ISOLATION OF THE NICOTINIC ACETYLCHOLINE RECEPTOR BY BIOSPECIFIC CHROMATOGRAPHY ON INSOLUBILIZED *NAJA NAJA* NEUROTOXIN

Evert KARLSSON
Institute of Biochemistry, Box 531, S-751 21 Uppsala.1, Sweden

and

Edith HEILBRONN and Lars WIDLUND
Research Institute of National Defence, Department 1, S-172 04 Sundbyberg 4, Sweden

Received 20 September 1972

1. Introduction

The electric organs of fishes belonging to the families of *Torpedinidae* and *Gymnotidae* are derived from striated muscle and cholinergically innervated. Their high content of "nicotinic" acetylcholine receptors, for *Torpedo* the number of about 10^{14} receptors sites per g fresh tissue has been given [1], makes them the best source known for purification of cholinergic receptor, but the actual amount of receptors is still very small as compared to that of other material present. An isolation procedure requiring many different purification steps is likely to give a low yield of the purified substance. *Biospecific (affinity) chromatography* to selectively concentrate and isolate the receptor should therefore be a convenient method.

Elapid and hydrophid venoms contain neurotoxins which block the nicotinic acetylcholine receptor, thus mimicking the action of curare [2]. These venom neurotoxins bind to the receptor and the binding is often regarded to be irreversible. However, mice given sublethal doses of neurotoxins ($0.8–0.9 \times LD_{100}$) and showing severe dyspnea may recover within a few hours [3]. This observation does not seem to be in accordance with an irreversible block. Furthermore, partial dissociation of toxin–receptor complexes and protection against toxin binding by cholinergic substances has been observed *in vitro* [4–6]. We describe here an isolation method for the nicotinic cholinergic receptor based on biospecific chromatography, adsorption of the receptor to insolubilized *Naja naja siamensis* neurotoxin and its subsequent desorption by carbamylcholine (carbachol).

2. Materials and methods

2.1. *Preparation of the biospecific adsorbent*

The principal neurotoxin of *Naja naja siamensis* (*kaouthia*) (Thailand cobra) was prepared by ion-exchange chromatography on Bio-Rex 70 and subsequent gel filtration of Sephadex G-50 [3]. It was covalently fixed to ECD-Sepharose 4B according to Axén et al. [7]. (ECD = epichlorohydrin cross-linked and desulphated). This gel has a better stability than non-treated gel and a very low amount of carbohydrates is dissolved out from the gel even at 100° [8]. The gel on a glass filter was washed with water and drained with a water pump. To 15 g of the drained gel (dry weight 0.6 g) was added 7.5 ml of water and 25 ml of a freshly prepared solution of cyanogen bromide (25 mg per ml). The gel was activated during 6 min by keeping the pH at 11 by continuous addition of 2 M NaOH, *immediately* transferred to a glass filter and *rapidly* washed with 50 ml cold (+ 4°) 0.2 M $NaHCO_3$ followed by 50 ml cold 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.4). The gel was then transferred to 20 ml of the above buffer containing 126 ml of the *siamensis* toxin and the mixture shaken for 12 hr at room temp. The gel was packed to a 2.0×4.6 cm

column and washed with water. The wash liquid was concentrated by rotatory evaporation and passed through a column of Sephadex G-25 in 0.2 M ammonium acetate. The void fraction was found to contain 20 mg of toxin as estimated by spectrophotometry ($A^{0.1\%}_{279}$ = 1.06). The column was washed further by pumping through for 12 hr at a flow rate of 25 ml per hr 0.2 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.4 and 1 M NaCl) followed by the same volume of 0.2 M sodium acetate buffer (pH 4.5 and 1 M NaCl). Before storage in a cold room, the adsorbent was always washed with 0.2 M acetic acid.

2.2. *Solubilization of membrane constituents*

A fresh electric organ of *Torpedo marmorata* was homogenized for 5 min in a Waring blendor. 0.10 M sodium phosphate buffer (pH 7.5, 1 M NaCl, 6% Triton X100, ionic strenth 1.30) was added to the homogenate until the detergent concentration was 2%. The pH was adjusted to 7.5 with 1 M NaOH. The mixture was stirred at room temp. for 3.5 hr, filtered through a cloth, centrifuged at 70,000 *g* for 1 hr, and the supernatant was recovered.

2.3. *Biospecific chromatography*

The adsorbent was washed with water, mixed with the supernatant and the mixture stirred at room temp. for 7 hr. The adsorbent was then packed to a column (2.0 × 4.6 cm) and the solution was run through. The column was washed with 0.10 M sodium phosphate buffer (pH 7.5 and 1% Triton). followed by elution for 1 hr with 14 ml total (one column volume) of the same buffer 1 M in NaCl to desorb electrostatically bound material. The column was washed free from NaCl with the phosphate detergent buffer, whereupon further elution was done with a linear gradient from 0 to 0.5 M of carbamylcholine chloride in the same buffer and eventually with 0.2 M acetic acid to desorb all remaining adsorbed material from the column. Before analysis for acetylcholine esterase (AChE) or protein carbachol had to be removed by dialysis.

2.4. *Protein content*

Alkaline hydrolysis and subsequent ninhydrin analysis was used to estimate the protein content of the various fractions. Human serum albumin was used as a standard.

2.5. *Acetylcholine esterase*

The enzyme activity was assayed according to Ellman et al. [9] using acetylthiocholine as the substrate.

2.6. *Binding of d-tubocurarine*

Samples containing equal amounts of protein were submitted to equilibrium dialysis in 0.20 M sodium phosphate buffer (pH 7.5 and 1% Triton) containing d-tubocurarine di([^{14}C]methyl) ether diiodide (108 μCi/mmole, Amersham Radiochemical Centre). Three different concentrations of the drug were used viz. 1, 5 and 10 μM.

2.7. *Estimation of the receptor equivalent weight*

Radioactive acetylated derivatives of the *siamensis* toxin were prepared as described elsewhere [10]. The specific radio activity was 1,000 Ci per mole and the LD_{100} (i.v.) was 150 μg per kg mouse as compared to 100 μg for the native toxin. The weight ratio μg receptor protein per μg bound toxin was determined from two experiments. Ca. 10 μg of labelled neurotoxin in 100 μl of ammonium acetate was added to an aliquot (100–200 μl) of a fraction. The sample was dialyzed for 6 hr at room temp. against a continuous flow of 0.10 M phosphate buffer (pH 7.5 and 1% Triton). The content of the tubing was then withdrawn with a syringe, the dialysis bag rinsed with 2 × 1 ml of buffer and the pooled solutions gel filtered on Sephadex G-75 in the above phosphate buffer containing 0.1% of Triton. This detergent concentration keeps the material solubilized, and allows a high flow rate. The toxin–receptor complex elutes in the void fraction, whereas the free toxin has an elution volume of about two and a half times that of the void volume. Radioactivity was measured after mixing 1 ml of relevant fractions with 5 ml of Instagel (Packard). 1 μg of radioactive toxin gave 50,800 cpm, and this value was used to calculate the amount of bound toxin.

Another aliquot (1.0 ml) of the same fraction was dialyzed against water with 0.1% detergent. The sample was then hydrolyzed with 6 M HCl for 24 hr at 110° and submitted to amino acid analysis to determine the total protein content. From these two experiments the ratio protein/bound toxin was determined. The protein equivalent weight was calculated from the formula weight, 7,861, of the radioactive monoacetyl derivative of the neurotoxin.

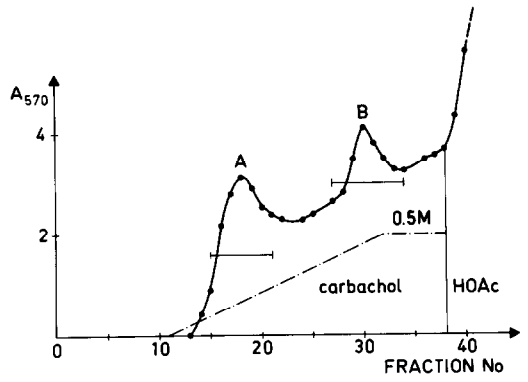

Fig. 1. Biospecific chromatography on Sepharose-bound *Naja naja siamensis* neurotoxin. Column (2.0 × 4.6 cm). Carbachol gradient initiated at zero, the breakthrough occurred in fraction 12. The line (– · – ·) indicates the change in the carbachol concentration from 0 to 0.5 M, and then the concentration was constant at 0.5 M for another 10 ml before the elution with 0.2 M acetic acid (HOAc) was started. The gradient volume (0 to 0.5 M) was 28 ml. At the breakthrough of the acetic acid the protein concentration increased rapidly as can be seen from the rapid increase of the ninhydrin colour (A_{570}). A (fractions 15–21) and B (26–33) were taken for further experiments.

Table 1
Protein content and acetylcholine esterase activity during isolation procedure.

	Protein		Enzyme activity		
			Total		Specific
Fraction	(mg)	(%)	(ΔA/min)	(%)	(ΔA/min × mg protein)
For adsorption	5500	100	52 000	100	9.5
Non adsorbed	5400	98	57 000	110	11
1 M NaCl effluent	4.3	0.08	2 900	5.6	670
Buffer effluent	1.7	0.03	1 100	2.1	650
Carbachol effluent:					
Total (tubes 11–38)	16	0.29			
Fraction A (fig. 1)	3.6	0.07	23	0.04	6.4
Fraction B (fig. 1)	5.4	0.10	27	0.05	5.0
0.2 M HOAc effluent	21	0.38	not assayed		

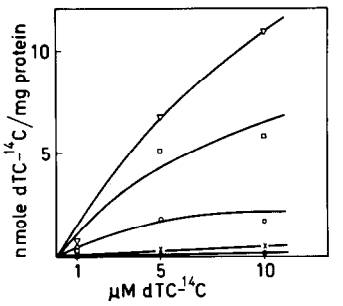

Fig. 2. Binding of d-tubocurarine di([^{14}C]methyl) diiodide (dTC–^{14}C). The different symbols denote samples from the following fractions (table 1): (□—□—□) A; (▽—▽—▽) B; (○—○—○) 1 M NaCl effluent; (×—×—×) for adsorption, (●—●—●) non adsorbed (effluent).

3. Results

3.1. *Isolation of the cholinergic receptor*

Pilot experiments performed at room temp. (ca. + 23°) and in a cold room (+ 5°), respectively, showed that 11 times more material able to bind radioactive toxin was eluted from the biospecific adsorbent by carbamylcholine chloride at the higher temperature. The main experiment was therefore carried out at room temp.

A clarified supernatant from an electric organ was incubated with the adsorbent. Fig. 1 shows the effluent profile obtained during the elution with the carbachol gradient and the final desorption with acetic acid. The protein content and the AChE activity at various stages of the isolation procedure are presented in table 1.

The material eluted with carbachol has a high affinity to curare (fig. 2) as well as to the *siamensis* neurotoxin. It is not desorbed from the insolubilized neurotoxin by high concentrations of NaCl. As seen from the chromatogram, there are at least two fractions with high affinity for curare eluting in the peaks A and B, respectively, B having the highest affinity. Both A and B are inhomogeneous (evident from the chromatogram). Their AChE activity is very low, about 0.05% of the total, and the traces of enzyme present have a low specific activity. The amino acid analysis shows A and B to be significantly different, but we prefer not to present any data on the amino acid composition of impure proteins. On the basis of the isolation method used and the binding studies we assume that the carb-

amylcholine effluent contains nicotinic cholinergic receptor protein. In A this material seems to be together with non binding protein.

3.2. *Equivalent weight and apparent molecular weight*

The ratio protein/bound toxin was 38.6 for the protein in peak A and 17.8 for that in peak B and the corresponding equivalent weights are 303,000 and 140,000, respectively. To estimate the *apparent* molecular weight, the material was incubated with radioactive neurotoxin and then gel filtered on Sepharose 6B (1.6 × 96.2 cm, void volume 60.6 ml) in 0.10 sodium phosphate buffer (pH 7.5 and 1% Triton). The elution volume of the protein A–toxin complex was (114.0 ± 0.5) ml (two determinations) and for the protein B–toxin complex 114.0 ml (one determination). The elution volume for lactate dehydrogenase from rabbit muscle (Worthington), molecular weight 142,000 [11], was (114.1 ± 1.1) ml (two determinations). The apparent molecular weight for both protein A and B would thus be about 140,000.

4. Discussion

The method described in this work offers a possibility for rapid isolation of milligram quantities of receptor proteins. We have obtained a 1,000-fold purification of two protein fractions with a high binding capacity for curare and neurotoxin. The apparent molecular weight of the carbachol eluted toxin binding material in the two fractions, as determined by gel filtration, is the same, but fraction A binds roughly about half the amount of toxin bound by fraction B. Also A and B differ in amino acid composition. Further, about 50% of the toxin binding material cannot be desorbed from the immobilized toxin with carbachol. These as well as some preliminary [12] results, unless caused by protein denaturation, may be understood if one assumes a receptor unit built from pieces of equal molecular weight, but with or without binding capacity for cholinergic and anticholinergic substances. Further studies will aim at the clarification of this point.

The binding between the neurotoxin and the receptor material is not of an electrostatic type as high salt concentrations do not dissociate the receptor–toxin complex. The electrostatic attraction is also generally weak in aqueous media due to the high dielectric constant of water. Hydrophobic interactions and hydrogen bonding contribute therefore mostly to the binding. The adsorbent, however, contains large amounts of positively charged neurotoxin and can therefore act as an anion-exchanger. AChE is obviously specifically bound in small amounts due to the anionic center present in that molecule. The low specific activity of the AChE in the carbachol fractions and their high curare-binding ability is in contrast to those of the preceding steps. In earlier experiments [12] AChE activity and curare-binding were also clearly separated. We therefore confirm other observations [6, 14], one of them from an attempt to use *Naja nignicollis* neurotoxin coupled to Sepharose that AChE and the cholinergic receptor are non-identical, though perhaps originally linked macromolecules.

Only a small amount of receptor protein was desorbed from the biospecific adsorbent when the experiment was carried out at 5°. One plausible explanation is that only a small amount of receptors was adsorbed, thus indicating a low affinity between the toxin and the receptor at this temperature. This would be in accordance with an observation by Lester [13] that the toxin induced postsynaptic block decreased abruptly below + 11°. If so, any toxin binding observed at cold room temperature would mostly be due to electrostatic attraction and only to a small extent to the specific toxin–receptor interaction.

Acknowledgement

This work was supported by the Swedish Natural Science and the Swedish Medical Research Council.

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