CERULEOTOXIN : AN ACIDIC NEUROTOXIN FROM THE VENOM OF *BUNGARUS CAERULEUS* WHICH BLOCKS THE RESPONSE TO A CHOLINERGIC AGONIST WITHOUT BINDING TO THE CHOLINERGIC RECEPTOR SITE

Cassian BON and Jean-Pierre CHANGEUX

Neurobiologie Moléculaire et Biochimie Cellulaire, Institut Pasteur, 28, rue du Docteur Roux – Paris 15 ème, France

Received 27 August 1975

1. Introduction

The toxins from the venoms of Elapid snakes have been extensively studied during the past few years and several of them are currently used to label pharmacological receptors and other membrane bound proteins [1,2]. On the basis of their structure and of their pharmacological action, Lee [1,3] has distinguished in these venoms the cardiotoxins, which, like the γ-toxin, the direct lytic factor or cobramine B, produce cardiovascular alterations responsible for local necrotic lesions [4], and the neurotoxins which block synaptic transmission. The neurotoxins themselves can be subdivided into two main categories : 1) the α-neurotoxin, such as α-bungarotoxin, naja toxin or cobrotoxin are basic polypeptides of 60–62 or 70–74 amino acids, which block synaptic transmission at the neuromuscular junction like curare [5] and bind with a high affinity to the nicotinic receptor site [2,5] and, 2) the β-neurotoxins, such as β-bungarotoxin and some phospholipases A have a presynaptic action and block the release of acetylcholine [1,6,7].

In this letter, we describe the purification and some of the structural and pharmacological properties of a toxin which does not fall into any of these categories. This toxin, that we shall refer to as ceruleotoxin, is present in the venom of the snake *Bungarus caeruleus*. It is an acidic protein which blocks the depolarisation of *Electrophorus* electroplaque by cholinergic agonists, even though it does not significantly bind to the nicotinic receptor site.

2. Materials and methods

The dried venom from *Bungarus caeruleus* was a gift of Dr Paul Boquet from the Service des Venins, Institut Pasteur, Garches.

The toxicity of the venom and of the protein fractions during purification was determined on adult male mice by intravenous injection of 0.2 ml of the tested solution per 20 g of body weight, and expressed as LD_{100}.

The homogeneity of the purified ceruleotoxin was tested by electrophoresis in polyacrylamide gels either in the absence of detergent by the method of Davis [8] (concentration of acrylamide 7.5%), or in the presence of 1% sodium dodecylsulfate as described by Fairbanks et al. [9] (concentration of polyacrylamide, 5.6%).

The apparent mol. wt of the purified ceruleotoxin was determined by filtration on a Bio-gel P-150 column equilibrated in 0.1 M ammonium acetate at pH 7.0, using globular proteins of known mol. wt as standards : creatine kinase from *Escherichia coli* K12 (81 000 daltons), ovalbumin (43 000 daltons) and bovine cytochrome *c* (12 700 daltons). The apparent mol. wt of the subunit(s) of ceruleotoxin was measured by electrophoretic migration on polyacrylamide gels in the presence of 1% sodium dodecyl sulfate, using : aspartokinase I - homoserine dehydrogenase I from *Escherichia coli* K12 (85 000 daltons), bovine serum albumin (67 000 daltons), ovalbumin (43 000 daltons), homoserine kinase from *Escherichia coli* K12 (30 000 daltons) and bovine hemoglobin (15 700 daltons) as standards.

North-Holland Publishing Company – Amsterdam

The pharmacological activity of the purified ceruleotoxin was measured on the isolated electroplaque from *Electrophorus electricus* by the method of Schoffeniels and Nachmansohn [10] and of Higman Podleski and Bartels [11] upon bath application of cholinergic agonist.

The effect of ceruleotoxin on the binding of ^3H α-toxin from *Naja nigricollis* was followed by the method of Weber and Changeux [12] with receptor-rich membrane fragments (spec. act.: 910 nmol in ^3H α-toxin sites per gram protein) prepared from homogenates of *Torpedo marmorata* electric organ by the method of Cohen, Weber and Changeux [13].

α-Bungarotoxin was purified from the venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, USA) by the method of Lee [14] and Mebs [15].

All chemical reagents were of analytical grade and from Merck and Koch-Light.

3. Results

3.1. *Purification of ceruleotoxin*

The procedure routinely used to purify *ceruleotoxin* is summarized in table 1. One gram of dried venom from *Bungarus caeruleus* was dissolved in 15 ml of 0.05 M Tris–HCl, pH 8.5 and the solution centrifuged at low speed to eliminate insoluble material. The supernatant (crude extract) was then added on the top of a QAE Sephadex A-50 column (2 × 15 cm) equilibrated with 0.05 M Tris–HCl, pH 8.5. A first toxic fraction which included the α-neurotoxin and other basic or neutral proteins did not adsorb to the column, and was released during the wash with the equilibration buffer. Then, a linear salt gradient (0 to 0.4 M NaCl in the equilibration buffer) was applied to the column, and a second toxic fraction was eluted at 0.125 M NaCl. This last toxic fraction contained the *ceruleotoxin*. It was concentrated by lyophilisation and purified to homogeneity by filtration on a Bio-gel P 150 (minus 400 mesh) column (1.1 × 80 cm) equilibrated with 0.1 M ammonium acetate pH 7.0.

3.2. *Homogeneity and molecular weight*

Fig.1 shows that the purified ceruleotoxin runs as a single band during polyacrylamide gel electrophoresis both in the absence and in the presence of sodium dodecyl sulfate. Enzymatic assays indicate however that the preparation contains trace amounts of acetylcholinesterase (4 nmol of acetylthiocholine × min^{-1} × mg^{-1} of protein) as determined by the method of Ellman [16] and of phospholipase (about 1% of the venom phospholipase activity was recovered in the ceruleotoxin fraction) as determined by the indirect

Table 1
Purification of ceruleotoxin from *Bungarus caeruleus* dried venom

	Protein (mg)	Toxicity (LD_{100})	Recovery of toxicity (%)	Specific toxicity ($LD_{100} \cdot mg^{-1}$)	Purification factor
Dried venom	1024	17 000	100	16.6	1
Crude extract	980	16 000	97.8	16.9	1.03
First toxic fraction from QAE-Sephadex column	810	12 000	70.7		
				117.8	
Second toxic fraction from QAE-Sephadex column	45	8000	47.1	178	10.6
Purified ceruleotoxin from Bio-gel P-150 column	6.3	6000	35.3	952	57.5

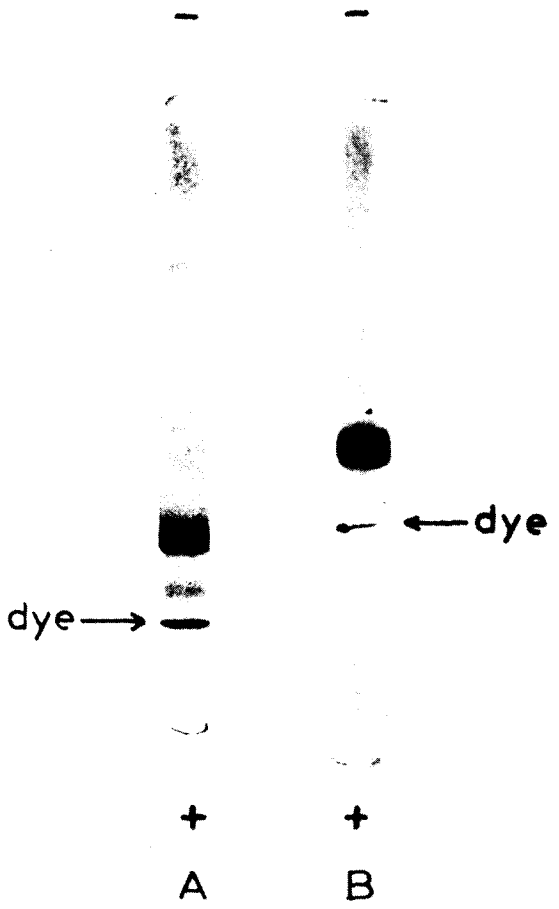

Fig.1. (A) Disc gel electrophoresis of purified ceruleotoxin in a 7.5% polyacrylamide gel following the technique of Davis [8]; 100 μg of purified toxin were added on top of the gel and Bromophenol blue was used as the tracking dye. The direction of migration was towards the anode. The gel was stained by Coomassie blue. (B) Electrophoresis in a 5.6% polyacrylamide gel in the presence of 1% sodium dodecyl sulfate following the technique of Fairbanks et al. [9] 25 μg of purified toxin were added on the top of the gel.

hemolytic method described by Yang, Huang and Tung [17].

Filtration of the purified ceruleotoxin on a Bio-gel P-150 column gives an apparent mol. wt for the native protein of 38 000 ± 4000 using globular proteins of known mol. wt as standards. In the presence of 1% sodium dodecyl sulfate, the ceruleotoxin moves as a single band with an apparent mol. wt of 15 000 ± 2000, using globular proteins as standards. The native toxin is therefore made up of several subunits.

3.3. *Pharmacological activity of ceruleotoxin*

The LD_{100} of purified ceruleotoxin determined by intravenous injection in mice is 0.052 μg/g body weight. The injected animals suffer from a flaccid paralysis of the skeletal muscles and die from a respiratory block. At all the concentrations tested, the ceruleotoxin kills the mice with a much longer delay than the α-toxins : depending on the dose from 3 to 48 h instead of 10 to 30 min.

Fig.2 further shows that exposure of the innervated face of *Electrophorus* electroplaque to 2.5×10^{-8} M (1 μg/ml) ceruleotoxin does not alter the resting potential of the cell, but blocks the depolarisation caused by bath application of 3×10^{-5} M carbamylcholine. The same figure illustrates that the blocking effect of ceruleotoxin develops with a much slower time course than that of α-toxins, for instance α-bungarotoxin [12].

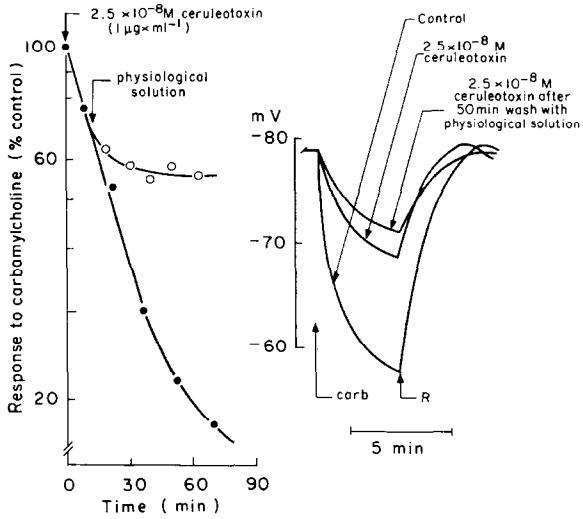

Fig.2. Ceruleotoxin blocks irreversibly the depolarisation of the isolated electroplaque from *Electrophorus electricus* by 3×10^{-5} M carbamylcholine applied in bath. Right: change of membrane potential caused by bath application of carbamylcholine. Left: the variation, as a function of time, of the amplitude of the depolarisation caused by bath application of 3×10^{-5} M carbamylcholine expressed as per cent of the control response. 1 μg per ml of ceruleotoxin was bath applied on the innervated face of the electroplaque at zero time. (•—•) The toxin was present during all the experiment. (○ - - ○) The toxin was washed after 12 min exposure.

Extensive washing of the treated cell with physiological solution does not modify the amplitude of the response. Within the time scale of the experiment the blocking effect of ceruleotoxin appears irreversible.

3.4. *In vitro effect of ceruleotoxin*

Ceruleotoxin was tested in vitro on *Torpedo marmorata* receptor-rich microsacs following either the efflux of $^{22}Na^+$ or the binding of ^3H α-toxin from *Naja nigricollis*. Preincubation for 5 min at room temperature of the excitable microsacs with ceruleotoxin at a concentration five-fold in excess over the concentration of receptor sites (determined as the concentration of ^3H α-toxin binding sites) *completely* blocks the increase of $^{22}Na^+$ caused by 10^{-4} M carbamylcholine. In addition, ceruleotoxin, at variance with α-bungarotoxin, shows some effects on $^{22}Na^+$ efflux at rest; it significantly decreases the rate of $^{22}Na^+$ release and increases the apparent volume.

In the same experiment and with the same membrane fragments the binding of *Naja nigricollis* ^3H α-toxin was also measured after preincubation with ceruleotoxin or α-bungarotoxin. It was found that preincubation with α-bungarotoxin reduces by 95% the total amount of *Naja nigricollis* ^3H α-toxin bound to the membrane fragments but that the same treatment with ceruleotoxin reduces the binding of the tritiated toxin by only 15%. Similar results were obtained when radioactive acetylcholine, instead of ^3H α-toxin, was used as a specific ligand of the nicotinic receptor site. The blocking of the response by ceruleotoxin therefore does not follow its occupancy of the cholinergic receptor site.

In fig.3 are compared in a more quantitative manner the effects of ceruleotoxin and of α-bungarotoxin on the binding of ^3H α-toxin from *Naja nigricollis* to receptor-rich membrane fragments from *Torpedo marmorata*. In both cases the membrane fragments were preincubated with the indicated concentration of toxins for 90 min at room temperature, then, the binding of the labelled toxin was measured. A concentration of α-bungarotoxin five-fold in excess over the concentration of nicotinic receptor sites blocks more than 90% of ^3H α-toxin binding sites, whereas the same concentration of ceruleotoxin has no significant effect on the binding of the labelled toxin. When a fifty-fold excess of ceruleotoxin was used, the binding of ^3H α-toxin decreased by only 15%; α-bungarotoxin and ceruleotoxin therefore do not compete for the same binding site on the membrane fragments.

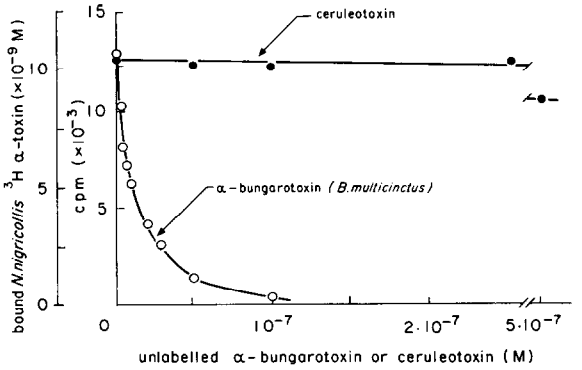

Fig.3. Absence of effect of ceruleotoxin on the binding of *Naja nigricollis* ^3H α-toxin to receptor-rich membrane fragments from *Torpedo marmorata*. Receptor-rich membrane fragments (910 nmol of ^3H α-toxin binding site per gram of protein; 1.1×10^{-8} M in ^3H α-toxin binding site) were incubated in *Torpedo* physiological solution for 90 min at room temperature with the indicated concentration of toxin. Binding of ^3H α-toxin was then measured by millipore filtration.
(○——○) α-Bungarotoxin from *Bungarus multicinctus*.
(●——●) Ceruleotoxin.

4. Discussion

Ceruleotoxin is a potent neurotoxin present in the venom of *Bungarus caerulus*, which can be purified in a homogenous form, and in milligram quantity. Even though ceruleotoxin primarily blocks the postsynaptic response to acetylcholine, it differs *markedly* both by its structure and by its pharmacological action from the classical α-toxins present in the same venom. It is an acidic protein made up of several subunits of about 14 000 daltons.

Ceruleotoxin is responsible for at least 35% of the total toxicity of the venom. However it acts more slowly than the α-toxin both on the mouse and on the isolated *Electrophorus electricus* electroplaque, most likely because of its higher mol. wt. This slow action of ceruleotoxin is responsible for the paradoxal 120% yield in toxin recovery which follows the purification step on the QAE-Sephadex column : the toxicity of ceruleotoxin being partially masked by the fast action of the α-toxins present in the crude venom.

Ceruleotoxin, at concentrations as low as 2.5×10^{-8} M, blocks almost completely and irreversibly the depolarisation of *Electrophorus* electroplaque by carbamylcholine. Moreover concentration of ceruleotoxin fivefold in excess over the concentration of cholinergic receptor site completely blocks the increase of $^{22}Na^{+}$ efflux caused by the same agonist. However the observation that ceruleotoxin does not compete with *Naja nigricollis* α-toxin or acetylcholine for the same site on receptor-rich membranes indicates that this acidic toxin does not significantly bind to the cholinergic receptor site. The identification of its molecular target in the subsynaptic membrane might bring some interesting information on the ion translocating device that acetylcholine regulates during synaptic transmission.

Acknowledgements

We thank Professor P. Boquet for supplying *Bungarus caeruleus* venom, Drs A. Menez, J. L. Morgat and P. Fromageot for the gift of *Naja nigricollis* ^{3}H α-toxin, Mrs Simone Mougeon for performing the experiments with the *Electrophorus* electroplaque, M. Weber and J. L. Popot for helpful discussions and Professor Georges N. Cohen for his encouragements and the staff of the Station Biologique d' Arachon (Director, Professor Boisseau) for supplying *Torpedo marmorata.*

This work was supported by funds from the Collège de France, The Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Commissariat à l'Energie Atomique and the National Institutes of Health.

References

[1] Lee, C. Y. (1970) Clinical Toxicology 3, 457–472.
[2] Changeux, J. P., Kasai, M. and Lee, C. Y. (1970) Proc. Natl. Acad. Sci. USA 67, 1241–1247.
[3] Lee, C. Y. (1970) in: Neuropoisons: Their pathophysiological actions (Simpson, L., ed.) Vol. 1, Plenum Press, New York.
[4] Lee, C. Y., Chang, C. C., Chiu, T. H., Chiu, P. J. S., Tseng, T. C. and Lee, S. Y. (1968) Naunyn-Schmiedeberg Arch. Pharmak. u. exp. Path. 259, 360–374.
[5] Chang, C. C. and Lee, C. Y. (1966) Brit. J. Pharmacol. 28, 172–181.
[6] Lee, C. Y. and Chang, C. C. (1966) Mem. Inst. Butantan. Simp. Internac. 33 (2), 555–572.
[7] Chang, C. C., Chen, T. F. and Lee, C. Y. (1973) J. Pharmacol. Exp. Ther. 184, 339–345.
[8] Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404.
[9] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[10] Schoffeniels, E. and Nachmansohn, D. (1967) Biochim. Biochim. Biophys. Acta 26, 1–15.
[11] Higman, H., Podleski, T. R. and Bartels, E. (1963) Biochem. Biophys. Acta 75, 187–193.
[12] Weber, M. and Changeux, J. P. (1974) Mol. Pharmacol. 10, 1–14, 15–34.
[13] Cohen, J. B., Weber, M. and Changeux, J. P. (1974) Mol. Pharmacol. 10, 907–932.
[14] Lee, C. Y., Chang, S. L., Kau, S. T. and Luh, S. H. (1972) J. Chromatogr. 72, 71–82.
[15] Mebs, D., Narita, K., Iwanaga, S., Samejima, Y. and Lee, C. Y. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 207–226.
[16] Ellman, G. L., Courteney, K. D., Andres, V. and Featherstone, R. (1961) Biochem. Pharmacol. 7, 88–95.
[17] Yang, C. C., Huang, L. C. and Tung, T. C. (1954) J. Formosan Med. Assoc. 53, 1–7.