BINDING OF ANTIBODIES TO ACETYLCHOLINE RECEPTORS IN *ELECTROPHORUS* AND *TORPEDO* ELECTROPLAX MEMBRANES

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ABSTRACT

Antisera against purified acetylcholine receptors from the electric tissues of *Torpedo californica* and of *Electrophorus electricus* were raised in rabbits. The antisera contain antibodies which bind to both autologous and heterologous receptors in solution as shown by an immunoprecipitation assay. Antibodies in both types of antisera bind specifically to the postjunctional membrane on the innervated surface of the intact electroplax from *Electrophorus* electric tissue as demonstrated by an indirect immunohistochemical procedure using horseradish peroxidase conjugated to anti-rabbit IgG. Only anti-*Electrophorus* receptor antisera, however, cause inhibition of the receptor-mediated depolarization of the intact *Electrophorus* electroplax. The lack of inhibition by anti-*Torpedo* receptor antibodies, which do bind, suggests that the receptor does not undergo extensive movement during activity.

The binding of anti-*Torpedo* antibodies to receptor-rich vesicles prepared by subcellular fractionation of *Torpedo* electric tissue was demonstrated by both direct and indirect immunohistochemical methods using ferritin conjugates. These vesicles can be conveniently collected and prepared for electron microscopy on Millipore filters, a procedure requiring only 25 μg of membrane protein per filter. In addition, it was possible to visualize the binding of anti-*Torpedo* receptor antibodies directly, without ferritin. These anti-*Torpedo* receptor antibodies, however, do not inhibit the binding of acetylcholine or of α-neurotoxin to receptor in *Torpedo* microsacs but do inhibit binding of α-neurotoxin to *Torpedo* receptor in Triton X-100 solution. It is likely that the principal antigenic determinants on receptor are at sites other than the acetylcholine-binding sites and that inhibition of receptor function, when it occurs, may be due to a stabilization by antibody binding of an inactive conformational state.

KEY WORDS   acetylcholine receptors ·
antireceptor antibodies · immunohistochemistry ·
electroplax · α-neurotoxin · ferritin

The acetylcholine receptors in electric tissue of *Torpedo* species and *Electrophorus electricus* are being intensively investigated at several levels of

biological organization by a variety of techniques (for reviews, see references 8 and 23). Antibodies to acetylcholine receptors purified from electric tissue have been raised in various animals. Two lines of evidence indicate that these antibodies can both bind to and affect the function of receptors *in situ*. Animals inoculated with receptor develop a dysfunction of neuromuscular transmission resembling myasthenia gravis (19, 31, 41, 42). Both the human disease and the effects of immunization of animals with receptor are ascribable largely to a decrease in the density of receptors in the postjunctional membrane (13, 14). Furthermore, antisera raised against *Electrophorus* receptor have an acute inhibiting effect on the response to carbamylcholine of the isolated, intact electroplax of *Electrophorus* (30, 32, 33, 41); human myasthenic sera, however, do not have an acute effect on isolated, normal human muscle (1). We report here the immunohistochemical demonstration of the binding of antireceptor antibodies to the plasmalemma of *Electrophorus* and *Torpedo* electroplax and examine the functional consequences of this binding. Preliminary reports of some of these findings have been published (20, 24).

MATERIALS AND METHODS

The dissection and electrophysiological recording of the responses of the *Electrophorus* electroplax, the purification of receptor from *Electrophorus electricus* and *Torpedo californica*, and the preparation of rabbit antireceptor antisera have been described previously (25, 43). All sera were held at 56°C for 30 min to inactivate complement. The globulin fraction was prepared by sodium sulfate precipitation (4). All antibody preparations gave single lines upon immunodiffusion against receptor (43). Horseradish peroxidase coupled to goat anti-rabbit IgG was obtained from Miles-Yeda, Ltd. (Rehovoth, Israel). Ferritin coupled to sheep anti-rabbit IgG, to rabbit anti-*Torpedo* receptor IgG, and to normal rabbit IgG were prepared by conjugation with toluene 2,4-diisocyanate (4, 39). Ferritin conjugates were filtered through a Millipore filter (HAWP 01300) (Millipore Corp., Bedford, Mass.).

Immunohistochemistry of Torpedo Membrane

Receptor-rich, vesiculated membrane fragments from *T. californica* electric tissue were prepared as described for *T. marmorata* (9). The vesicles were trapped by filtration on Millipore filters (HAWP 02500). In the direct procedure, 25 μl of vesicle suspension containing 1-2 mg of protein per milliliter and about 1 nmole of α-

neurotoxin binding sites per milligram protein were mixed with 1 ml of 50 mM Na phosphate buffer (pH 7.0) and 50 μl of normal rabbit serum and kept at room temperature for 15 min. 50 μl of ferritin-conjugated rabbit IgG (either normal or immune) at a protein concentration of about 20 mg/ml were added, and the mixture was kept at room temperature for 1 h. A Millipore filter mounted on a Gelman filter holder base (without the funnel) (Gelman Instrument Co., Ann Arbor, Mich.) was washed by filtration first with 4 ml of phosphate buffer and then with 1 ml of normal rabbit serum diluted fivefold with phosphate buffer. The incubation mixture in 200-μl aliquots was pipetted slowly onto the center of a filter under suction. Each aliquot was allowed to pass the filter before the next was added. The vesicles were thus confined to a small area in the center of the filter. The filter was then washed with 10 one-milliliter portions of phosphate buffer applied to the center of the filter. The filter was removed from the filter holder and placed in 5 ml of 2% glutaraldehyde in phosphate buffer and kept at room temperature for 1 h. In the indirect procedure, 25 μl of vesicles, 700 μl of phosphate buffer, and 250 μl of serum (either normal or immune) were mixed and kept at room temperature for 1 h. The mixture was filtered on a Millipore filter, and the filter was washed as in the direct procedure. The stem of the filter holder base was then stoppered, the funnel was screwed onto the base, and 1 ml of ferritin-conjugated sheep anti-rabbit IgG (about 0.5 mg protein/ml) in phosphate buffer was placed on the filter and occasionally shaken gently during 1 h of incubation. The stem of the filter holder was opened, and the excess ferritin conjugate was filtered, and the filter was washed and placed in glutaraldehyde as before.

As described by Baudhuin et al. (5), the filters were removed from glutaraldehyde, rinsed in phosphate buffer, placed in 1% osmium tetroxide in phosphate buffer (pH 7.2) for 1 h, stained with uranyl acetate (16), dehydrated in ethanol followed by propylene oxide, and embedded in Epon.

Immunohistochemistry of Electrophorus Electroplax

Electroplax dissected from the organ of Sachs (38) were gently shaken at room temperature in the following solutions: 1 ml of heat-treated normal goat serum diluted 10-fold in eel Ringer's solution (ER) (25) for 30 min; 5 ml of ER, three times for 10 min each time; 1 ml of heat-treated rabbit serum (normal or immune) diluted 10-fold in ER for 60 min; 5 ml of ER, three times for 30 min each time; 1 ml of horseradish peroxidase conjugated goat anti-rabbit IgG at about 1.2 mg/ml in ER for 30 min; and 5 ml of ER, three times for 30 min each time. The electroplax were fixed in 2% glutaraldehyde in ER at 4°C for 2 h and washed twice in 5 ml of ER for 10 min at room temperature and again in 5 ml of ER for 16 h at 4°C. The washed electroplax were incubated for

30 min in the diaminobenzidine medium of Graham and Karnovsky (17) to demonstrate sites of peroxidase activity, postfixed for 1 h in 1% osmium tetroxide in 50 mM sodium phosphate buffer (pH 7.2), and embedded in Epon. The electroplax shown in Figs. 13, 14, 16, and 17 were in addition initially exposed to 1 ml of collagenase (Worthington CLS grade; Worthington Biochemical Corp., Freehold, N. J.) at 1 mg/ml in ER for 30 min at room temperature.

The histochemical experiments on intact electroplax were repeated three independent times with similar results. Both direct and indirect labeling of vesicles were each repeated at least five independent times, with appropriate controls. The results were highly reproducible.

RESULTS

Titers of Antisera

Antisera raised against three types of receptor preparations, *Electrophorus* receptor and *Torpedo* receptor in Triton X-100 solution and *Torpedo* receptor dissociated in sodium dodecyl sulfate

(SDS) solution, have high titers for the heterologous antigens (Table I). *Torpedo* receptor in Triton and *Electrophorus* receptor in Triton cross-react to the extent of 1–10% of the reaction with autologous antisera. The titers of the antisera determined with receptor radioactively labeled with maleimidobenzyltrimethylammonium iodide (25), a small affinity label (mol wt 372), or with *Naja naja siamensis* α-neurotoxin (mol wt 7,820) are similar, supporting the suggestion (29, 30) that the principal antigenic sites are not in the immediate vicinity of the acetylcholine-binding sites.

Visualization of the Binding of Antibodies to Receptor-Rich Membrane

The binding of anti-*Torpedo* receptor antibodies to receptor-rich, vesiculated plasma membrane fragments from *Torpedo* electroplax is readily demonstrated by both the direct and the indirect

TABLE I

Antireceptor Antibodies: Comparison of Titers for Receptors in Solution, of Binding to Intact Electroplax, and of Inhibition of Receptor-Mediated Depolarization

Immunogen*	Rabbit	Titers‡			Binding to electroplax§	Inhibition of response#
		R_T/Triton	R_T/SDS	R_E/Triton		
	No.	nmol/ml	nmol/ml	nmol/ml		%
R_T/Triton	8	6	3	0.6	Yes	5 ± 2 (5)
	27	1	ND¶	ND	Yes	8 ± 4 (5)
R_T/SDS	1	4	8	0.06	No	14 ± 3 (2)
	2	2	10	ND	No	−10 ± 5 (3)
R_E/Triton	16	0.004	0	0.4	Yes	34 ± 4 (4)
	17	0.3	0.1	2	Yes	41 ± 1 (8)
None	22	0	0	0	No	0 ± 0 (3)
	28	0	0	0	No	11 ± 4 (6)

* Rabbits were injected subdermally in the back with 25–100 μg of purified receptor emulsified in Freund's complete adjuvant; a second injection of receptor in Freund's incomplete adjuvant was made after 25 days. The receptor preparations injected were: *Torpedo* receptor in Triton X-100 solution (R_T/Triton), *Torpedo* receptor in SDS solution (R_T/SDS), and *Electrophorus* receptor in Triton solution (R_E/Triton).

‡ Titers of antisera were determined from the initial slope of the immunoprecipitation curves (32, 43), using receptors labeled with maleimidobenzyltri-[³H]methylammonium iodide (25). Titers are expressed as nmoles of α-neurotoxin-binding sites precipitated per milliliter of serum.

§ Binding of antibodies to intact *Electrophorus* electroplax was determined immunohistochemically with horseradish peroxidase-conjugated goat antirabbit IgG (see Figs. 10–18).

The globulin fractions of the antisera, prepared by sodium sulfate precipitation and containing all of the antireceptor activity of the sera, were applied to the intact electroplax at a concentration of 1 mg/ml for 30 min at a flow rate of 0.3 ml/min. The electroplax was washed for two 15-min periods, and the response to 40 μM carbamylcholine was determined after each period. The final response was compared with the average of two control responses obtained before the addition of antibody. The average inhibition, the SE of the mean, and the number of electroplax tested (in parentheses) are shown.

¶ Not determined.

ferritin methods. Typically, 50–75% of the vesicles incubated with anti-*Torpedo* receptor antibody, in the direct (Figs. 1 and 6; cf. Figs. 2 and 8) or the indirect (Figs. 3 and 7; cf. Fig. 4) method, are surrounded by a densely packed layer of ferritin particles. As expected (39, 40), the electron-dense core of the ferritin is located some distance from the surface of the trilaminar membrane structure. In the direct method this distance is typically 10–13 nm. In the indirect method, the dense core of the ferritin is about 15–20 nm from the trilaminar membrane surface. Binding of antibodies seems to enhance the visibility of the globules or palisade structure described (36) as extending from the outer surface of the electroplax membrane (Fig. 7, inset). It seems reasonable that the antibodies might interact with a portion of the receptor that may protrude from the surface of the phospholipid bilayer; this could contribute to the fact that the spacing of the ferritin from the membrane surface is somewhat greater (10–13 nm with the direct method) than the 8 nm reported for ferritin-labeled antibodies bound to other membranes (40).

Vesicles treated with antireceptor antibodies not coupled to ferritin show a moderately electron-dense layer about 10–12 nm thick on the outside surface (Figs. 5 and 9). This layer is not seen with normal rabbit serum (Figs. 2 and 8) or with other antisera that, like the normal serum, do not produce subsequent binding of ferritin when used in the indirect method (Fig. 4).

In control preparations for direct and indirect methods (e.g., Figs. 2, 4, and 8), one must look at many grid squares before encountering a single vesicle with more than one or two small patches of ferritin or a scattering of a few individual ferritin granules associated with it. Membranes identifiable as nonplasmalemmal, such as the mitochondria occasionally seen in the vesicle fractions, show a similarly very sparse labeling in all preparations, both experimental and control. As additional controls, the direct and indirect methods were applied to a membrane fraction of *Torpedo* liver and the indirect method was used on *Torpedo* erythrocyte ghosts. Once again, only scattered grains of ferritin were bound to the membranes. The little ferritin which is bound in such preparations is generally not separated from the membrane by a distinct space, such as that characteristic of the antibody preparations described above. However, in all types of control preparations, we have, on very rare occasions,

encountered individual vesicles that do show such spacing. Their frequency is so low as to stymie adequate investigation. We believe that they may reflect the occasional persistence of the extracellular coat on the membranes since we have observed nonspecific binding of ferritin to the extracellular coat of intact electroplax that would produce images of the sort that we obtain with these vesicles.

The binding of anti-*Electrophorus* receptor antibodies to *Torpedo* vesicles is barely detectable by the indirect ferritin method. With antibodies raised against *Torpedo* receptor in SDS, the binding of ferritin with the indirect method is not detectably greater than in normal serum controls (Fig. 4).

To control for the possibility that the Millipore filter procedure might selectively retain vesicles which bind antibody, we filtered untreated vesicles and applied ferritin coupled to anti-*Torpedo* receptor IgG to the vesicles on the filter. In another procedure, vesicles were collected by centrifugation instead of filtration. In all cases, the results are similar to those obtained with our routine methods.

The binding of rabbit antireceptor antibodies to intact electroplax from *Electrophorus* was visualized by an indirect immunohistochemical procedure using horseradish peroxidase coupled to antirabbit IgG. With all sera, normal and immune, there is appreciable staining of the noninnervated side of the cell (Figs. 10–12). This staining is localized in the extracellular coat associated with the membrane and extends into the extensive tubular invaginations found on this side of the cell (Fig. 19). Such nonspecific staining of the extracellular coat is much less marked on the innervated side of the cell; the basis of this difference is not evident. There is prominent staining of the innervated cell surface after exposure of the cell to either anti-*Electrophorus* (Figs. 13 and 16) or anti-*Torpedo* (Figs. 15 and 18) antisera. This specific staining is largely confined to the regions of the innervated membrane directly apposed to nerve terminals containing synaptic vesicles, but there is some staining of the surface lateral to the synaptic region (Figs. 13, 16, and 18). Especially with anti-*Electrophorus* antiserum, sometimes there is also light staining of the electroplax surface at some distance from synaptic contacts (Figs. 13 and 16), but this is too light and variable for us to interpret confidently. If it does reflect the presence of a low concentration
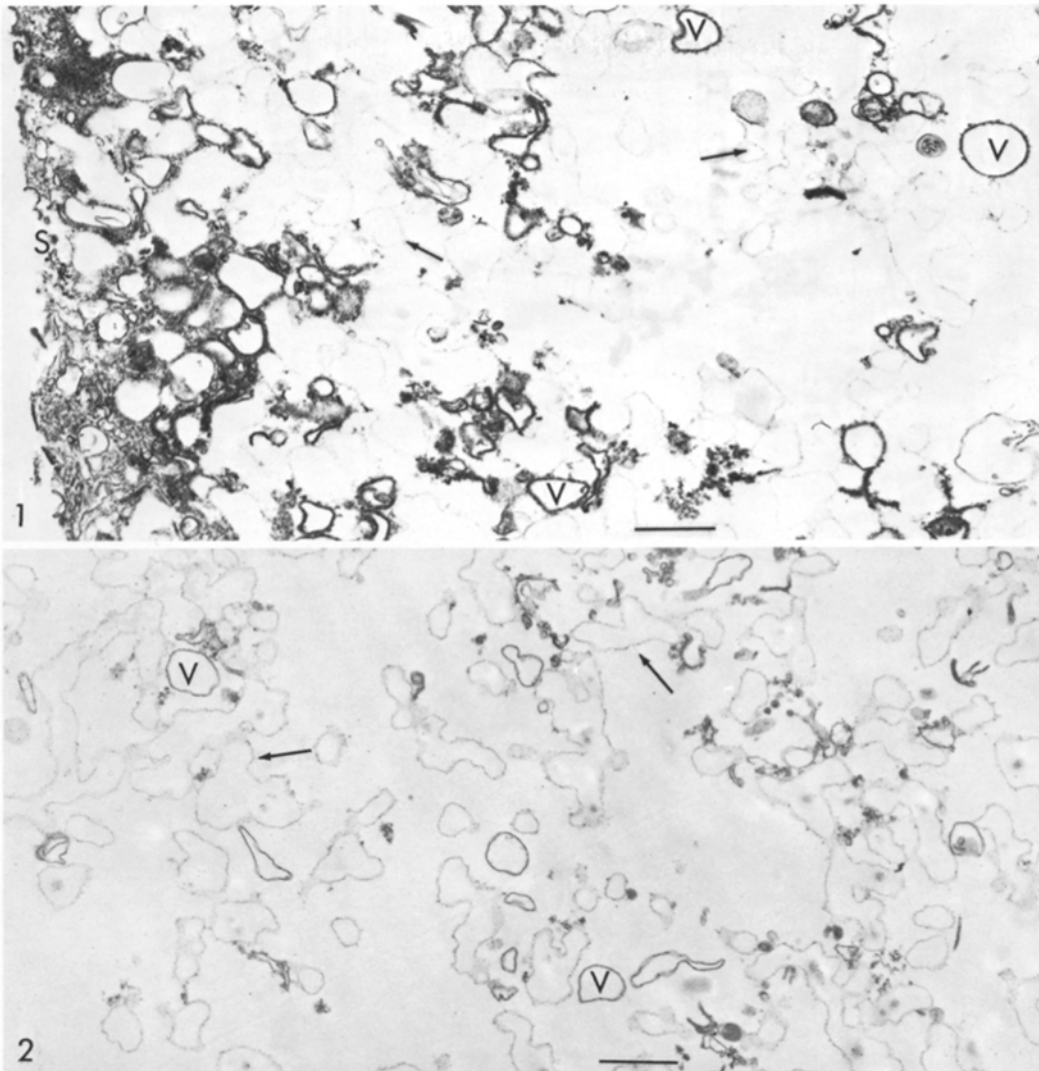
FIGURES 1–2   Figs. 1 and 2 are from the same experiment. The thin sections were stained with uranyl acetate and lead citrate (44). Bars, 1 μm.

FIGURE 1   *Torpedo* vesicles labeled with ferritin conjugated anti-*Torpedo* receptor IgG by the direct procedure. S indicates the surface of the filter, near which the vesicles and ferritin form a compact layer. A great deal of ferritin is trapped nonspecifically in this layer, so we have generally focused our attention on the more dispersed vesicles (V) present deeper within the filter; for each type of preparation studied, we have confirmed that these vesicles and those at the surface show the same pattern of labeling, or lack thereof. In the present preparation, many of the vesicles show a layer, appearing as a fuzzy outline at low magnification, which can be seen at higher magnification (Fig. 6) to represent bound ferritin-antibody conjugates. Most of the filter is dissolved by propylene oxide, although a residue remains, represented by thin outlines of films that probably contain proteins cross-linked by the fixatives and to which some ferritin binds (arrows; see also Figs. 8 and 9). × 11,000.

FIGURE 2   Vesicles from a direct method control preparation which was identical to that in Fig. 1 except that ferritin-conjugated normal rabbit IgG was used. V indicates vesicles which, in this case, lack the fuzzy layer seen in Fig. 1 (Fig. 8 is a higher magnification view of one such vesicle). The arrows indicate the residual outline of the filter. × 10,000.
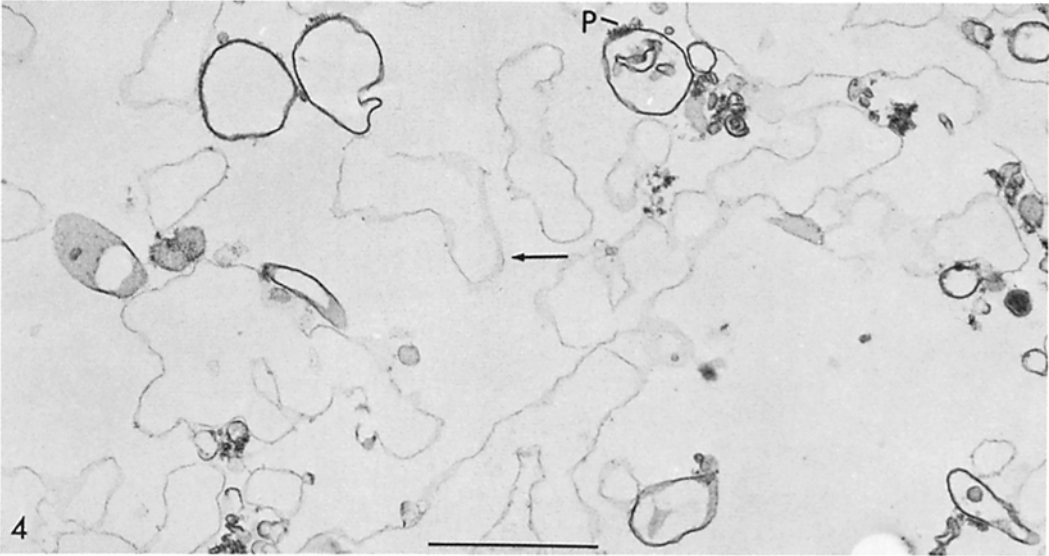
of nonjunctional receptor, it is interesting that this is not detectable with anti-*Torpedo* antiserum (Figs. 15 and 18). There is no staining of the innervated cell surface with normal sera (Figs. 14 and 17) and very little with antisera to *Torpedo* receptor in SDS.

We were unable to localize binding of antireceptor antibodies to electroplax using ferritin conjugates, apparently because of poor penetration to the plasmalemma.

Pretreatment with collagenase seems to enhance the intensity and uniformity of the immunohistochemical staining, but the distribution of reaction product is not detectably altered. Collagen fibers still are readily seen after the treatment, but it may be that the enzyme "loosens" the connective tissue or perhaps alters portions of the extracellular coat, and thus facilitates penetration of the antibodies.

Functional Effects

The rabbit antisera and their globulin fractions, and normal sera, were tested for their effects on the depolarizing response to carbamylcholine of the intact *Electrophorus* electroplax and on the binding of acetylcholine and of *Naja naja siamensis* α-neurotoxin to *Torpedo* vesicles. Only anti-*Electrophorus* antisera and IgG produce a stable inhibition of the response of the electroplax (Table I and Fig. 20). A maximum stable inhibition of about 50% is obtained after about 1 h of application of a large excess of antisera (Fig. 20). The extent of inhibition stabilizes after about 30 min

of washing and two applications of carbamylcholine. Analysis of the dependence of the response on the concentration of carbamylcholine (Fig. 21) indicates that antibody binding causes a decrease in the maximum response and a slight increase in the concentration of carbamylcholine eliciting a half-maximal response, in agreement with reference 41; there is, however, no change in the Hill coefficient of the response (21). The antisera raised against *Torpedo* receptor either in Triton X-100 or dissociated in SDS do not cause a stable inhibition of the response of the electroplax. This is so even though the first type of antiserum binds to receptor in intact *Electrophorus* electroplax (Figs. 15 and 18); both types form immune complexes with solubilized *Electrophorus* receptor, and both were applied to the electroplax in at least 1,000-fold excess of antibody-combining sites over the number of receptor sites.

Autologous antisera partially inhibited the binding of α-neurotoxin to receptor in detergent solution (Table II and cf. reference 2). In contrast, the autologous antisera tested had little if any effect on the binding of α-neurotoxin by *Electrophorus* or *Torpedo* electroplax membrane fractions (Table II and reference 10). The anti-*Electrophorus* receptor antiserum tested was shown to bind to receptor in intact electroplax (Figs. 10, 13, and 16) and to inhibit the depolarizing response of the electroplax to carbamylcholine (Table I). The two anti-*Torpedo* receptor antisera tested were shown to bind to receptor in *Torpedo* membrane vesicles (Figs. 1, 3, 5, 6, 7, and 9).
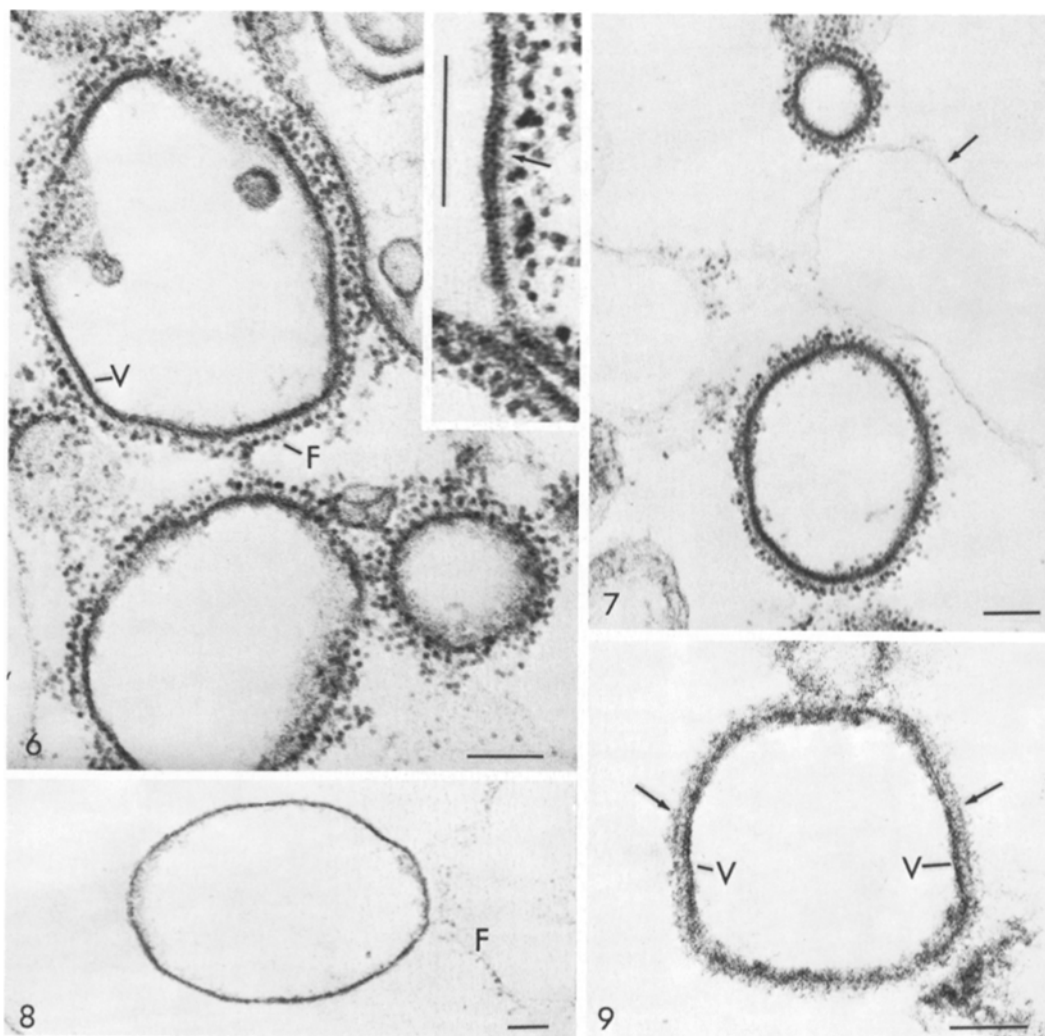
FIGURES 3–5 The thin sections for Figs. 3–5 were stained with uranyl acetate and lead citrate. Bars, 1 μm.

FIGURE 3 *Torpedo* vesicles from an indirect preparation made with anti-*Torpedo* receptor antiserum. Most of the vesicles (V) show an adherent layer of ferritin; a higher-power view of such vesicles is provided in Fig. 7. The arrow indicates the outline of a portion of the filter. × 24,000.

FIGURE 4 *Torpedo* vesicles from an indirect preparation made with an antiserum raised against *Torpedo* receptor dissociated in SDS. Most of the membrane surface is free of ferritin. The occasional small patches of ferritin that are seen (P) probably represent nonspecific trapping rather than specific binding, since in "blind" comparisons we could not distinguish vesicles treated as in this figure from those exposed instead to normal rabbit serum. The arrow indicates the outline of a portion of the filter. In addition to their intrinsic interest, such preparations serve as controls for the indirect method that supplement the normal-serum controls. The antiserum used here has a high titer of antibodies against receptor in solution (Table I, no. 2), but, despite this, fails to bind to receptor in membrane. × 22,000.

FIGURE 5 *Torpedo* vesicles from a preparation exposed to anti-*Torpedo* receptor antiserum but not to ferritin-conjugated antibodies. Many of the vesicles show a moderately electron-dense layer (L); a higher-power view is provided in Fig. 9. The arrow indicates the outline of a portion of the filter. × 23,000.

FIGURES 6-9   The thin sections for Figs. 6-9 were stained with uranyl acetate and lead citrate. Bars, 0.1 μm.

FIGURE 6   Vesicles from the same direct-method preparation used for Fig. 1. V indicates vesicle membrane and F, the layer of ferritin associated with one of the vesicles.The *inset* at the upper right shows a higher-magnification view of the surface of a vesicle from the same preparation. At the arrow are seen structures strongly reminiscent of the globules or palisade structure described in reference 36 as extending from the outer surface of the electroplax membrane. × 100,000; inset, × 190,000.

FIGURE 7   Vesicles from the same indirect-method preparation used for Fig. 3. The vesicles show a densely packed layer of ferritin aligned along their surfaces. Some ferritin is also present inside of the larger vesicle. The arrow indicates the outline of a portion of the filter. × 78,000.

FIGURE 8   Vesicle from the same direct-method, control preparation used for Fig. 2. There is no adherent layer of ferritin along the membrane (cf. Fig. 6). A few ferritin grains adherent to the protein film that outlines the filter (see Fig. 1, legend) are seen at F. × 58,000.

FIGURE 9   Vesicle from the same preparation used for Fig. 5. The vesicle shows a moderately electron-dense layer (arrows) associated with its membrane (V). × 100,000.
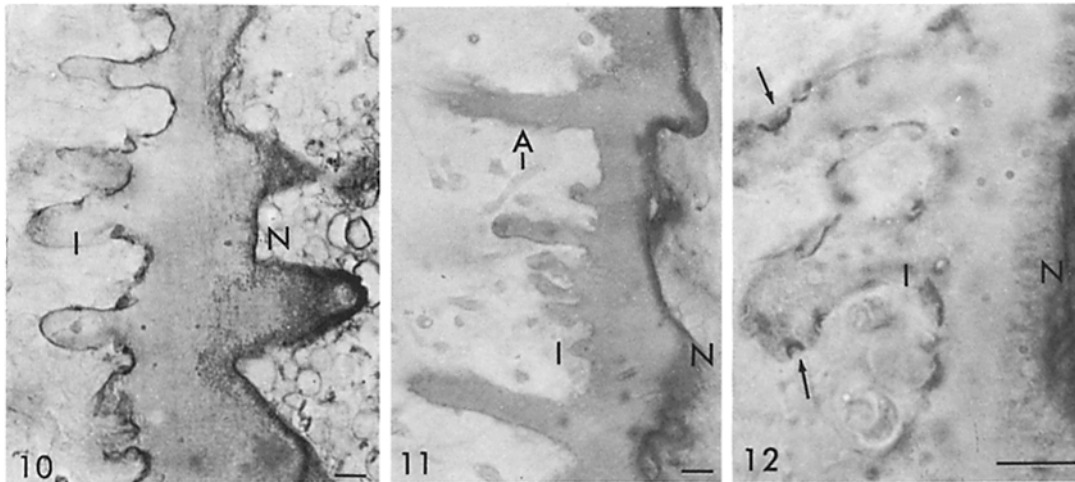
Figures 10-12   Figs. 10-12 are phase-contrast micrographs of thick sections of *Electrophorus* electroplax stained by the indirect immunohistochemical peroxidase procedures. Bars, 10 μm.

Figure 10   This preparation was made with anti-*Electrophorus* receptor antiserum. Peroxidase reaction product is seen on both cell surfaces, the innervated (*I*) and the noninnervated (*N*). × 420.

Figure 11   This preparation was made with normal rabbit serum. Peroxidase reaction product is seen only along the noninnervated cell surface (*N*). *A* indicates an axon near the innervated surface (*I*). The slight density along the innervated surface is due to the phase-contrast effect; examination with ordinary illumination reveals very little, if any, of the brown reaction product. × 420.

Figure 12   This preparation was made with anti-*Torpedo* receptor antiserum. Peroxidase reaction product is present along the noninnervated surface of the cell (*N*). Product is also present along the innervated surface (*I*), but here it is restricted to the troughlike invaginations (arrows), where axons make synaptic contact with the electroplax. × 1,000.
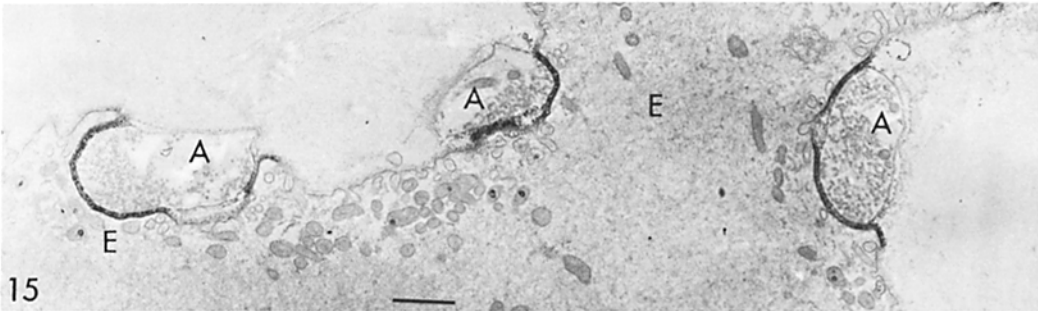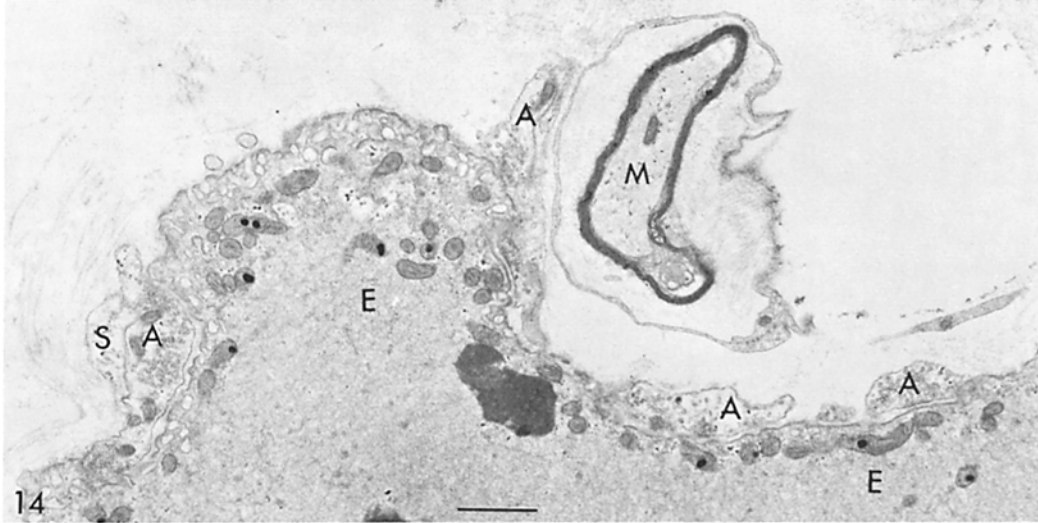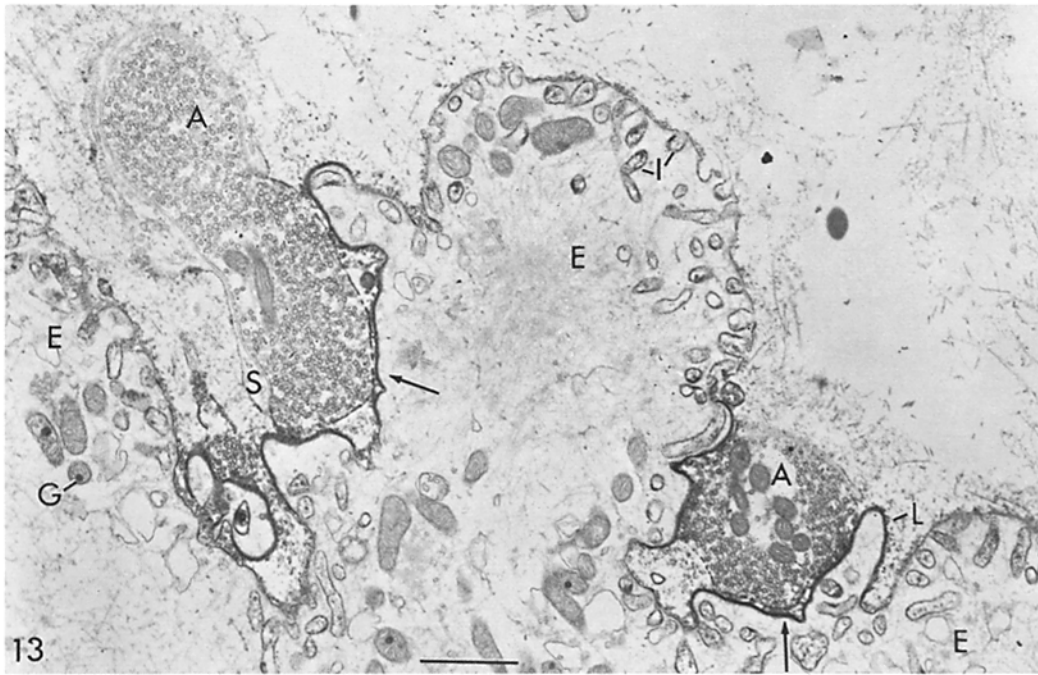
The binding of [³H]acetylcholine by receptor in *Torpedo* membrane (in the presence of 0.1 mM diisopropylfluorophosphate) was determined by equilibrium dialysis at 25°C near the half-saturating concentration of unbound acetylcholine (30 nM) (10). The quantity of acetylcholine bound to membrane preincubated for 1 h with a 70-fold excess of anti-*Torpedo* receptor IgG was 104 ± 15% (two experiments with the IgG from one antiserum) of that bound to membrane preincubated with IgG from normal rabbit serum.

DISCUSSION

The localization of the binding sites of antireceptor antibodies in *Electrophorus* electroplax is consistent with the localization of α-neurotoxin-binding sites in electroplax (7) and in muscle (3, 6, 11, 15, 18, 34) demonstrated through the use of radioactively tagged or fluorescent or peroxidase tagged α-neurotoxin or through the use of antibodies to α-neurotoxin. It is reassuring that despite the fact that the α-neurotoxin-binding sites

and the antibody-binding sites on the receptor apparently differ, the cytochemical localizations of receptor by the two approaches are the same. The demonstration of the binding of antireceptor antibodies to the intact electroplax supports the notion that such antibodies bind to receptor in myasthenia gravis. The suggestion that such binding is manifested in myasthenic muscle as a fuzzy coat on the crests of the postjunctional folds (35) is supported by our demonstration that exposure of receptor-rich *Torpedo* membrane to antireceptor antibody can produce a similar layer.

The localization of antibody binding to vesicles by immunoferritin techniques allows the determination of the proportion of membrane in a subcellular fraction that contains receptor. The Millipore filter technique that we have developed requires only 25 μg of membrane protein and is thus quite sensitive. This approach should also prove useful in the determination of the disposition of different antigenic determinants, particularly those of the individual polypeptide components of the recep-

tor, in the native membrane. As is clear, for example, from the lack of binding to *Torpedo* vesicles of antibodies raised against *Torpedo* receptor in SDS, not all the antigenic determinants accessible in solution are accessible from the extravesicular side of the vesicles. This surface corresponds to the extracellular side of the plasmalemma, since, for example, there is equal binding of the impermeable α-neurotoxin to closed vesicles and to a Triton extract of vesicles. With appropriate antibodies and by suitably opening the vesicles, we should be able to determine whether an antigenic determinant is accessible from the outside, the inside, or not accessible at all (cf. reference 28).

The mechanism of inhibition of the response of the electroplax by anti-*Electrophorus* receptor antisera is unknown. The direct determination of the effect of antibody binding on the binding of acetylcholine to receptor in *Electrophorus* membrane is technically difficult, and reliable data are not available. However, even in large excess, the autologous antireceptor antisera that we have tested seem not to inhibit appreciably the binding of α-neurotoxin to *Electrophorus* or to *Torpedo* membranes, even though antibodies contained in the sera do bind to these membranes. Furthermore, with *Torpedo* membranes, in which receptor concentration and affinity for acetylcholine are high, we have found that the antiserum that we tested does not inhibit acetylcholine binding, complementing the results with α-neurotoxin. Since α-neurotoxin and acetylcholine are thought to bind to the same sites, mechanisms other than the inhibition of acetylcholine (or carbamylcholine) binding should be considered to explain the effect of anti-*Electrophorus* receptor antibodies on the response of the electroplax. For example, the closed conformation of the acetylcholine receptor channel might be stabilized relative to the open form, such that the maximum permeability change obtained on saturation with agonist would be decreased without much change in the binding of agonist. Such a mechanism might be reflected in the decreased maximum response with little change in the concentration of agonist eliciting a half-maximal response that we (Fig. 21), and others (41) observe with the intact electroplax.

In contrast to our findings, Sanders et al. (37) have found that anti-*Torpedo* receptor antiserum does block acetylcholine binding to receptor in *Torpedo* membrane fragments about 50%. Such differences among antibody prepfarations are not surprising; similar differences seem to occur in anti-Na, K-ATPase antibody preparations (28). We conclude on the basis of our observations only that the binding of antibody to receptor need not necessarily block or perturb the acetylcholine- and α-neurotoxin-binding sites. Furthermore, even when the binding of antibodies inhibits receptor function, as in the case of anti-*Electrophorus* receptor antibodies acting on the intact electroplax, the effect is not necessarily due to inhibition of binding. Our observation that antireceptor antisera can block α-neurotoxin binding to detergent-solubilized receptor is consistent with
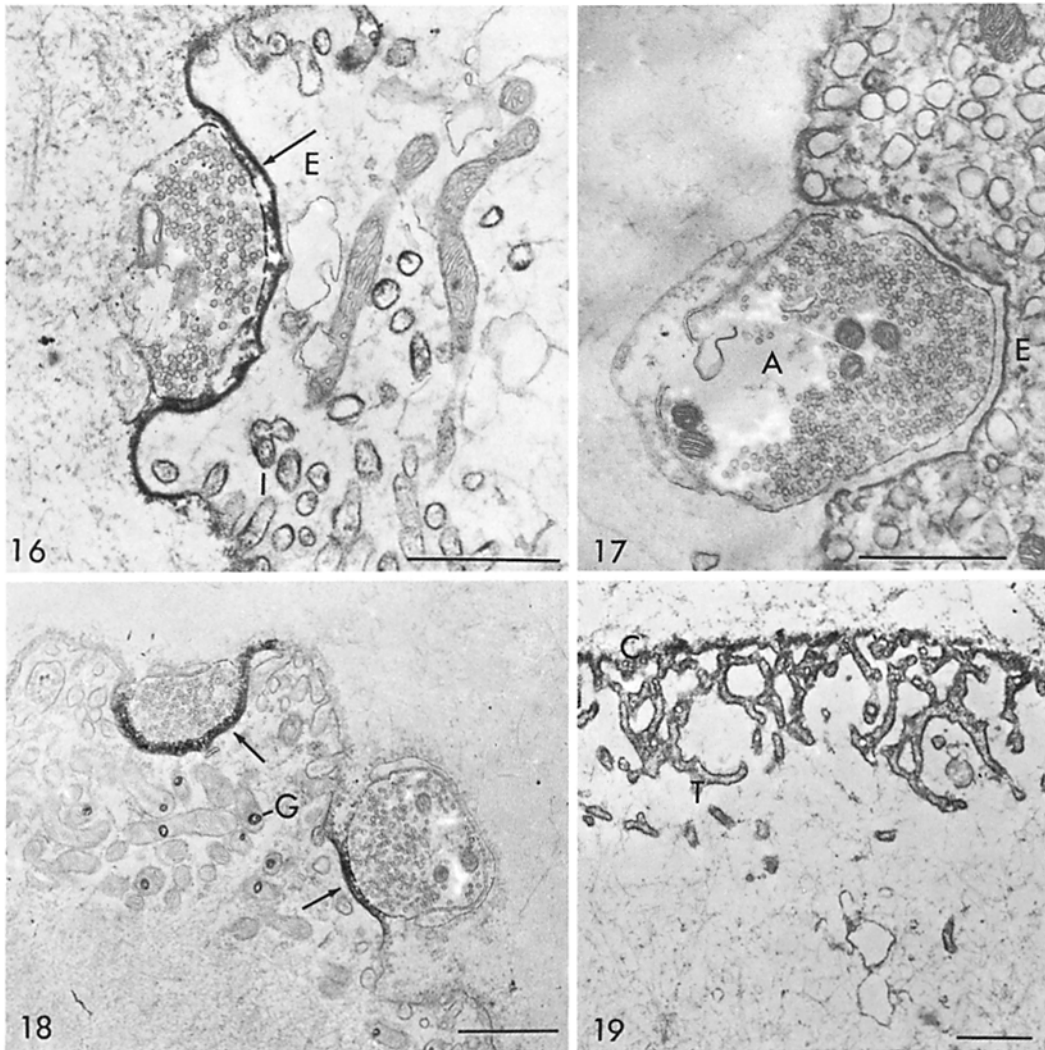

FIGURES 13–15 Figs. 13–15 are from the innervated surfaces of *Electrophorus* electroplax stained by the indirect immunohistochemical peroxidase procedures. The thin sections were stained with lead citrate. Bars, 1 μm.

FIGURE 13 This preparation was made with anti-*Electrophorus* receptor antiserum. *E* indicates portions of the electroplax; *A*, axon regions containing numerous synaptic vesicles; and *S*, a Schwann cell process. Peroxidase reaction product is seen along the electroplax surfaces where axons make synaptic contact (arrows) and along the surfaces immediately lateral to these contacts (*L*). Much less product is seen along the electroplax surfaces more distant from the axons, although some is present within some of the invaginations of the surface (*I*). As is evident from the subsequent micrographs, the electron-dense granules (*G*) within mitochondria are seen regularly in preparations made with all of the antisera, both normal and antireceptor. × 13,000.

FIGURE 14 This preparation was made with normal rabbit serum. *E* indicates portions of the electroplax; *A*, axon regions rich in synaptic vesicles; *S*, a Schwann cell process; and *M*, a myelinated region of an axon. No reaction product is seen along the electroplax surface. × 10,000.

FIGURE 15 This preparation was made with anti-*Torpedo* receptor antiserum. *E* indicates portions of the electroplax and *A*, axon regions. rich in synaptic vesicles. Peroxidase product is seen where the axons make synaptic contact with the electroplax. × 8,000.

FIGURES 16–19 Figs. 16–19 are from *Electrophorus* electroplax stained by the indirect immunohisto-chemical peroxidase procedures. Figs. 16–18 show the innervated surfaces of the electroplax whereas Fig. 19 shows a noninnervated surface. The thin sections were stained with lead citrate. Bars, 1 μm.

FIGURE 16 Portion of the same anti-*Electrophorus* antiserum preparation as was used for Fig. 13. Reaction product is seen along the electroplax surface (*E*), where an axon, rich in vesicles, makes synaptic contact (arrow). Product is also present along the surface lateral to this zone. The structures at *I* are sections through infoldings of the cell surface (see Fig. 13). × 20,000.

FIGURE 17 Portion of the same normal-serum preparation as was used for Fig. 14. No peroxidase activity is demonstrable. *A* indicates an axon, and *E*, the electroplax. × 20,000.

FIGURE 18 Portion of the same anti-*Torpedo* antiserum preparation as was used for Fig. 15. Peroxidase reaction product is seen along the electroplax surface where axons make synaptic contacts (arrows). *G* indicates a mitochondrial granule of the type mentioned in the legend to Fig. 13. × 13,000.

FIGURE 19 Portion of the noninnervated surface of a preparation made with anti-*Electrophorus* receptor antiserum. Reaction product is present in the extracellular coat (*C*) and, like the coat, it extends into the elaborate system of tubular infoldings of the surface (*T*). × 10,000.
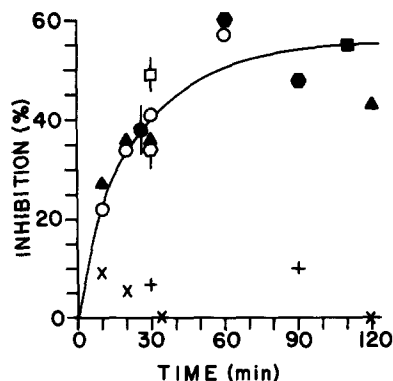
FIGURE 20 The inhibition after exposure to anti-*Electrophorus* receptor antisera and IgG of the carbamylcholine-elicited depolarization of the innervated membrane of the *Electrophorus* electroplax. The inhibition was determined as in Table I. The abscissa represents the time period of exposure to serum or IgG. The open symbols represent IgG fraction and the closed symbols heat-treated serum as follows: anti-serum from rabbit no. 16 (see Table I), diluted 1:10, (●); IgG fraction of no. 16 antiserum at 1 mg/ml, (○); antiserum from rabbit no. 17, diluted 1:20, (▲); 1:10, (●); 1:5, (■); IgG fraction of no. 17 antiserum at 1 mg/ml, (○); at 2 mg/ml, (□); normal rabbit serum diluted 1:10, (×); IgG fraction of normal rabbit serum at 1 mg/ml, (+).
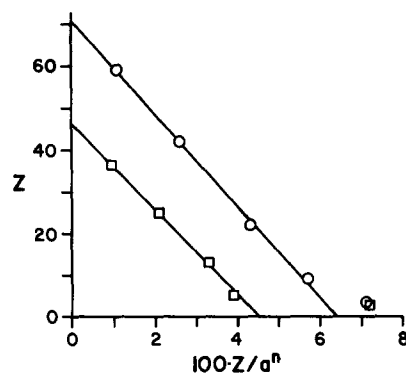
FIGURE 21 The carbamylcholine-elicited depolarization of the innervated membrane of the *Electrophorus* electroplax as a function of the concentration of carbamylcholine both before (○) and after (□) exposure of the membrane to anti-*Electrophorus* receptor antiserum (heat-treated) diluted 10-fold with eel Ringer's solution for 50 min. The depolarization 80 s after addition of carbamylcholine in mV (Z) is plotted against Z divided by the carbamylcholine concentration (a) in micromolar raised to the n power. In accordance with the equation $Z = Z_{max} - Z(K/a)^n$, the intercept on the ordinate is Z_{max}, and the slope is the concentration of agonist eliciting a half-maximal response (K) to the nth power. The Hill coefficient, n, is obtained from the slope of the plot of log $[Z/(Z_{max} - Z)]$ versus log a, and is in this case 1.69 before antiserum and 1.62 after antiserum. The maximum responses are 70 mV before and 47 mV after, and K is 60 μM before and 74 μM after. The data after antiserum were obtained starting 40 min after the antiserum had been removed and after several responses to carbamylcholine had been obtained in order to reach a state of stable inhibition (see text).

the report of Sanders et al. (37) on acetylcholine binding. It is possible that when receptor is embedded in its native membrane environment, the binding sites for acetylcholine and α-neurotoxin are stabilized, relative to the solubilized state, against the perturbing effects of antibody binding.

Similarly, the lack of an effect of anti-*Torpedo* receptor antisera on the response of the *Electrophorus* electroplax clearly indicates that the binding of antibody, demonstrable immunohistochemically, does not necessarily perturb function. Since *Electrophorus* receptor and *Torpedo* receptor differ even in gross characteristics such as polypeptide composition (23), it is to be expected that they will differ to some extent in antigenic determinants. The lack of inhibition of transport due to antibody binding in the cases of Na, K-ATPase (28) and Ca-ATPase (12) has been taken as evidence that these transport proteins do not undergo extensive movements such as, in the extreme, rotation through the membrane. It has previously been inferred that the flux of cations through the acetylcholine receptor in the active state was too great to be consistent with a mobile carrier and required a more or less fixed channel

that spanned the postsynaptic membrane (22). Our results with anti-*Torpedo* receptor antibodies support this inference.

The direct demonstration by the immunohistochemical methods of this paper that the receptor spans the membrane has, however, thus far eluded us. Although within occasional vesicles exposed to anti-*Torpedo* receptor antibodies there is a scattering of ferritin along the inner surface of the membrane (Fig. 7), such configurations are encountered too infrequently in our preparations to be interpreted reliably in terms of the location of antigenic sites, especially since the study of control preparations indicates that some non-specific trapping of antibodies, or ferritin, within vesicles may occur.

We assume that the histochemical binding and the functional perturbations that we observe are due to antireceptor antibodies rather than to

TABLE II

Effects of Autologous Antireceptor Antiserum on the Binding of α-Neurotoxin by Receptor in Membrane and in Triton X-100 Solution

	Torpedo	Electrophorus
	% of normal serum	% of normal serum
Membrane	99 ± 6 (3)	88 ± 1 (2)
solution	48 ± 2 (3)	36 ± 12 (2)

An aliquot of membrane fraction suspended in buffer ("membrane") or dissolved in Triton X-100 ("solution"), containing 1-3 pmol of α-neurotoxin-binding sites, was mixed with 25-50 μl of autologous, heat-treated antireceptor antiserum. After 1 h of 25°C, 50 μl of [α-^{3}H]-neurotoxin (25) (7 pmol, 10 Ci/mmol) was added and after 1 h, the mixture was diluted and filtered through two DEAE-cellulose filters (Whatman DE81) (27). To the filters in a counting vial were added 0.5 ml of 1 N HCl and 10 ml of Scintisol (Isolab, Inc., Akron, Ohio). Determinations were made in duplicate and were compared with binding to membrane mixed with an identical quantity of normal rabbit serum. The number of independent determinations is in parentheses. In the case of Torpedo, two sera were tested, one of them twice; in the case of Electrophorus, one serum was tested twice.

Torpedo membrane was prepared according to reference 9 and Electrophorus membrane according to reference 26. These fractions were diluted either with buffer solution containing 100 mM NaCl, 10 mM morpholino-propane sulfonic acid (MOPS) (pH 7.4) or with the same buffer containing 1% Triton X-100. The ratio of the number of antibody-combining sites for receptor, calculated from the serum titer (Table I), to the number of toxin-binding sites in the incubation mixtures was about 30 in the case of Torpedo and about 100 in the case of Electrophorus.

antibodies against some impurities in the receptor preparations that we used as immunogens. The receptor preparations were as highly purified as any that have been reported and contain the more or less accepted polypeptide components (23). The small quantity of receptor required to produce the immune response, the single lines obtained on immunodiffusion (43), the cross-reactivity between purified receptors of two different species, the specificity of the immunohistochemical binding to subsynaptic membrane, and the lack of binding to liver, erythrocyte, and mitochondrial membranes are consistent with our assumptions.

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