BBA 75947

BINDING OF α-BUNGAROTOXIN TO THE CHOLINERGIC RECEPTOR PROTEOLIPID FROM *ELECTROPHORUS* ELECTROPLAX

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(Received December 28th, 1971)

SUMMARY

1. This investigation was initiated to study the similarities or differences between the cholinergic proteolipid first isolated in our laboratory from *Electrophorus* electroplax and the protein–detergent–α-bungarotoxin complex isolated by others.

2. The binding of α-[^{131}I]bungarotoxin to the proteolipid extracted from electric tissue or electroplax membranes was studied using column chromatography and a partition method.

3. The saturation curve obtained revealed a single site of binding per molecule of proteolipid of molecular weight 37000.

4. The binding of α-bungarotoxin could not be displaced with acetylcholine or decamethonium.

5. It is concluded that the cholinergic proteolipid extracted with organic solvents is similar to the protein–α-bungarotoxin complex separated by the use of strong detergents.

INTRODUCTION

Since 1967 work from our laboratory has shown that special proteolipids (*i.e.* hydrophobic lipoproteins) isolated from gray matter or from nerve-ending membranes of the central nervous system have high affinity for binding cholinergic drugs[1–3], serotonin[4] and adrenergic blocking agents[5,6]. Furthermore from peripheral tissues such as the electric organ of *Torpedo* and *Electrophorus*[7,8] and the electroplax membranes[9] a cholinergic "receptor" proteolipid was isolated which showed high affinity binding for acetylcholine and other cholinergic ligands. This proteolipid, after incorporation into artificial lipid bilayer membranes, induced conductance changes upon interaction with the transmitter[10].

In recent years Lee and Chang[11] have found that α-bungarotoxin, a basic polypeptide of mol. wt 8000 extracted from the venom of an elapid snake from Taiwan *Bungarus multicintus*, acts much like curare. In fact it produces a permanent blockade of the neuromuscular junction[11] and of the electroplax[12], probably by

Abbreviation: DNETMA, dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate.

reacting with the cholinergic receptor. In both cases it was found that the binding of α-bungarotoxin could be protected with (+)-tubocurarine. The binding of α-bungarotoxin has been recently used to isolate a protein–toxin complex from electric tissue of *Electrophorus*[13] or *Torpedo*[14]; in both cases strong detergents were used.

The purpose of this investigation was to demonstrate that the receptor proteolipid, first isolated by La Torre *et al.*[7] from the electric tissue of *Torpedo* and *Electrophorus*, corresponds to the protein–detergent–α-bungarotoxin complex separated by other authors[13,14].

MATERIAL

Proteolipid extraction

Proteolipids from the electric organ of *Electrophorus electricus* were extracted with chloroform–methanol (2:1, by vol.) and purified by column chromatography in Sephadex LH 20 (ref. 7). As previously shown (see Fig. 1 of De Robertis *et al.*[8]) three protein peaks are eluted with chloroform and two with chloroform–methanol (4:1, by vol.). To study the interaction with α-bungarotoxin, Peak I, a non-receptor proteolipid, and Peak III, the cholinergic receptor proteolipid, were used.

Cholinergic ligands

α-Bungarotoxin was generously provided by Prof. C. Y. Lee from the University of Taiwan. Batches of this toxin dissolved in 0.16 M NaCl in 0.2 M phosphate buffer (pH 7.5) were labeled with carrier free ^{131}I by Dr Mitta of the Argentine Atomic Energy Commission, using the method of Greenwood *et al.*[15]. The α-[^{131}I]bungarotoxin was purified by passage through Amberlite or Sephadex G-25 columns and had an initial specific activity of 4.3 mCi/mg protein. After 2 days no liberation of free ^{131}I was observed. A few experiments were carried out with α-[^{3}H]bungarotoxin kindly provided by Prof. E. Barnard from the University of Buffalo.

[*Me*-^{14}C]Acetyl choline chloride (10.4 mCi/mM) was from Amersham/Searle, (+)-di[*Me*-^{14}C]tubocurarine ether iodide (83.3 mCi/mM) from Nuclear Chicago, [*Me*-^{3}H]decamethonium chloride (178 mCi/mM) from Amersham/Searle. The radioactivity of the α-[^{131}I]bugarotoxin was determined with a Gamma counter and that of the ^{14}C- and ^{3}H-labeled ligands with a Nuclear Chicago Scintillation Counter.

METHODS AND RESULTS

Interaction of α-[^{131}I]bungarotoxin with the receptor proteolipid

Two methods were used to study the interaction:

(*a*) Peaks I and III of proteolipid from *Electrophorus* were separated, concentrated, made up in chloroform–methanol (2:1, by vol.) and 5 μg of α-[^{131}I]bungarotoxin in a 25-μl aliquot of 0.2 M borate buffer (pH 8.0) was added to both peaks. After 60 min of constant stirring they were rechromatographed on a Sephadex LH 20 column (2 cm × 17 cm). In Peak I there was no binding of α-[^{131}I]bungarotoxin and, as in the case of the free ligand, the radioactivity was retained by the column. The receptor Peak III behaved differently and the protein was eluted from the column

together with 16% of the radioactivity added to the column. The amount bound was 114600 cpm, equivalent to 0.8 μg of α-[^{131}I]bungarotoxin (Fig. 1).

(*b*) A more practical method was an adaptation of that recently described by Weber *et al.*[16] for the study of the binding of the cholinergic fluorescent probe: dimethylaminonaphtalene-5-sulfonamidoethyltrimethylammonium perchlorate (DNETMA). Essentially this method is based on a partition between the chloroform phase, which contains the proteolipid, and a water phase saturated with chloroform and containing 100 mM Tris–HCl buffer (pH 7.2) into which the α-[^{131}I]bungarotoxin is added. In a control without proteolipid it was found that practically all the α-[^{131}I]bungarotoxin remained in the upper phase; the partition coefficient for α-bungarotoxin being 99.3/0.7 in favor of water. In Table I, to account for the differences in protein content and the radioactive decay, the results are expressed in ng of α-bungarotoxin bound per μg of proteolipid protein. With increasing concentrations of α-bungarotoxin in the upper phase there was practically no binding with the non-receptor proteolipid. For example with $3.1 \cdot 10^{-7}$ M α-[^{131}I]bungaro-

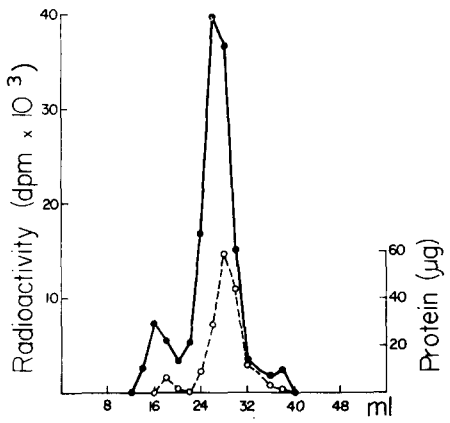

Fig. 1. Rechromatography in Sephadex LH 20 of the cholinergic proteolipid (Peak III) of *Electrophorus* with addition of α-[^{131}I]bungarotoxin. ●—●, α-[^{131}I]bungarotoxin; ○---○, protein.

TABLE I

BINDING OF α-[^{131}I]BUNGAROTOXIN TO THE CHOLINERGIC PROTEOLIPID FROM *Electrophorus*

The binding was done using the technique of Weber *et al.*[16] (see description in the text). [α-Bungarotoxin$_i$], initial molar concentration of α-bungarotoxin in the upper phase; [α-bungarotoxin$_f$], free molar concentration of α-bungarotoxin in the upper phase; [α-bungarotoxin$_b$], α-bungarotoxin bound to the proteolipid.

[α-*Bungarotoxin*$_i$] (*M*)	[α-*Bungarotoxin*$_f$] (*M*)	[α-*Bungarotoxin*$_b$] (*ng/µg protein*)
$3.1 \cdot 10^{-7}$	$2.8 \cdot 10^{-7}$	26.0
$6.2 \cdot 10^{-7}$	$5.8 \cdot 10^{-7}$	69.0
$1.2 \cdot 10^{-6}$	$1.1 \cdot 10^{-6}$	130.0
$2.5 \cdot 10^{-6}$	$2.3 \cdot 10^{-6}$	190.0
$3.1 \cdot 10^{-6}$	$2.9 \cdot 10^{-6}$	230.0
$3.7 \cdot 10^{-6}$	$3.5 \cdot 10^{-6}$	210.0
$5.0 \cdot 10^{-6}$	$4.8 \cdot 10^{-6}$	215.0

toxin in the upper phase only 2.7 ng/μg protein were fixed by Peak I against 26.0 ng/μg protein fixed by Peak III. When the receptor proteolipid (Peak III) is present in the lower phase, the α-[^{131}I]bungarotoxin penetrates in increasing amounts in relation to the concentration of ligand (Table I). A few experiments done with α-[^{3}H]bungarotoxin gave similar results to those with α[^{131}I]bungarotoxin. At saturation there are about 215 ng α-bungarotoxin bound per μg of proteolipid, which corresponds to approximately one molecule of α-bungarotoxin per one molecule of proteolipid protein of approximately 37000 mol. wt.

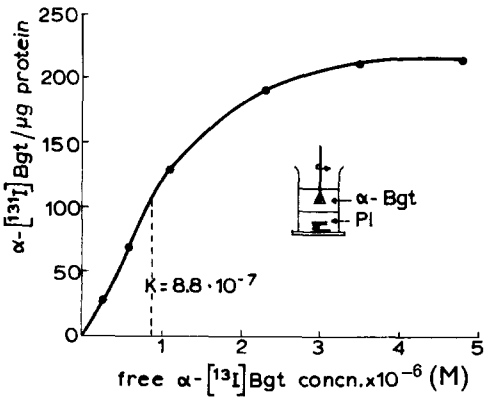

Fig. 2. Binding curve of α-[^{131}I]bungarotoxin to the cholinergic proteolipid (Peak III) of *Electrophorus*. α-Bgt = α-bungarotoxin; Pl, proteolipid.

Fig. 2 shows the saturation curve obtained by plotting the concentration of free α-[^{131}I]bungarotoxin against the amount of toxin bound. The shape of this curve is hyperbolic, indicating a single site of binding for α-bungarotoxin with an apparent dissociation constant of $8.8 \cdot 10^{-7}$.

Competition of α-bungarotoxin with other cholinergic ligands

The competition between α-bungarotoxin and other cholinergic ligands was studied using the technique of Weber *et al.*[16]. The receptor proteolipid of Peak III, in 2 ml chloroform, was submitted to binding in the cold with 50 μg of α-bungarotoxin in 2 ml of water saturated with chloroform at pH 8.0 in the upper phase. After 24 h the upper phase was removed and replaced with water saturated with chloroform (pH 7.2) and with 100 mM Tris–HCl buffer. Into the phase [^{14}C]acetylcholin, [^{3}H]decamethonium or (+)-[^{14}C]tubocurarine were added. After 4 h of stirring of the two-phase aliquots of the upper and lower phases were removed and counted. Controls in which the binding was carried out without previous treatment with α-bungarotoxin and blanks in which the ligands were partitioned between water and chloroform were carried out simultaneously. The results shown in Table II demonstrate that α-bungarotoxin had completely inhibited the binding of [^{14}C]acetylcholine and [^{3}H]decamethonium. With (+)-[^{14}C]tubocurarine the results were not as clear cut because in the control a much higher binding occurred. α-Bungarotoxin inhibited only 5.6 and 6.7 % of this binding which could be of a non-specific type.

TABLE II

COMPETITION BETWEEN α-BUNGAROTOXIN AND OTHER CHOLINERGIC LIGANDS

2 ml of proteolipid (PL) of Peak 3 containing 13 μg/ml protein in chloroform were in the lower phase. The upper phase consisted of 2 ml of water (pH 7.2) with 100 mM Tris buffer, saturated with chloroform (see description in the text).

	Blank (nmoles)	*Control (nmoles)*	*α-Bungarotoxin treated (nmoles)*	*% Inhibition by α-bungarotoxin*
	water	*water*	*water*	
	chloroform	*chloroform + PL*	*chloroform + PL*	
[^{14}C]Acetylcholine	66.64	70.26	70.41	100
	0.25	0.49	0.26	
[^{3}H]Decamethonium	3.20	2.58	3.47	100
	0.02	0.17	0.02	
	3.13	2.84	3.64	100
	0.02	0.24	0.03	
(+)-[^{14}C]Tubocurarine	5.26	0.73	1.20	6.7
	0.10	5.02	4.69	
	4.76	0.36	0.55	5.6
	0.28	4.53	4.28	

Binding of α-bungarotoxin to electroplax membrane

30 g of frozen electric tissue were homogenized in 40 ml 0.2 M sucrose and submitted to cell fractionation according to Changeux *et al.*[17]. Previous studies has shown that a preparation of membranes rich in acetylcholinesterase and receptor proteolipid could be separated with this procedure[8]. These membranes were resuspended in 3 ml of 100 mM Tris–HCl buffer (pH 7.2) and 2.5 nmoles of α-[^{131}I]bungarotoxin were added. After 30 min incubation at 4 °C and centrifugation at 40000 rev./min for 30 min, the sediment was separated and washed twice by centrifugation. In these membranes 0.49 nmoles of α-[^{131}I]bungarotoxin per mg protein were bound. When the pellet of membranes was treated with chloroform–methanol (2:1, by vol.), to extract the proteolipid, the radioactivity remained with the residue. The following pieces of evidence led us to think that this treatment had produced the rupture of the α-[^{131}I]bungarotoxin receptor proteolipid complex and that the toxin, because of its solubility properties, had remained with the protein residue. (a) The same amount of proteolipid protein was extracted from a control membrane fraction and from another previously incubated with α-bungarotoxin. For example in the pellet control from 27 g fresh tissue 151 μg proteolipid protein was extracted while from an α-bungarotoxin-treated pellet, corresponding to 12 g fresh tissue, 70 μg of proteolipid protein were extracted. (b) Preliminary results indicate that membrane from which proteolipid were previously extracted bind considerably less α-[^{131}I]bungarotoxin than the control membranes. For example in one case the pellet control had bound 0.49 nmole α-[^{131}I]bungarotoxin per mg protein while the one extracted with chloroform–methanol fixed 0.08 nmole per mg protein. (c) The proteolipid extracted from electroplax membranes binds α-[^{131}I]bungaro-

toxin. From lyophilized electroplax membranes corresponding to 27 g fresh tissue 151 μg of proteolipid were extracted in chloroform–methanol (2:1, by vol.). After addition of half the volume of chloroform the extract was concentrated to 1.5 ml and treated with 20 μg of α-[^{131}I]bungarotoxin in 0.2 M borate buffer (pH 8.0) for 1 h with constant stirring. Then the extract was loaded upon a column of Sephadex LH 20 (1 cm × 17 cm). Upon elution with chloroform the radioactivity appeared in a sharp peak coinciding with a peak of protein. At the height of the peak the specific radioactivity corresponded to the binding of 100 ng of α-bungarotoxin per μg of proteolipid protein (Fig. 3).

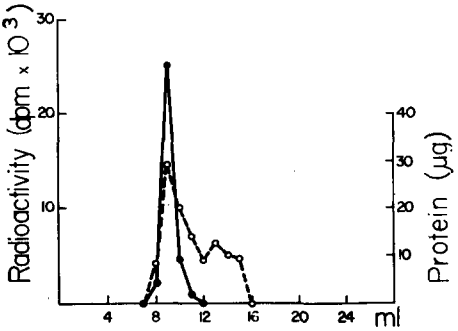

Fig. 3. Chromatography in Sephadex LH 20 of an extract from electroplax membranes of *Electrophorus*. α-[^{131}I]Bungarotoxin was added to the extract. ●—●, α-[^{131}I]bungarotoxin; ○---○, protein.

DISCUSSION

Dr G. G. Lunt (University of Bath, United Kingdom), first drew our attention to some quantitative relationships between the number of binding sites per g fresh tissue that may be demonstrated with α-bungarotoxin[12,14] in *Torpedo* and *Electrophorus* and the number of receptor proteolipid molecules that were extracted by us from the same tissues. La Torre *et al.*[7] found that there were some 65 μg of receptor proteolipid per g fresh tissue in *Torpedo* and about 26 μg/g in *Electrophorus*[8].

The molecular weight of the cholinergic receptor proteolipid was calculated by De Robertis *et al.*[8] to be about 40000 by the binding of [^{14}C]acetylcholine. The presence of one binding site of high affinity and about 9 binding sites of low affinity per molecule was suggested. More recently, by measuring the binding of DNETMA, Weber *et al.*[16] calculated that 1 mole of DNETMA was bound per 5000 g of receptor proteolipid at complete saturation. This indicated a molecular weight of about 50000 for the proteolipid. Assuming a molecular weight of 40000 (ref. 8) it can be calculated that there are some $6.8 \cdot 10^{14}$ receptor proteolipid molecules per g tissue in *Torpedo* and about $2.7 \cdot 10^{14}$ in *Electrophorus*. La Torre *et al.*[7] suggested that the higher content of receptor in *Torpedo* could be due to the richer innervation and to the fact that in these species there are no spikes and bioelectrogenesis originates only by postsynaptic potentials[18]. It seemed more than a coincidence that Miledi *et al.*[14] found $6.6 \cdot 10^{14}$ binding sites of α-bungarotoxin per g fresh tissue in *Torpedo* and Changeux *et al.*[12] $2.4 \cdot 10^{14}$ binding sites per g in *Electrophorus* (calculated).

It is also worth mentioning that the number of acetylcholinesterase active sites is approximately the same as that of receptor binding sites[12,14]; however,

De Robertis and Fiszer de Plazas[9] were able to separate quantitatively the enzyme from the receptor proteolipid. Changeux *et al.*[12] have isolated a "receptor enriched" protein fraction in which macromolecules in the range of 540000 daltons were reported[19] and the protein–Triton X-100–α-bungarotoxin complexes isolated by Miledi *et al.*[14] had a molecular weight of 80000, while larger aggregates were also found. In our case[7,8] we are probably dealing with isolated receptor molecules whose number agrees with that of cholinergic binding sites in both the *Torpedo* and the *Electrophorus*.

The extraction procedure used by both the other groups of investigators indicate that they are concerned with a rather hydrophobic protein (or lipoprotein) which is very difficult to solubilize and needs high concentrations of Triton X-100 (ref. 14) or deoxycholate[13] to become partially dissolved in the aqueous solution. It seems possible that such detergents, interacting with the protein and or with the lipid moiety of the proteolipid, result in the formation of a complex which is to some extent soluble in aqueous media. Preliminary work done in our laboratory suggests that Triton X-100 renders the receptor proteolipid soluble in water.

The results presented here demonstrate that α-bungarotoxin interacts with the receptor proteolipid and that this binding is rather strong. In fact it is not displaced with acetylcholine or decamethonium in the concentrations used, which correspond to the saturation of the high affinity site[8].

At an α-bungarotoxin concentration of $3.7 \cdot 10^{-6}$ M, in which the binding reaches 215 ng per μg of receptor proteolipid (Table I), there is 100% inhibition of the binding of acetylcholine and decamethonium to the receptor proteolipid (Table 2).

The type of saturation curve obtained with α-[^{131}I]bungarotoxin suggests that the binding results in a stoichiometric complex with the receptor proteolipid, and that a 1:1 relationship is reached with a molecular weight of 37000 for the protein moiety of the proteolipid.

These findings confirm the suggestion advanced by De Robertis and Barrantes[20] that α-bungarotoxin only binds to the site of high affinity of the proteolipid. The strength of binding that α-bungarotoxin makes with the cholinergic receptor proteolipid is evidenced by the apparent dissociation constant of $8.8 \cdot 10^{-7}$, which may be calculated from the saturation curve (Fig. 2).

The fact that labelled α-bungarotoxin is not extracted with organic solvents after it has been bound to electroplax membranes may be used as an argument against the similarity of the proteolipid to the protein–detergent–α-bungarotoxin complex[12,14]. However, an explanation suggested by our experiments is that the chloroform–methanol extraction destroys the bungarotoxin-α-receptor complex and that the label remains with the residue probably because the partition coefficient of the toxin favors the water. In fact similar amounts of proteolipid are extracted from the control or from the α-bungarotoxin treated membranes and, after extraction, the protein residue shows little binding for α-[^{131}I]bungarotoxin. Furthermore the presence of a receptor proteolipid which binds α-[^{131}I]bungarotoxin may be demonstrated in the extract (Fig. 3). Allowing for the volume differences of the chromatographic beds, this proteolipid appears in the column in the same volume of elution as the rechromatographed Peak III bound to α-[^{131}I]bungarotoxin (Fig. 1).

In summary: We think that the proteolipid Peak III from *Electrophorus*, which has been shown to bind with high affinity [^{14}C]acetylcholine, [^{14}C]hexamethonium and *p*-[^{3}H](trimethylammonium)benzene diazonium fluoroborate[7], as well as DNETMA[16] and now [^{3}H]decamethonium and α-[^{131}I]bungarotoxin is the same protein as the one separated with strong detergents and bound with this neurotoxin[13,14].

ACKNOWLEDGEMENTS

The authors are very grateful to Prof. C. Y. Lee from the University of Taiwan for his generous supply of α-bungarotoxin. We express our gratitude to Dr A. Mitta from the Argentine Atomic Energy Commission for the labelling of the neurotoxin with ^{131}I and to Dr E. Barnard for providing a sample of α-[^{3}H]bungarotoxin.

This work has been supported by grants from the Instituto de Farmacología (Legajo 4699/70) Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina and the National Institutes of Health, U.S.A. (5 Roi NS 06953-06 NEUA).

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