Studies on Purified Eel Acetylcholine Receptor and Anti-Acetylcholine Receptor Antibody

(electroplax/neurotoxin/affinity chromatography)

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ABSTRACT Rabbit antiserum against purified *Electrophorus electricus* acetylcholine receptor is studied using an immunoprecipitin assay to measure either antibody titer or concentration of toxin-binding sites in solubilized receptor preparations. This antiserum, unlike control serum, blocks the electrophysiological response of the electroplax to carbamylcholine. Toxin (α -neurotoxin, *Naja naja*) and several cholinergic ligands produce partial inhibition of the reaction of antiserum with purified acetylcholine receptor. Evidence is presented that some of the toxin-binding sites on receptor, purified by affinity chromatography on toxin-agarose conjugates, are occluded by toxin. In addition, evidence is presented that antireceptor antiserum will cause precipitation of more toxin-binding sites present in an initial extract than in purified receptor preparations.

Over the past few years, considerable effort has been directed towards understanding the molecular biology of the acetylcholine receptor (AChR), a large portion of which has been concerned with its purification (1-8). Implicit in these efforts is the assumption that knowledge of the structural properties of purified receptor will provide insight into its function *in situ*. In order to generate a probe of receptor structure and function which could be used to compare receptor purified and *in situ*, we stimulated anti-AChR antibody production in rabbits. As we have reported (9), the injection of rabbits with purified receptor resulted in the formation of antireceptor antibodies and subsequently the appearance of flaccid paralysis leading to death. In this paper, we present properties of one antiserum thus obtained and use this antiserum and antitoxin antiserum to study purified eel acetylcholine receptor.

MATERIALS AND METHODS

Preparation of Neurotoxin. Toxin was purified from the venom of the Indian Cobra *N. Naja Naja*, as previously described (10). Purified toxin was iodinated with Na 125 I (New England Nuclear Corp., carrier-free) to specific activities of 30-40 Ci/mmol (11).

Preparation of Acetylcholine Receptor. Acetylcholine receptor was purified from the electric eel, *Electrophorus electricus*, by affinity chromatography on columns of toxin-agarose conjugates (6). Protein concentrations determined by the Lowry reaction (12) were corrected to dry-weight protein by stan-

dardizing the reaction to results obtained by amino acid analysis of purified receptor. The preparations used in these experiments had specific activities of 4000-6500 nmol of specific toxin-binding sites per gram of protein. Purified acetylcholine receptor was tritiated with [3 H]acetic anhydride (Amersham Searle, 3 Ci/mmol) by the method of Ostrowski (13). Acetylated receptor was separated from the [3 H]acetate by dialysis, followed by sedimentation on a 5-20% sucrose gradient. The specific activities of acetylated receptor ([3 H]-acetyl receptor) ranged from 0.5 to 1.0×10^8 cpm/mg of protein.

Preparation of Antisera. Anti-acetylcholine receptor antiserum was prepared as previously described (9). Antitoxin antiserum was prepared by injecting rabbits with a toxin-keyhole limpet hemocyanin complex. Toxin and hemocyanin were mixed on an equal weight basis and coupled with 2.5% glutaraldehyde (14). The insoluble complex was washed and emulsified with Freund's complete adjuvant. Each rabbit received 2 mg of the complex subcutaneously, another 2 mg after 2 weeks, and was bled 2 weeks later. Goat anti-rabbit immunoglobulin G antiserum (GAR) was prepared using purified rabbit immunoglobulin G. Goats were injected subcutaneously with 5 mg of antigen emulsified in Freund's complete adjuvant, boosted with the same dose after 2 weeks, and bled 2 weeks later. GAR was fractionated by ammonium sulfate precipitation. Protein precipitating between 0 and 35% saturation was collected, dissolved in phosphate buffered saline and dialyzed overnight against phosphate buffered saline. This preparation was titered on normal rabbit serum and used at the ratio of GAR to normal rabbit serum found to give maximum precipitation of protein.

The Precipitation Assay. Antibody titer and, in some experiments, receptor concentration, was determined by radioimmunoassay. Iodinated toxin was allowed to bind to receptor, and the toxin-receptor complex reacted with anti-AChR antiserum. The toxin-receptor-antibody complex was then precipitated with GAR using normal rabbit serum as carrier. The reactions were conducted in the cold in Eppendorf microcentrifuge tubes. In a typical experiment, a series of tubes were prepared each containing 100 ng of purified receptor, 500 ng of [125 I]toxin, 10 μ l of normal rabbit serum, and different volumes of anti-AChR antiserum at an appropriate dilution (usually 1:100 in phosphate buffered saline containing 1% Triton X-100). Each tube was then made to contain 175 μ l of reaction mixture by addition of phosphate buffered saline + 1% Triton X-100. After overnight incubation at 4°, 25 μ l of

Abbreviations: GAR, goat anti-rabbit immunoglobulin G antiserum; AChR, acetylcholine receptor; Toxin, α -neurotoxin, *Naja naja*.

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GAR was added. After an additional overnight incubation at 4°, the immune precipitate was sedimented by centrifugation in an Eppendorf microcentrifuge and the resulting pellet was washed twice with 1 ml volumes of phosphate buffered saline + 1% Triton X-100. After the second wash, the pellet was dissolved in 200 μ l of 1% acetic acid and 100 μ l of the solution was counted in a liquid scintillation counter. Background radioactivity trapped in the pellet was determined by control experiments in which the antireceptor antibody was completely replaced with normal rabbit serum. The results are expressed as pmol of toxin-binding sites in the precipitate.

Electrophysiological Recording. Electric eels, *Electrophorus electricus*, were obtained from Paramount Aquarium (Ardsley, N.Y.). Single cells were dissected out of slices taken from the tail in the region of the organ of Sachs. Cells were mounted in a chamber similar to that described by Schoffeniels (15), and the membrane potential across the innervated face was recorded with a KCl filled microelectrode inserted into the cell and an agar filled electrode in the solution bathing the innervated face. Potentials were amplified with a Grass P18 microelectrode amplifier and recorded on a Brush recorder. The electroplax, during dissection and during experiments, was bathed with eel Ringer's solution (16) containing 0.15% glucose.

RESULTS

Antibody titer and toxin-binding site concentration were determined using the radioimmunoassay described in *Materials and Methods*. A series of reaction mixtures, each containing the same amount of [125 I]toxin-receptor complex, were reacted with different volumes of anti-AChR antiserum, followed by precipitation of the antibody-receptor-toxin complex with GAR (Fig. 1). Two separate measurements are made in this assay. The plateau portion of the curve is a measure of the number of toxin-binding sites added to the reaction mixture, whereas the initial slope is a measure of antibody titer. Clearly, the determination of toxin-binding site concentration and serum titer in this manner is only valid to the extent that the toxin binding is specific and the reaction of antibody with receptor is not competitive with toxin binding. The initial slope of the curve must also depend upon the specific activity of the receptor used in the assay since the presence of inactive toxin-binding sites could decrease the amount of toxin binding but have no effect on the ability of the receptor to react with antibody.

In an effort to examine these parameters, we first tested the degree to which we could use precipitation of [125 I]toxin by

FIG. 1. Titration curve of purified receptor with anti-AChR antiserum using [125 I]toxin. The assay was performed as described in *Materials and Methods*.

FIG. 2. Determination of the specificity of the radioimmunoassay. Two receptor ligands, *d*-tubocurarine and benzoquinonium, and one receptor inactive quaternary ammonium compound, choline, were tested for their ability to inhibit coprecipitation of [125 I]toxin with the receptor-antireceptor-GAR complex. The figure shows the percent inhibition produced by each compound at the indicated concentration. ●—●, Benzoquinonium; ○—○, *d*-tubocurarine; ■—■, choline.

anti-AChR antiserum as a measure of acetylcholine receptor toxin-binding sites. If the toxin precipitated is bound to receptor, its binding, and hence precipitation, should be inhibited by receptor antagonists such as *d*-tubocurarine and benzoquinonium, but not by the receptor inactive compound choline. The results in Fig. 2 show that this is the case. We can compute a dissociation constant for toxin-receptor binding from each of the inhibition curves in Fig. 2. Assuming dissociation constants of 1.4×10^{-8} M and 2×10^{-7} M for benzoquinonium-receptor binding and *d*-tubocurarine-receptor binding, allows calculation of toxin-receptor dissociation constants of 8.8×10^{-10} M and 7.0×10^{-10} M, respectively. These compare well with the value of 3×10^{-10} M found by direct binding assay at 4° (6).

Recovery of toxin-binding sites from affinity columns is always less than 100%, usually ranging from 40 to 60% (5-7, 17). Two obvious explanations are incomplete elution of receptor from the column, and/or elution of receptor molecules either wholly or partially deficient in toxin-binding activity. Comparison of the antiserum titer on purified receptor and on receptor initially extracted from the membrane should help clarify this point. If it is assumed that the same antigenic determinants were present on both purified receptor and receptor present in the initial membrane extract, then differences in the slopes of the initial portion of the titration curves represent differences in activity of these receptor preparations. We, therefore, titrated a number of the receptor preparations solubilized by Triton (before they were applied to the affinity column) and a number of the preparations of purified receptor eluted from the affinity column. The results in Table 1 show that 1 ml of antiserum will precipitate an average of 3.44 nmol of toxin-binding sites in an initial membrane extract but only about 2.7 nmol of toxin-binding sites when purified receptor is used in the assay. One interpretation of these results is that 22% of the toxin-binding sites in the purified receptor preparation are no longer capable of binding toxin. Alternatively, although purified receptor may be fully active with respect to toxin binding, it may have lost a portion of its antigenic determinants during purification. In either case, we cannot account for the low yield from the affinity column by inactivation.

TABLE 1. Comparison of purified receptor with initial extract

Preparation	Crude extract		Purified receptor	
	Concentration* (nM)	Activity† (nmol/ml)	Concentration (nM)	Activity (nmol/ml)
1	8.50	3.23	7.40	2.50
2	7.30	3.44	12.7	2.55
3	4.95	3.52	5.15	2.99
4	5.95	3.57	3.70	2.81
Average		3.44 ± 0.09		2.7 ± 0.20

* Concentration of toxin-binding sites in the immunoprecipitation reaction.

† The number of nmol of toxin-binding sites that can be precipitated by 1 ml of anti-AChR antiserum.

An alternative explanation which could partially or completely account for incomplete recovery of toxin-binding sites from the affinity column is that receptor is eluted as a toxin-receptor complex. In this form, it may not be detected by an assay employing [125 I]toxin. The toxin might be present as native toxin, an active fragment of native toxin, or as a toxin-agarose complex. These possibilities could be tested using antitoxin antiserum.

In order to detect immunoprecipitation of receptor without relying on added toxin, a radioimmunoassay was developed which was identical to that described in *Materials and Methods* with the exception that [3 H]acetyl receptor replaced [125 I]-toxin and cold receptor. Before proceeding with this assay we

FIG. 3. Immunoprecipitation of [3 H]acetyl receptor, comparison with the [125 I]toxin-binding assay. [3 H]Acetyl receptor (100 ng) was incubated with the indicated quantities of anti-AChR antiserum, in the presence and absence of [125 I]toxin, and precipitated with GAR. ●—●, pmol of toxin-binding sites precipitated as determined from [125 I]toxin; ○—○, [3 H]acetyl receptor precipitated. Insert: Dilution of [3 H]acetyl receptor with unlabeled receptor. A constant amount of [3 H]acetyl receptor was added to a series of tubes with increasing quantities of unlabeled purified receptor, and anti-AChR antiserum sufficient to precipitate 40% of the [3 H]acetyl receptor. The receptor-antibody complex was precipitated with GAR. The curve shows the [3 H]acetyl receptor precipitated, in percent of the control, as a function of amount of unlabeled receptor added.

determined the degree to which precipitation of tritium was a measure of precipitation of acetylcholine receptor. In a duplicate set of tubes we used anti-AChR antiserum to precipitate [3 H]acetyl receptor in the presence and absence of [125 I]toxin and compared the titration curves for precipitation of toxin-binding sites and precipitation of tritium. As seen in Fig. 3, the two curves are very similar and clearly saturate over the same range of antibody concentrations. These results indicate that essentially all the tritiated material binds toxin or is co-precipitated with material which binds toxin. In addition, we determined that acetylation does not alter the ability of receptor to react with antibody. The results of a typical dilution experiment, seen in Fig. 3, indicate that [3 H]acetyl receptor and native receptor behave in the same fashion with respect to antibody.

With the availability of a radioimmunoassay which did not depend upon toxin binding for detection of receptor precipitation, it was possible to determine whether antitoxin antiserum was able to precipitate purified receptor. The curve in Fig. 4 shows precipitation of [3 H]acetyl receptor by increasing quantities of antitoxin antiserum. It might be argued that the observed precipitation is a consequence of crossreaction between antitoxin and purified receptor. Two facts make this unlikely: First, at high antitoxin concentrations even limited crossreaction should result in precipitation equal to that produced by anti-AChR. However, the maximal precipitation was only 50% of that observed with anti-AChR antiserum. Second, the addition of benzoquinonium inhibits precipitation by approximately 80%, presumably by competing with toxin for sites on the receptor. The failure to achieve 100% inhibition by benzoquinonium, even after prolonged incubation, may reflect either a very tightly bound fraction of toxin or precipitation by antitoxin of some toxin which was labeled during acetylation of the toxin-receptor complex. This latter reaction would be insensitive to benzoquinonium, as is the precipitation of [125 I]toxin by antitoxin antiserum. In summary, acetylcholine receptor purified on toxin-agarose con-

FIG. 4. Precipitation of [3 H]acetyl receptor with antitoxin antiserum. [3 H]Acetyl receptor, 100 ng, was incubated with the indicated quantities of antitoxin antiserum and precipitated with GAR. ●—●, Radioactive material precipitated with antitoxin; ○—○, radioactive material precipitated in the presence of mM benzoquinonium; ■—■, radioactive material precipitated in the presence of both mM benzoquinonium and 500 ng of toxin.

jugates exists in part as a toxin-receptor complex, and this complex may be precipitated by antitoxin antiserum.

As previously mentioned, rabbits injected with eel acetylcholine receptor developed paralysis and died. We examined the possibility that some of the antibody species present in the serum were directed against the active site of the receptor. Duplicate sets of tubes were prepared to titrate [^3H]acetyl receptor with anti-AChR antiserum. In one set of tubes we added cold toxin to test its ability to inhibit precipitation of [^3H]acetyl receptor. The results in Fig. 5 show that the initial slope, but not the plateau, is altered in the presence of toxin. These results are expected if the presence of bound toxin inhibits the binding of some species of antibody molecules present. The plateau values should be the same if toxin does not inhibit the binding of all the antibody species present. Benzoquinonium (mM), *d*-tubocurarine (mM), carbamylcholine (10 mM), and decamethonium (mM), but not choline (mM) are also effective inhibitors of precipitation of [^3H]acetyl receptor by anti-AChR antiserum. The observed inhibition of precipitation by cholinergic ligands is not a property of all immunoprecipitation reactions. As mentioned above, the precipitation of [^{125}I]toxin by antitoxin antiserum was unaffected by mM benzoquinonium.

There remain other explanations for the effect of toxin on the antibody titration curve. A portion of the [^3H]acetate might have reacted with toxin bound to receptor. In this case the presence of cold toxin or receptor ligands would serve to displace [^3H]acetyl toxin thus decreasing the tritium precipitated by anti-AChR antiserum. The fact that the maximum amount of labeled material precipitated is independent of the presence of receptor ligands makes this explanation unlikely. It might however be argued that the anti-AChR antiserum is contaminated with antitoxin activity and that this activity contributes to the precipitation of [^3H]acetyl receptor. Although we do find a slight antitoxin activity in the serum used above, precipitation of [^3H]acetyl toxin, whether free or bound to receptor by contaminating antitoxin activity is ruled out because: (1) the antitoxin activity is too low by a factor of 100; (2) sera essentially devoid of antitoxin activity have the same effect; and (3) during competition with cold toxin, the antitoxin activity would be almost exclusively active on cold

FIG. 5. Toxin inhibition of precipitation of [^3H]acetyl receptor by anti-AChR antiserum. [^3H]Acetyl receptor was precipitated with anti-AChR antiserum in the absence and presence of toxin (500 ng). The [^3H]acetyl receptor precipitated in each case is shown as a function of the quantity of antiserum added. O—O, Without toxin; ●—●, with toxin.

FIG. 6. Inhibition by anti-AChR antiserum of chemical excitation in the electroplax. Isolated electroplax were prepared and mounted as described in *Materials and Methods*. The depolarization produced by 10^{-4} M carbamylcholine was determined and taken as 100%. A 1–40 dilution of fractionated control serum or antiserum was then added to the chamber bathing the innervated face. After a given time, the cell was washed free of unbound antibody using Ringer's solution and the response to carbamylcholine determined. The figure shows the percent of the original response remaining after incubation for the indicated times. ●—●, Fractionated control serum; O—O, fractionated antiserum.

toxin. The simplest explanation remains that of inhibition, either steric or allosteric, by receptor ligands of some antibodies. It should be stressed that the effect, while small, is reproducible with a given serum and has been seen with all sera tested thus far.

Given the observations that receptor-immunized rabbits develop paralysis and that a portion of the antibody activity obtained from these rabbits is inhibited by cholinergic ligands, we tested for ability of antiserum to block chemical excitation in the isolated electroplax. Initial experiments showed that normal rabbit serum blocked depolarization induced by carbamylcholine. We, therefore, fractionated several of our control sera and antisera using ammonium sulfate. Protein precipitating between 0 and 35% saturation was recovered, suspended in eel Ringer's solution to a volume one-half that of the original serum, dialyzed against eel Ringer's solution, and tested on the electroplax at a dilution of 1/40. The results in Fig. 6 show that antiserum but not control serum inhibits the response to carbamylcholine. The inhibition of carbamylcholine-induced depolarization by anti-AChR antiserum is not a general property of sera obtained from immunized rabbits. A similar fraction of antitoxin antiserum had the same effect as control serum.

DISCUSSION

The availability of purified eel acetylcholine receptor made possible the preparation of antireceptor antibodies, and led to the observation that the generation of antireceptor antibodies in rabbits is associated with flaccid paralysis. In this paper, we described a system used to assay the activity of these antisera and have noted some properties of receptor interaction with antibody.

During purification of acetylcholine receptor by affinity chromatography on toxin columns, there is a 40–60% loss of toxin-binding sites. Although incomplete elution of receptor probably accounts for most of the loss, it is important to consider other factors as well. We have shown that anti-AChR antiserum is more efficient in precipitating toxin-binding sites from an initial extract of receptor than from a preparation of

purified receptor. This result might be a consequence of purification of some inactive toxin-binding sites no longer capable of binding toxin or of some loss, during purification, of antigenic determinants on the receptor molecule. In addition, we have found that some of the toxin-binding sites on purified receptor are occupied by toxin or toxin conjugated to a fragment of agarose. It seems likely that affinity chromatography using other ligands will also result in purification of a complex between acetylcholine receptor and the ligand used in the column. Since the ligand may be coupled to a small portion of the support medium, it may not be possible to rely on dialysis to separate receptor from its contaminating ligand. These considerations are particularly pertinent in studies of binding sites using equilibrium dialysis and in studies of receptor function in artificial membranes. Carbohydrate analysis of purified receptor containing a ligand-agarose complex might be misleading.

A portion of the antibody activity directed against purified receptor can be blocked by toxin, decamethonium, carbamylcholine, *d*-tubocurarine, and benzoquinonium, but not choline. The maximal inhibition observed ranged from 6 to 15%. These values might depend upon the extent to which purified receptor is contaminated with toxin from the affinity column. If, as in Fig. 4, we can precipitate 50% of the receptor molecules with antitoxin antiserum it seems that at least 50% of the molecules have at least one site occupied by toxin. If each molecule has only one binding site, then the observed inhibition underestimates by a factor of two the maximum possible inhibition. Clearly, the underestimation becomes small very quickly as the number of binding sites per molecule increases. It is possible to estimate the degree to which the maximal observed inhibition reflects the contribution these antibody species make to the whole serum activity. If we assume that only one antibody molecule must bind to receptor to produce a complex that is precipitated by GAR, we calculate that about 25% of the antibody molecules are incapable of binding receptor in the presence of cholinergic ligands.

Anti-AChR antiserum blocks the electrical response of the monocellular electroplax to carbamylcholine. This constitutes a strong argument that the purified acetylcholine receptor preparation contains physiologically significant receptor molecules. It seems probable that the antibody species responsible for the effect on the electroplax also contribute to the paralysis observed in the injected rabbits. The ability to block acetyl-

choline receptor mediated depolarization may be a consequence of those antibody species whose binding is inhibited by receptor ligands. On the other hand, there may be present in the serum some antibody species capable of inhibiting receptor control of ion permeability without altering its ability to bind cholinergic ligands.

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