IMMUNOLOGICAL CHARACTERISATION OF THE CHOLINERGIC RECEPTOR PROTEIN FROM *ELECTROPHORUS ELECTRICUS*

Hiroyuki SUGIYAMA, Philippe BENDA*, Jean-Claude MEUNIER and Jean-Pierre CHANGEUX
Unité de Neurobiologie Moléculaire Institut Pasteur, Paris 15e, France

and

* *Laboratoire de Biologie Moléculaire Collège de France, Paris 5e, France.*

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1. Introduction

The recent developments of the studies on the cholinergic receptor protein have raised two important questions. Affinity columns of different nature are used to purify the receptor protein from crude extracts: some with cholinergic arms [1, 2], others with snake venom α-toxins coupled to Sepharose [3–5]. Do they yield the same final product? Marked differences have been noticed between the "real" affinity of the purified protein for cholinergic agonists and the "apparent" affinity of the excitable cell for the same agonists. One wonders, therefore, if the material selected by the affinity column is directly involved in the physiological response to acetylcholine?

To answer these questions, the protein purified on a column with cholinergic arms was injected into rabbits. We report here that the serum of an immunized rabbit blocks the response of the isolated electroplax to bath applied carbamylcholine. The same serum precipitates, *in vitro*, the receptor protein purified on the cholinergic column. This last result agrees with some of those reported independently by Patrick and Lindstrøm [6] after immunization against a protein purified with a column of *Naja naja* α-toxin coupled to Sepharose.

2. Materials and methods

2.1. *Crude extracts of cholinergic receptor protein*

Detergent extraction of the acetylcholine receptor (AcChR) protein from *Electrophorus electricus* was performed by the method of Olsen et al. [1]; that from *Torpedo* in the following manner: 5 g of frozen electric tissue of *Torpedo marmorata* was homogenized in 10 ml of 0.16 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2.5 mM phosphate-buffer, pH 7.0 (Ringer's solution) with a Polytron homogenizer at power setting 6 for about 20 sec and the homogenate centrifuged at 20 000 *g* for 10 min in a Spinco 30 rotor. The resulting pellet was resuspended in 2 ml of buffer containing 0.1 M NaCl, 0.1 M Tris–HCl, pH 7.4, 0.02% (w:v) Na-azide, and 1% Triton X-100, and stirred gently at 4°C overnight. After extraction, the mixture was centrifuged for 60 min at 100 000 *g* (30 000 rpm in a Spinco 30 rotor), and the supernatant used for the immunoprecipitation experiment. A crude extract from chick embryonic muscle was obtained by homogenization of 10 lyophilized leg muscles from 13 days-old chick embryos in 10 ml of eel Ringer's solution, washing, extracting, and centrifuging in the same way as in the case of *Torpedo*. The crude extracts thus obtained contained in typical cases 140, 480, and 8.4 pmol/ml of [^{3}H]α-toxin binding sites, and 4.1, 2.6 and 4.0 mg proteins/ml for *Electrophorus*, *Torpedo*, and chick embryo muscle, respectively.

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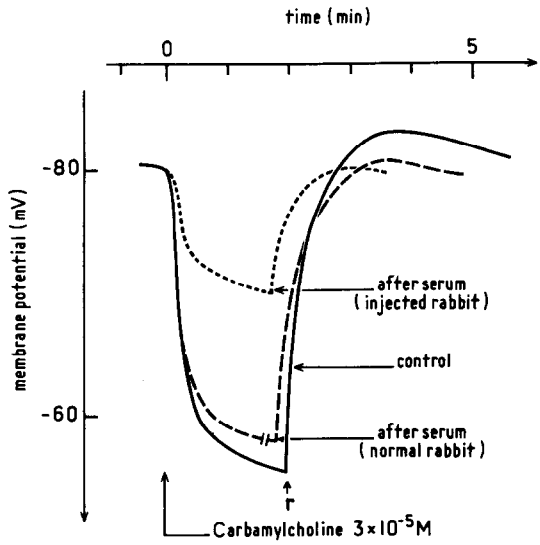

Fig. 1. Effect of anti-AcChR serum on the change of membrane potential caused by bath application of carbamylcholine [13] on the isolated electroplax from *Electrophorus*.

2.2. *Purification of the cholinergic receptor from Electrophorus*

The receptor protein was purified from 1% Triton X-100 extracts of frozen electric organs by an affinity column with a cholinergic arm following the method described by Olsen et al. [1]. After the column, the protein was further purified by centrifugation in a sucrose density gradient in the presence of 0.5% Na cholate [7]. The preparation of receptor protein used for immunization had a specific activity of 4000 nmoles of [^{3}H]α-toxin binding sites (measured by the Millipore filtration assay [1]) per g protein (measured by the method of Lowry with Folin reagent).

2.3. *Immunization*

0.4 ml of the receptor protein solution containing 0.5% Na-cholate (1 mg protein/ml) was emulsified with an equal volume (0.4 ml) of Freund's complete adjuvant, and injected into a rabbit subcutaneously at several spots in the back. Sixteen days later the rabbit received a second similar injection. Three days after the booster, the rabbit developed the flaccid paralysis described by Patrick and Lindstrøm [6] and was immediately sacrificed.

2.4. *Immunoprecipitation*

In the case of *Electrophorus* and *Torpedo*, 10 μl of 1% Triton X-100, 0.1 M NaCl, 0.1 M Tris–HCl, pH 7.4 and 0.02% Na-azide, containing appropriate amounts of receptor protein from a crude extract or a purified preparation was mixed with 10 μl of rabbit anti-AcChR serum appropriately diluted with a rabbit normal serum. When the chick-embryo muscle was tested, 50 μl of crude extract instead of 10 μl was directly used. The mixture was incubated for 20 hr at room temp., and then centrifuged with a Beckman Microfuge Model 152 for 15 min. The supernatant was assayed for [^{3}H]α-toxin binding [1].

Different conditions of the incubation for the antigen–antibody reaction were tried; for example, incubation for 24 hr at room temp. followed by 3 days incubation at 4°C, but no significant differences in the precipitation curves were noticed.

2.5. *Electrophysiological experiments*

The single electroplax was dissected by the method of Schoffeniels and Nachmansohn [8], cholinergic agents were bath applied to the innervated face of the cell and membrane potential recorded with intracellular KCl-filled microelectrodes.

3. Results

Three days after the booster injection of purified AcChR from *Electrophorus* the three rabbits injected developed a flaccid paralysis. Two of them died, the third one was killed and its serum studied. A similar phenomenon was observed independently by Patrick and Lindstrøm [6] with a preparation of AcChR purified on a column of *Naja naja* α-toxin and was interpreted by them as an autoimmune reaction to *Electrophorus* receptor protein.

Fig. 1 shows the effect of anti-AcChR serum on the response of *Electrophorus* electroplax to bath applied carbamylcholine. The innervated face was exposed for 20 min to a 10-fold dilution of serum in Ringer's solution; the membrane potential did not change; then the cell was rinsed for 10 min and the response to carbamylcholine was tested. When the serum came from an immunized rabbit the depolarisation caused by carbamylcholine was reduced by about

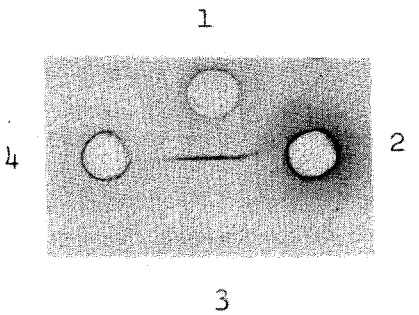

Fig. 2. Ouchterlony double diffusion in 1.2% agar gel and 0.025 M veronal buffer, pH 8.2. 1) anti-AcChR serum; 2) crude extract from *Electrophorus;* 3) purified AcChR from *Electrophorus* (about 1 mg/ml); 4) crude fraction unbound by the affinity column. In 2, 3 and 4, preparations are in 0.1 M NaCl, 0.1 M Tris–HCl buffer, pH 7.4, 0.02% NaN_3 and 1% Triton X-100. Holes 2 and 4 were filled three times with the solutions. The concentration of AcChR in the crude extract (no. 2) was close to the threshold value for the formation of a visible precipitate; its presence is revealed by the asymmetrical deviation induced in the pattern of the precipitation line.

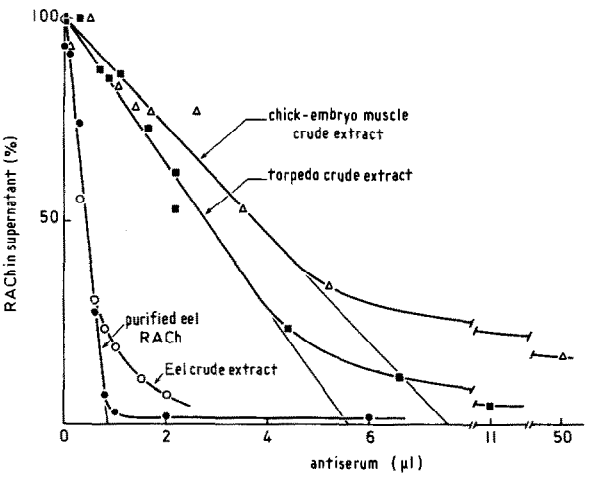

Fig. 3. Quantitative precipitation reaction of various preparations of cholinergic receptor with the anti-AcChR serum. 100% on the ordinate axis corresponds to 0.74 pmole [^{3}H]α-toxin binding sites. The actual amounts of receptor at the initial points were 0.74, 0.31, 0.70 and 0.46 pmole for the purified AcChR, crude extracts from eel, *Torpedo,* and chick embryonic muscle, respectively, and normalized to 0.74 pmole. To a given amount of AcChR, the indicated amount of anti-AcChR serum diluted in 10 µl of normal serum was added. For the two points over 10 µl serum, indicated amounts (after the normalization) of the anti-AcChR serum were directly added.

70%, when it came from a normal rabbit, the effect was negligible. Little if any recovery occurred after extensive rinsing of the cell with physiological solution (less than 10% after 1 hr). Increasing anti-AcChR serum concentrations (up to 1/3 dilution) and exposure for longer periods of time did not markedly change the amplitude of the block. Analysis of the dose–response curve to carbamylcholine after exposure to the anti-AcChR serum showed both a decrease of the maximal response and a slight increase of the *apparent* dissociation constant.

The antiserum was tested *in vitro* against the purified receptor protein by the double diffusion method of Ouchterlony. Fig. 2 shows that a single precipitation band occurs between anti-AcChR serum and eel purified receptor (specific activity 2400 nmole/g protein). In the case of the figure, the gel did not contain any detergent. When the gel contained 1% Triton X-100 essentially no difference was observed, except that the optimum concentration of AcChR which gave the sharpest precipitation band was a little lower.

A slight precipitation band was also noticed between anti-AcChR serum and crude extract but not between serum and the crude fraction unbound by the affinity column.

The anti-AcChR serum was further characterized by the quantitative precipitin reaction (fig. 3). Increasing amounts of the anti-AcChR serum were added to a constant amount of AcChR, and after overnight incubation at room temp. the mixture was centrifuged and the supernatant assayed for [^{3}H]α-toxin binding. Fig. 3 shows the results obtained with purified eel AcChR (specific activity 2400 nmoles/g protein) and crude extracts from *Torpedo* and chick embryonic muscle. The anti-AcChR serum precipitates all the toxin binding component from a preparation of purified eel AcChR. The same experiment done with crude extracts from *Electrophorus* electric tissue yielded the same results and in both cases almost exactly the same amount of serum was needed to precipitate the same quantity of toxin binding material (table 1). Therefore, the component which binds *N. nigricollis* α-toxin in crude extract cannot be distinguished immunologically from purified AcChR.

Table 1

Preparation of cholinergic receptor	Antigen precipitated at equivalence by anti-eel AcChR serum*
Purified AcChR from *Electrophorus*	0.89 ± 0.08
Complex α-toxin purified AcChR (*Electrophorus*)	0.35 ± 0.08
Crude extract from *Electrophorus*	0.87 ± 0.05
Crude extract from *Torpedo*	0.14 ± 0.02
Crude extract from chick embryonic muscle	0.10 ± 0.03

* Unit: nmol of [^{3}H]α-toxin binding site precipitated by 1 ml of antiserum.

The anti-AcChR serum directed against eel receptor also precipitates the toxin binding component present in crude extracts of *Torpedo* electric tissue and chick embryonic muscle. The efficiency of precipitation as measured by the quantity of toxin binding material precipitated at equivalence by 1 ml of anti-AcChR serum is smaller with the heterologous antigens (table 1).

The specificity of the interaction between purified eel AcChR and the homologous antibody was studied by examining the precipitin reaction in the presence and absence of cholinergic effectors or α-toxin. Decamethonium (2.5×10^{-3} M) and d-tubocurarine (2.5×10^{-4} M) do not change the precipitation curve. However, precipitation of the complex [^{3}H]α-toxin and receptor, after separation from unbound α-toxin by Sephadex G-50 filtration, requires, at equivalence, a much larger quantity of anti-AcChR serum than the free receptor (table 1). Binding of the α-toxin therefore interferes with the reaction of the anti-AcChR antibodies with their homologous antigenic protein.

Antibodies [9–10] directed against the globular forms of acetylcholinesterase purified by the methods of either Leuzinger and Baker [11] or Massoulié et al. [12] do not precipitate the cholinergic receptor protein prepared from the same tissue. Conversely, the anti-AcChR serum shows little reaction with acetylcholinesterase. The slight precipitation observed is associated with a small fraction of antibodies specific to acetylcholinesterase present in anti-AcChR serum (less than 1% of the anti-AcChR antibodies) and raised in the rabbit by the enzyme present in the preparation of receptor protein (less than 1 catalytic site of acetylcholinesterase per 100 [^{3}H]α-toxin binding sites [1]). Therefore under the present experimental conditions, no cross reaction was evident between cholinergic receptor protein and acetylcholinesterase.

4. Discussion

The antibodies directed against the toxin binding protein purified on a column with cholinergic arm block the response of the electroplax to bath applied carbamylcholine. This protein therefore participates directly in the electrogenic action of acetylcholine; it is without ambiguity the cholinergic receptor protein. The purified protein binds carbamylcholine *in vitro* with a dissociation constant close to 2×10^{-6} M [7]; nevertheless, the same molecule mediates, *in vivo*, the response to the same agonist with an "apparent" dissociation constant of 4×10^{-5} M [13].

Since direct application of serum blocks the cell response, the receptor protein must be exposed, at least in part, on the surface of the cell membrane; studies by freeze etching of receptor rich membrane fragments from *Torpedo* lead to the same conclusion [14].

The serum directed against the purified receptor protein precipitates quantitatively the toxin binding material present in crude extracts. Thus, purification by affinity chromatography does not cause a marked alteration or loss of a major component which would modify the immunoreactivity of the receptor protein.

The antibodies against eel cholinergic receptor cross react with nicotinic receptors from groups as distant as elasmobranchs, bony fishes and birds but no cross reaction occurs with a closely related membrane protein: acetylcholinesterase. This exceptional stability for the receptor structure in the course of vertebrate evolution might be relevant to its critical function for the survival of the organism.

The fact that the α-toxin partially interferes with the interaction antibody–AcChR suggests that some of the antibodies present in the serum binds to an antigenic site on the AcChR molecule which is part of, or close to, the α-toxin binding site. Others should be directed against different parts of the protein and might be used to map the surfaces of the receptor protein occluded in the membrane phase.

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References

[1] Olsen, R.W., Meunier, J.C. and Changeux, J.P. (1972) FEBS Letters 28, 96.
[2] Schmidt, J.S. and Raftery, M.A. (1972) Biochem. Biophys. Res. Commun. 49, 572.
[3] Karlsson, E., Heilbronn, E. and Widlund, L. (1972) FEBS Letters 28, 107.
[4] Lindstrøm, J. and Patrick, J. (1973) in press.
[5] Reich, E. (1973) The 5th International Congress on Pharmacology, San Francisco, July 1972, in press.
[6] Patrick, J. and Lindstrøm, J. (1973) Science, in press.
[7] Meunier, J.C. and Changeux, J.P. (1973) FEBS Letters 33, 143.
[8] Schoffeniels, E. and Nachmansohn, D. (1967) Biochim. Biophys. Acta 26, 1.
[9] Benda, P., Tsuji, S., Daussent, T. and Changeux, J.P. (1969) Nature 225, 1149.
[10] Riéger, F., Benda, P., Bauman, A. and Rossier, J. (1973) FEBS Letters 32, 62–68.
[11] Leuzinger, W. and Baker, A. (1967) Proc. Natl. Acad. Sci. U.S. 57, 446.
[12] Massoulié, Riéger, F. and Bon, S. (1971) European J. Biochem. 21, 542.
[13] Higman, H., Podleski, T.R. and Bartels, E. (1963) Biochim. Biophys. Acta 75, 187.
[14] Cartaud, J., Benedetti, L., Cohen, J.B., Meunier, J.C. and Changeux, J.P. (1973) FEBS Letters 33, 109–113.