PROPERTIES OF ASSOCIATION OF CARDIOTOXIN WITH LIPID VESICLES AND NATURAL MEMBRANES

A fluorescence study

Jean-Pierre VINCENT, Marco BALERNA and Michel LAZDUNSKI
Centre de Biochimie, Faculté des Sciences, Université de Nice, Parc Valrose, 06034 Nice, France

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1. Introduction

Neurotoxins and cardiotoxins isolated from snake venom are parent proteins originating from a commor ancestral gene [1,2]. The essential features of the molecular mechanism of the association of snake neurotoxins with the membrane-bound acetylcholine receptor are now well understood (review [3]). Cardiotoxin inhibits the sodium–potassium activated adenosine triphosphatase activity of both excitable [4] and non-excitable cells [5]. Binding studies of radioactivity-labelled cardiotoxin to axonal membranes led us to propose that cardiotoxin associates to the lipid phase of membranes [4]. The present work analyses the specificity and some of the physicochemical properties of the interaction between cardiotoxin and phospholipids.

2. Materials and methods

Cardiotoxins were purified from *Naja mossambica mossambica* venom as described [4]. The present work was carried out with cardiotoxin C which corresponds to cardiotoxin V_I^{II} in the nomenclature adopted [6]. Neurotoxin I from the same venom was prepared as described [7].

Abbreviations: PC, phosphatidyl choline; SM, sphingomyelin; PS, phosphatidyl serine; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PA, phosphatidic acid; EDTA, ethylene diamine tetraacetic acid

Commercial phospholipids and gangliosides (Serdary, Canada) were of the following origin: PC, SM and gangliosides, beef brain; PS, bovine brain; PE and PI, pig liver; PA, egg lecithin. Fatty acids (oleate and palmitate) were obtained from Fluka.

Axonal membranes from the crab *Cancer pagurus* were prepared in this laboratory as described [8]. Phospholipid vesicles were prepared as in [9].

Fluorescence measurements were performed in a Perkin Elmer fluorescence spectrophotometer Model MPF-3 (excitation wavelength: 280 nm; excitation and emission slits: 7 nm; temp. 25°C).

3. Results

3.1. *Interaction of cardiotoxin with phospholipids*

The fluorescence emission spectrum of cardiotoxin is shown in fig.1A. The emission maximum is centered around 343 nm indicating that the spectrum is mainly due to the only tryptophane residue of cardiotoxin (Trp_{11} [10]) and that the indole side-chain is partially buried [11,12].

Addition of vesicles of negatively charged phospholipids to the cardiotoxin solution produces two main changes: a shift of the wavelength at the maximum from 343–333 nm and an increase in the maximal fluorescence intensity. Both effects reflect the change of environment of Trp_{11} to a less polar one and indicate an association between cardiotoxin and phospholipids. Simultaneously, a large increase in turbidity is observed, showing that phospholipid vesicles are

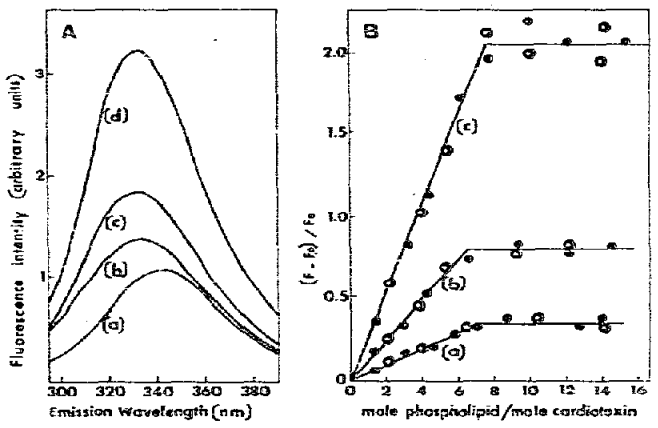

Fig.1. Interaction of cardiotoxin with PS, PA and PI. Cardiotoxin was dissolved at 25°C in a 50 mM Tris–HCl buffer at pH 8.0 containing 1 mM EDTA to inhibit any trace of phospholipase activity. Aliquots of stock solutions of negative phospholipid vesicles were added and the fluorescence emission spectrum was recorded immediately after mixing. 1A: Spectrum of free cardiotoxin (a) and of complexes formed between cardiotoxin and PI (b), PA (c) and PS (d). 1B: titration of cardiotoxin with PI (a), PA (b) and PS (c). The relative increase in fluorescence at 333 nm, $(F-F_0)/F_0$, is plotted against the molar ratio between negative phospholipid and cardiotoxin. F is the fluorescence intensity measured at 333 nm for a given amount of negative phospholipid added, F_0 is the fluorescence intensity of cardiotoxin at 333 nm. The titrations were carried out with two different cardiotoxin concentrations: 0.67 μM (⊙) and 6.7 μM (•).

destroyed by cardiotoxin. All these effects are instantaneous and can be observed immediately after mixing (10 s) of the lipid–protein solution.

The amplitude of the blue shift does not depend upon the type of negative phospholipid used (PS, PA or PI). Conversely, large differences are observed between increases in fluorescence intensities with different phospholipids (fig.1A). The PS–cardiotoxin complex is characterized by a maximal fluorescence intensity of 305 ± 15% as compared to cardiotoxin alone (100%). Corresponding values for PA– and PI–cardiotoxin complexes are 180 ± 5% and 135 ± 5%, respectively.

Figure 1B shows that when increasing amounts of negative phospholipid are added to cardiotoxin, the maximal intensity increases linearly until a plateau value is reached. The same pattern is observed when the cardiotoxin concentration is varied from 0.67–6.7 μM. One mol cardiotoxin is able to bind 7 ± 1 mol PS, PA or PI.

Complexation between cardiotoxin and negatively-charged phospholipids is reversible. Ca^{2+} ions are able to displace PS from the PS–cardiotoxin complex (fig.2A). 50% PS is displaced from the complex at 12 mM Ca^{2+}, total displacement occurs at Ca^{2+} concentrations higher than 30 mM. Figure 2B shows that after formation of the PS–cardiotoxin complex, PA

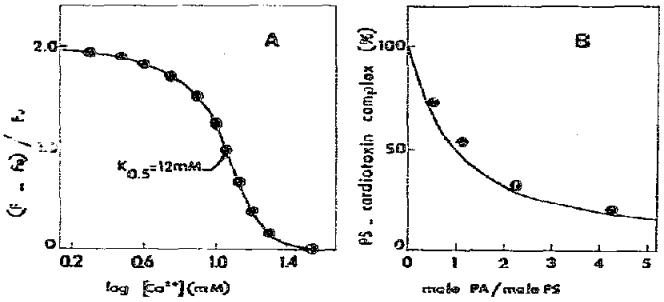

Fig.2. Reversibility of the cardiotoxin–PS interaction. 2A: Displacement of PS from its association to cardiotoxin by Ca^{2+} ions. Cardiotoxin (6.7 μM) was first associated with a 4-fold molar excess of PS. Ca^{2+} ions were then added and the dissociation of the cardiotoxin–PS complex was followed by monitoring the decrease in relative fluorescence intensity, $(F-F_0)/F_0$. F represents the fluorescence intensity at 333 nm for a given Ca^{2+} concentration and F_0 is the fluorescence intensity of cardiotoxin alone at 333 nm. It has been checked that Ca^{2+} ions do not perturb the fluorescence spectrum of free cardiotoxin. 2B: Displacement of PS from its association to cardiotoxin by PA. Cardiotoxin (6.7 μM) was first saturated by an 8-fold molar excess of PS. PA was then added and replacement of PS by PA in the complex was followed by monitoring the decrease in fluorescence intensity at 333 nm. This decrease is due to the fact that the cardiotoxin–PS complex is characterized by an higher fluorescence intensity (305%) than the cardiotoxin–PA complex (180%). The % PS–cardiotoxin complex, x, is calculated from the relationship:

$$y \cdot 100 = 305 \cdot x + 180(1-x)$$

where y is the measured fluorescence intensity at 333 nm, expressed in % fluorescence intensity of free cardiotoxin at 333 nm. The experimental points (•) measured as described are compared to the theoretical curve of displacement which can be calculated from the known [PS] and [PI] concentrations, assuming that both phospholipids exhibit identical affinities for cardiotoxin

$$(\%\ \text{PS–cardiotoxin complex} = 100 \times [PS]/([PS]+[PA])$$

is able to replace PS in the complex. Similar experiments were carried out to test the reversibility of the PA— and PI—cardiotoxin associations and gave similar results. In all cases, displacements are instantaneous (less than 10 s).

When vesicles of PC, SM or PC(50%)–PE(50%) are added to cardiotoxin, no change of the fluorescence spectrum is observed. The cardiotoxin—neutral phospholipid mixtures remain clear even after long periods of incubation (> 1 h). Moreover, none of these zwitterionic phospholipids are able to compete with negatively-charged phospholipids for their association to cardiotoxin. Therefore, neutral phospholipids do not seem to associate with cardiotoxin.

Table 1 summarizes the results of studies in which mixed vesicles (i.e., vesicles containing more than one phospholipid) were allowed to associate with cardiotoxin. Vesicles made from 50% PS and 50% PC behave like vesicles of pure PS. Cholesterol does not influence the PS—cardiotoxin interaction. Mixed vesicles made from 50% PA and 50% PI give the same stoichiometry of association to cardiotoxin as each one of these phospholipids alone, the increase in fluorescence being the arithmetic mean of increases observed with vesicles made from pure PA and PI. The same is true for mixed PA–PS and PI—PS vesicles. Vesicles containing the same phospholipid proportions as Fraction II of axonal membranes [8] have also been prepared. In one case, cholesterol was added in the proportion it has in axonal membranes, in the other case, cholesterol was omitted. Both types of vesicles shift the wavelength of maximal fluorescence intensity to the same extent that vesicles made from a single negatively charged phospholipid (table 1). The

Table 1

Association of cardiotoxin with phospholipids, gangliosides, fatty acids and axonal membranes: fluorescence characteristics and stoichiometric data

Lipids	λ_{max}[a] (nm)	I_{max}[b] (%)	Stoichiometry (mol negative lipid/ mol cardiotoxin)
PS	333	305 ± 15	7.5 ± 1
PA	333	180 ± 5	6.5 ± 1
PI	333	135 ± 5	7.0 ± 1
PS(50)-PA(50)	333	240 ± 10	7.5 ± 1
PS(50)-PI(50)	333	220 ± 10	7.2 ± 1
PA(50)-PI(50)	333	155 ± 5	7.0 ± 1
PS(50)-PC(50)	333	307 ± 15	7.7 ± 1
PS(50)-Cholesterol(50)	333	270 ± 10	8.0 ± 1
'Axonal membrane' vesicles with cholesterol[c,d]	333	150 ± 5	4.2 ± 0.5
'Axonal membrane' vesicles without cholesterol[c]	333	150 ± 5	6.8 ± 1
Oleate	333	220 ± 10	10.8 ± 1
Palmitate	333	210 ± 10	11.5 ± 1
Gangliosides	339	175 ± 5	12.2 ± 1
Axonal membranes, I	333	320 ± 15	4.3 ± 0.5
Axonal membranes, II	333	320 ± 15	3.8 ± 0.5

[a] λ_{max} is the wavelength of maximal fluorescence intensity of the cardiotoxin–lipid complex

[b] I_{max} is the maximal fluorescence intensity of the complex at λ_{max}, expressed in % fluorescence intensity of cardiotoxin alone at the same wavelength

[c] The phospholipid composition of axonal membranes (Fraction II) is (w/w): PE, 36.39%; PS, 13.55%; PC, 30.53%; PI, 1.01%; SM, 18.20%; PA, 0.75% [8]

[d] The cholesterol/phospholipid ratio (mol/mol) is 0.672 [8]

maximal increase in intensity is 150 ± 5% in both cases. However, the stoichiometry of association is 4.2 ± 0.5 mol negatively-charged phospholipid bound/mol cardiotoxin when cholesterol is present instead of 6.8 ± 1 in the absence of cholesterol.

3.2. *Interaction of cardiotoxin with other lipid constituents of natural membranes*

Lyso compounds do not perturb the fluorescence spectrum of cardiotoxin in any way. Conversely, oleate and palmitate which are the most abundant fatty acids in natural phospholipids [13], provoke the same blue shift as negative phospholipids on the fluorescence spectrum of cardiotoxin (table 1). Both fatty acids increase the maximal fluorescence intensity by 220 ± 10% and give similar stoichiometries of association: 10–11 mol fatty acid/mol cardiotoxin. Gangliosides are also able to associate with cardiotoxin (table 1). They provoke a small blue shift from 343–339 nm and an increase in fluorescence of 175 ± 5%. Saturation occurs when 12–13 mol gangliosides are bound/mol cardiotoxin.

3.3. *Interaction of cardiotoxin with axonal membranes*

Purification of axonal membranes from the crab *Cancer pagurus* led to the isolation of two fractions, I and II, which sedimented at 17.5% and 19.5% sucrose, respectively [8]. The phospholipid, ganglioside, cholesterol and protein contents of both fractions are available [8]. Fractions I and II are able to induce the same changes on the fluorescence spectrum of cardiotoxin as synthetic vesicles of PS: a wavelength shift from 343–333 nm and an intensity increase of 320 ± 15% (table 1). The stoichiometry of association, calculated on the basis of the negative phospholipid content of axonal membranes, is 4 mol negative phospholipid of the membrane/mol cardiotoxin.

3.4. *Interaction of neurotoxin I with phospholipids*

The fluorescence spectrum of neurotoxin I is mainly due to its only tryptophane residue, Trp_{29} [14]. The spectrum is insensitive to the addition of vesicles made from negative or zwitterionic phospholipids. Moreover, neurotoxin I is unable to compete with cardiotoxin for its association to negative phospholipids. The competition experiment was carried out as follows: neurotoxin I was first mixed with a solution of PS vesicles. The neurotoxin/PS ratio was chosen so that, if the neurotoxin were able to bind to PS just like cardiotoxin, all PS molecules would be associated to neurotoxin. The neurotoxin–PS mixture was then used to measure the association of PS to cardiotoxin. Results of this experiment were found to be identical with those in which no neurotoxin was added to the solution of PS. Replacement of PS by PA, PI or gangliosides in the competition experiment described above gave identical results. Neurotoxin I does not seem to possess any affinity for lipids.

4. Discussion

This paper directly demonstrates that cardiotoxin interacts with lipids in natural and synthetic membranes. Only negatively-charged lipids (PS, PA, PI and gangliosides) are able to associate with cardiotoxin. A binding of fatty acids with the toxin is also observed, but is probably of less physiological interest since natural membranes do not contain appreciable amounts of free fatty acids [15].

The decrease in polarity of the Trp environment observed upon formation of the cardiotoxin–negative lipid complexes may be due either to a masking of the indole side-chain by the apolar moiety of the lipids or to a conformational change of the protein. The former explanation is more probable because the blue shift amplitude, i.e., the masking of Trp_{11} in cardiotoxin, depends upon the apolar character of the lipid associated to the toxin. Binding of PS, PA, PI or fatty acids induces a blue shift of about 10 nm on the fluorescence spectrum of cardiotoxin. Gangliosides, which are less apolar because of their sugar moiety, only induce a shift of 4 nm (table 1).

Neutral phospholipids do not produce any change in the fluorescence spectrum of cardiotoxin and are unable to compete with negative lipids for their association to cardiotoxin. We therefore conclude that there is no interaction between this class of lipids and cardiotoxin.

The binding of cardiotoxin to negative lipids is stoichiometric: as long as the toxin is not saturated, each new molecule of added lipid binds to cardiotoxin (i.e., the amount of free lipid is negligible). This

result means that the dissociation constants (K_d) of complexes formed between cardiotoxin and negative lipids are much lower than the toxin concentrations used (1–10 μM). Although no precise value can be given, K_d values are certainly $<10^{-7}$ M.

Ca^{2+} ions are able to dissociate the cardiotoxin–negative lipid complexes (fig.2A). Half-dissociation occurs at 12 mM Ca^{2+}. This result is not surprising since such Ca^{2+} concentrations are known to antagonize the effect of cardiotoxin on nerve conduction [16] and to prevent the binding of a tritiated derivative of cardiotoxin to axonal membranes [4]. A competition exists between Ca^{2+} ions and cardiotoxin for binding to lipids. Hauser recently reported an apparent dissociation constant for the Ca^{2+}–phospholipid interaction of about 30 mM [17].

The selectivity of cardiotoxin towards negatively charged constituents of membranes strongly suggests that the positive charges borne by cardiotoxin are implicated in the interaction. The association probably involves ion pairs between the positive charges of lysine and arginine side-chains of cardiotoxin and the negative charges of lipids. Cardiotoxin C contains 9 Lys, 2 Arg and 1 N-terminal α-amino group, i.e., a total of 12 positive charges [10]. This number is higher or equal to the stoichiometries of association observed in our experiments (table 1). Each one of the positive charges of cardiotoxin may be a site of fixation for negative phospholipids, gangliosides or fatty acid. Neurotoxin I from the same venom, which shows some homology with cardiotoxin and which also bears a great number of positive charges, does not associate with negative lipids. However, although neurotoxin I contains 4 Lys, 7 Arg and 1 N-terminal α-amino group, i.e., 12 positive charges like cardiotoxin, it also contains 5 Glu, 2 Asp and 1 C-terminal carboxylate, i.e., 8 negative charges [14]. In the cardiotoxin sequence, there exists only 1 Asp and 1 C-terminal carboxylate [10]. Moreover, comparison of the sequences of both toxins [10,14] shows that only 5 positive charges are placed in homologous position, comprising the N-terminal amino group. A Lys residue in cardiotoxin C is replaced by a Glu in neurotoxin I in 4 positions of the sequences. Formation of ion pairs does not probably suffice to explain the high affinity of cardiotoxin for negative lipids ($K_d < 10^{-7}$ M). It is probable that these initial interactions are further stabilized by hydrophobic associations between the apolar parts of both cardiotoxin and lipids. In this regard, it is interesting to note that cardiotoxin contains more apolar residues than the neurotoxin. There are 6 Pro, 2 Ala, 3 Val, 3 Ile, 5 Leu 2 Tyr, 1 Phe and 1 Trp in cardiotoxin C [10] as compared to 3 Pro, 0 Ala, 1 Val, 1 Ile, 2 Leu, 2 Tyr, 0 Phe and 1 Trp in neurotoxin I [14]. Structural differences noted above are probably responsible for the very different modes of action of neuro and cardiotoxins. Moreover, ^1H NMR studies have already shown [18] that in spite of homologies in the sequences of the two types of toxins, there are conformational differences. The molecular structure of cardiotoxin is more flexible than that of neurotoxin.

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