, pH = 7.5. ○———○, cardiotoxin II; ●———●, snake neurotoxin III; ▽———▽, snake neurotoxin I; + ——— +, scorpion neurotoxin I.

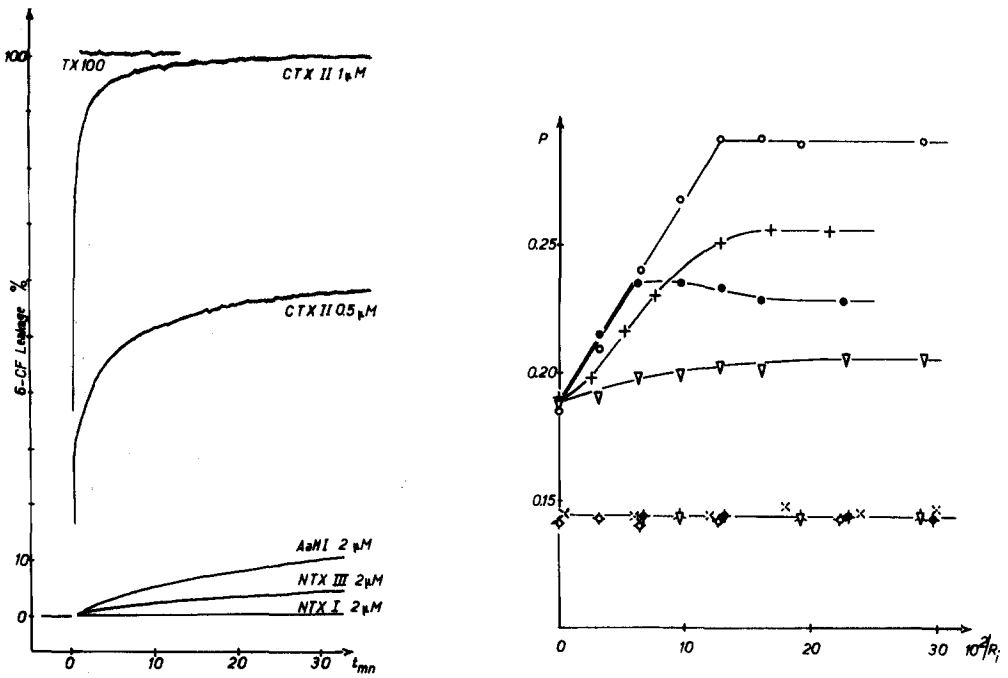

Fig. 3. Kinetics of the release of 6-carboxyfluorescein trapped inside phosphatidylserine single-shelled vesicles. At zero time, amounts of the various toxins are added to the solution containing the vesicles: [PS] = 20 μM; dye concentration inside the vesicles, 200 mM. The experiments are done at 6°C, excitation wavelength 490 nm, emission 520 nm. Control experiments with Triton X-100 allow for each kinetic to obtain the full release of the dye. CTX II, cardiotoxin II; NTX I, snake neurotoxin I; NTX III, snake neurotoxin III; Aah I, scorpion neurotoxin I.

Fig. 4. Variation of the degree of polarization, P, of diphenylhexatriene embedded in lipid vesicles upon addition of toxins. Effect on phosphatidylserine vesicles: ○——○, cardiotoxin II; ●——●, snake neurotoxin III; ▽——▽, snake neurotoxin I; + ——+, scorpion neurotoxin I. Effect on egg phosphatidylcholine vesicles: —◇——◇—, cardiotoxin II; —✳——✳—, snake neurotoxin III; —▽——▽—, snake neurotoxin I; —✳——✳—, scorpion neurotoxin I.

this type of measurement. However, it is clear from the results obtained that cardiotoxin II, which is a well-known direct lytic factor, always induced very significant release of the dye (Fig. 3). Recently, using a paramagnetic probe, Hsia et al. [19] obtained similar results. On the other hand, the effects induced by neurotoxins are much weaker: for scorpion neurotoxin I, the release is always less than 10%; for snake neurotoxin III it never exceeds a few percent; with snake neurotoxin I no effect at all is observed (Fig. 3).

Fluorescence polarization measurements

The variation of the degree of polarization, P, of diphenyl hexatriene embedded in phosphatidylserine vesicles upon addition of the proteins is plotted in Fig. 4 versus $10^2/R_i$. The P value, which is equal to 0.190 for pure vesicles, is notably enhanced with all the proteins used, except snake neurotoxin I. Since in all cases, the fluorescence intensity of the probe also increases, one can conclude that the mobility of diphenylhexatriene is reduced when cardiotoxin II, scorpion neurotoxin I and snake neurotoxin III are added to

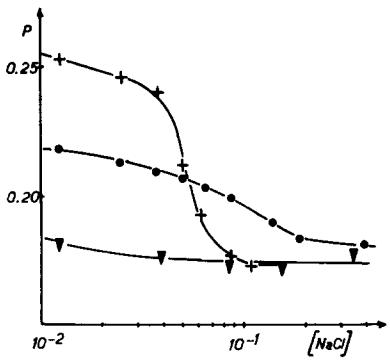

Fig. 5. Variation of the degree of polarization, P, with NaCl concentration: ●———●, snake neurotoxin III-phosphatidylserine complexes, $R_i = 4.5$; + ——— +, scorpion neurotoxin I-phosphatidylserine complexes, $R_i = 4.5$; ▼———▼, phosphatidylserine alone.

phosphatidylserine vesicles. With egg phosphatidylcholine vesicles is detected no effect at all on the P value, whatever the toxin used (Fig. 4).

The observed effects on P can be reversed by an increase in ionic strength, as can be seen in Fig. 5. The initial P values are recovered for NaCl concentrations of about 100—200 mM in the case of scorpion neurotoxin I and snake neurotoxin III, whereas it has previously been shown that 1 M NaCl is needed to dissociate the cardiotoxin II-phosphatidylserine complexes [1].

Discussion

Four techniques have been used in order to detect whether neurotoxins bind to phospholipids; the results traduce perturbation of the protein, of the lipid matrix or of the whole vesicular structure. One can conclude unambiguously that at least two neurotoxins, neurotoxin III from *Naja mossambica mossambica* and neurotoxin I from *Androctonus australis* Hector, interact with negatively charged lipids. The number of lipid molecules bound per neurotoxin is close to that previously determined for cardiotoxin II, one protein for seven phospholipid molecules [1]. For snake neurotoxin I, it seems that either a very weak interaction or no interaction at all occurs, in agreement with a previous work [2]. Finally, no effect is observed when neurotoxins are added to egg phosphatidylcholine vesicles, indicating that towards negative lipids they exhibit the same specificity as cardiotoxin II [1].

Both cardiotoxin II and neurotoxins lead to a decrease of the fluidity of the lipid bilayer. However, a general breakdown of the vesicular structure does not seem to occur with neurotoxins, since trapped 6-carboxyfluorescein is only very partially released in that case. So, the reversible increase in turbidity observed with snake neurotoxin III could be due simply to the aggregation of the lipid vesicles.

A very important point concerning the biological significance of these results is the stability of the lipid-protein complexes. As has already been shown [1], the affinity of cardiotoxin II for negative lipids is very high ($K_a >> 10^6$ M) and its complex with phosphatidylserine is half-dissociated only by 1 M NaCl or 12

mM Ca^{2+}. On the contrary, even if the K_a values are also rather large, the binding occurring in the micromolar range, the neurotoxin-phosphatidylserine complexes are totally dissociated by 100—200 mM NaCl or less than 1 mM Ca^{2+}. As a consequence, this lipid-protein interaction should not play a major role in most of the biological experiments, since ionic strength in physiological conditions is high enough to prevent them.

A possible explanation of the different binding properties between snake cardio- and neurotoxins can be inferred from analysis of their sequences [17] and of the preliminary X-ray results on the structure of short snake neurotoxins [20,21]. As pointed out by Dufton and Hider [22], an important feature of cardiotoxins is that the first loop 7—16 which contains Trp has a marked hydrophobic character. The hydrophobic index of the sequence 9—14 of cardiotoxin II calculated as quoted by Segrest [23] is $HI_{9-14} = 3.8$. Moreover, up to five ammonium groups are located close to this hydrophobic part. So, the binding of this cardiotoxin occurs probably in two steps: (i) basic residues would interact with either phosphate or carboxyl groups of lipids (which could explain the specificity towards negative lipids); (ii) the penetration of hydrophobic residues into the bilayer would afterwards lead to the stabilization of the complex (and also to the drastic changes in the fluorescence spectrum).

In the case of snake neurotoxins, the same first loop is, on the contrary, rich in hydrophilic residues, $HI_{9-14} = 0.7$, and moreover, there is nowhere in the sequence any hydrophobic area [22]. So, only the first electrostatic step would occur upon binding, which could explain the weaker stability of the complexes. The absence of any hydrophobic area does not allow us to define which part of the protein interacts with lipids. The effects observed on fluorescence spectra of Trp residues located respectively in position 29 for neurotoxin I and 20, 29 for neurotoxin III, could be due to a conformational change as well as to a direct interaction of Trp with lipids. However, the weakness of the fluorescence changes, compared to those obtained with cardiotoxin, seems to support the first hypothesis.

The behaviour of scorpion neurotoxin I is difficult to compare to that of other toxins since its sequence is quite different. However, it seems also possible to conclude that only electrostatic interactions are involved in this case, leading to some conformational change which could explain the observed quenching of the tryptophan fluorescence.

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