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Immunochemical Similarities between Subunits of Acetylcholine Receptors from *Torpedo*, *Electrophorus*, and Mammalian Muscle†

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ABSTRACT: Polypeptide chains composing acetylcholine receptors from the electric organs of *Torpedo californica* and *Electrophorus electricus* were purified and labeled with ^{125}I. Immunochemical studies with these labeled chains showed that receptor from *Electrophorus* is composed of three chains corresponding to the α, β, and γ chains of receptor from *Torpedo* but lacks a chain corresponding to the δ chain of *Torpedo*. Experiments suggest that receptor from mammalian muscle contains four groups of antigenic determinants corresponding to all four of the *Torpedo* chains. Binding of ^{125}I-labeled chains was measured by quantitative immune precipitation and electrophoresis. Antisera to the following immunogens were used: denatured α, β, γ, and δ chains of *Torpedo* receptor, native receptor from *Torpedo* and *Electrophorus* electric organs and from rat and fetal calf muscle, and human muscle receptor (from autoantisera of patients with myasthenia gravis). The four chains of *Torpedo* receptor were immunologically distinct from one another and from higher molecular weight chains found in electric organ membranes. Antibodies to these chains reacted very efficiently with native *Torpedo* receptor, but the reverse was not true. Antibodies to native receptor from *Torpedo* and *Electrophorus* reacted slightly with each of the chains of the corresponding receptor. However, cross-reaction between chains and antibodies to any native receptor was most obvious with the α chain of *Torpedo* or the corresponding α' chain of *Electrophorus*. Antiserum to α chains exhibited higher titer against receptor from denervated rat muscle. Antibodies from myasthenia gravis patients did not cross-react detectably with ^{125}I-labeled chains from electric organ receptors. Most interspecies cross-reaction occurred at conformationally dependent determinants whose subunit localization could not be determined by reaction with the denatured chains.

Acetylcholine receptor (AcChR)[1] purified from the electric organ of the marine elasmobranch *Torpedo californica* is composed of four glycopeptide chains in the apparent mole ratio $\alpha_2\beta\gamma\delta$ (Lindstrom et al., 1979; Weill et al., 1974; Raftery et al., 1975; Hucho et al., 1978; Chang & Bock, 1977; Froehner & Rafto, 1979; Vandlen et al., 1979). Receptor purified from the electric organs of the fresh water teleost *Electrophorus electricus* appears on polyacrylamide gel electrophoresis in $NaDodSO_4$ to be composed of two sharp bands of apparent molecular weights approximating 41 000 and 52 000 (Lindstrom & Patrick, 1974) as well as a more diffuse band approximating 60 000 (Karlin & Cowburn, 1973). Both the α chains of *Torpedo* (38 000 apparent molecular weight) and the 41 000 apparent molecular weight chain of *Electrophorus* are specifically labeled by an affinity labeling reagent directed at the acetylcholine binding site ([^{3}H]MBTA (Karlin & Cowburn, 1973; Weill et al., 1974). The function of the other chains in either AcChR is unknown, though it is suspected that they may be components of the ion conductance channel regulated by binding of acetylcholine, because it is known that pure AcChR can be prepared under conditions in which it retains full ion conductance activity (Moore et al., 1979; Lindstrom, Einarson, Anholt, and Montal, unpublished experiments). Previously, it had not been possible to determine whether these chains in the AcChR from each species were even structurally analogous. Substantial variations in the apparent molecular weights of the chains from either species further hampered this determination. Here we report that antisera to the α, β, γ, and δ chains from *Torpedo* AcChR can be used to identify the three chains from *Electrophorus* AcChR by cross-reaction as α', β', and γ', corresponding in immunochemical structure and presumably function to the corresponding chains from *Torpedo*.

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[1] Abbreviations used: AcChR, acetylcholine receptor; [^{125}I]-α-BGT, ^{125}I-labeled α-bungarotoxin; $NaDodSO_4$, sodium dodecyl sulfate; MG, myasthenia gravis; EAMG, experimental autoimmune myasthenia gravis; [^{3}H]MBTA, [^{3}H]-4-(*N*-maleimido)-α-benzyltrimethylammonium iodide.

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Biochemical studies of AcChR from muscle are difficult due to the limited amounts of material available and the more severe threat of proteolysis. AcChR purified from rat muscle (Froehner et al., 1977b; Nathanson & Hall, 1979) or a mouse muscle cell line (Boulter & Patrick, 1977) is composed of four prominent chains. Antigenic determinants specifically cross-reacting with each of the four *Torpedo* chains have been found in AcChR from human muscle (Lindstrom et al., 1978b). Two of the chains from rat muscle AcChR are labeled with [^{3}H]MBTA (Froehner et al., 1977a; Nathanson & Hall, 1979), suggesting that muscle AcChR contains α-like chains but that its structure may be more complex. Here we report that a single chain of AcChR from fetal calf muscle AcChR is labeled with [^{3}H]MBTA, that antisera to calf and rat AcChR cross-react with α and α', and that antisera to α can distinguish between AcChR from normal muscle and AcChR from denervated rat muscle. These results indicate the existence and importance of an α-like subunit in AcChR from muscle. Here we also report antigenic determinants in native fetal calf AcChR which specifically cross-react with antisera to the other *Torpedo* chains, but we have not yet extended this analysis to identifying corresponding chains as we have with *Electrophorus* AcChR.

The data we present suggest that AcChR from widely divergent species including both fish electric organs and mammalian muscle is composed of a multisubunit structure in which structural and functional analogies can be found between corresponding subunits. This is especially important in view of reports that AcChR can be purified from electric organs (Sobel & Changeux, 1977; Sobel et al., 1978) and muscle (Shorr et al., 1978; Merlie et al., 1977) which is composed only of α chains. The data presented here are consistent with the view that these reports are the result of proteolytic or other artifacts.

The specificities of antibodies to AcChR are especially interesting because these antibodies are the primary pathological agents in myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG) [reviewed in Lindstrom (1979)]. Immunization of rats with purified receptor (Lindstrom et al., 1976c) or its subunits (Lindstrom et al., 1978b) results in high titers of anti-AcChR antibodies, which, through their slight cross-reaction with AcChR in muscle, trigger a complex series of pathological mechanisms (Lindstrom et al., 1976a,b; Heinemann et al., 1977; Engel et al., 1977; Lindstrom & Einarson, 1979; Merlie et al., 1979) that impair neuromuscular transmission, causing weakness and death. In patients with MG, changes in an individual's anti-AcChR titer correlate well with changes in his disease severity (Dau et al., 1977; Newsome-Davis et al., 1978), but, in groups of patients, concentrations of anti-AcChR do not correlate well with severity (Lindstrom et al., 1976d). This has lead to the suggestion that specificity of the anti-AcChR antibody may be important. Knowledge of the specificity of anti-AcChR antibodies may also help to identify the unknown immunogen in MG. Here we report several observations on the specificity of anti-AcChR antibodies and their ability to induce EAMG.

Antisera to the purified AcChR macromolecule have been used to localize (Karlin et al., 1978; Tarrab-Hazadai et al., 1978; Klymkowsky & Stroud, 1979), quantitate (Lindstrom et al., 1976a), and impair the function of AcChR (Patrick et al., 1973; Heinemann et al., 1977; Karlin et al., 1978) as well as to affect AcChR metabolism (Heinemann et al., 1977, 1978) and produce an autoimmune disease model (Patrick & Lindstrom, 1973). Here we use antisera to AcChR subunits as templates to compare the structure of AcChR from various species. This begins development of a library of antibodies to specific antigenic determinants on *Torpedo* AcChR for use as templates for comparing structures of other AcChR and as probes for examining the functional role of these structures.

Materials and Methods

AcChR's from electric organs and muscle were purified by affinity chromatography on toxin–agarose as previously described (Lindstrom et al., 1978b). In order to avoid proteolysis of AcChR from fetal calf muscle, we maintained the temperature at all times at $\leq$4 °C, applied the extract to an affinity column and washed it within 1 day, included phenylmethanesulfonyl fluoride (10^{-3} M) and 5 mM EGTA to inhibit proteases, and used 10 mM NaN_3 to inhibit bacterial growth. Crude extracts of muscle AcChR for use as antigens in antibody assays were prepared as previously described (Lindstrom et al., 1976a).

The polypeptide chains composing *Torpedo* and *Electrophorus* AcChR were purified by preparative electrophoresis in $NaDodSO_4$ as described in the preceding paper (Lindstrom et al., 1979).

Polypeptide chains were iodinated by using lactoperoxidase coupled to agarose (David, 1971) kindly given by Dr. Gary David. Polypeptide chains (1.4×10^{-9} mol) were lyophilized in microfuge tubes and redissolved in 20 μL of water. Then 2.5 mCi of ^{125}I was added in 10 μL of water, followed by 5 μL of lactoperoxidase conjugated to agarose at 50 μg/mL. Centrifugation for 30 s ensured that all reactants were mixed. Reaction was initiated with 5.24 μL of a 1/20000 dilution of 30% H_2O_2. After vortexing and centrifugation, we applied this to a 1 × 10 cm column of Sephadex G-25 along with a 50-μL wash. The column was eluted with 0.1% $NaDodSO_4$ in 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, and 10 mM NaN_3. ^{125}I-Labeled peptide eluted in the void volume well resolved from free ^{125}I. From 40 to 80% of the total ^{125}I was incorporated in the peptide. Protein concentration was determined by A_{280}. Specific activities ranged from 1.5×10^{18} to 3×10^{18} cpm/mol.

Rats were immunized with *Torpedo* AcChR peptides (Lindstrom et al., 1978b) or native AcChR emulsified in 200 μL of complete Freund's adjuvant as previously described (Lennon et al., 1975). The AcChR content of these rats, their serum anti-AcChR antibody concentration, and the concentration of antibody-bound AcChR were measured as previously described (Lindstrom et al., 1976a).

Antisera were assayed by indirect immune precipitation using [^{125}I]-α-BGT-labeled native AcChR as previously described (Patrick et al., 1973) or using ^{125}I-labeled chains as the antigen. In both cases, antigen was used at 1×10^{-9} M. Native AcChR's were solubilized in 0.5% Triton X-100, whereas ^{125}I-labeled chains were solubilized in 0.5% Triton X-100 and 0.1% $NaDodSO_4$.

Anti-antibodies were conjugated to agarose to make reagents for precipitating immune complexes to be run on gels. These conjugates were far superior to the use of anti-antibody alone for two reasons. One, the antigen could be solubilized very efficiently, and, two, anti-antibody was not dissociated from the agarose, permitting much or all of the sample to be applied to a gel. Goat antihuman IgG was affinity-purified. Sepharose was activated with 100 mg/mL CNBr (Cuatrecasas & Anfinsen, 1971), reaction with antibody was in 0.2 M sodium carbonate buffer, pH 9.5, and the reaction was quenched after 11 h at 4 °C with 0.5 M glycine. Eighty-six percent of the antihuman IgG was coupled with Sepharose C14B, giving 13 mg/mL final yield. One-third activity was retained after

conjugation; thus, 140 μL of a 1:1 slurry was sufficient to bind the antireceptor antibody in 5 μL of serum. Goat antirat IgG serum (40% ammonium sulfate cut) was conjugated to Sepharose C14B by the same method to 10 mg/mL with 99% efficiency in 3 h. All activity was retained after conjugation; thus, 130 μL of a 1:1 slurry bound all the antireceptor antibody in 5 μL of serum.

Immune complexes with ^{125}I-labeled polypeptide chains as the antigen were prepared for electrophoresis as follows. Antisera (≤5 μL) were incubated in microfuge tubes overnight at 4 °C with 1 mL of 0.5% Triton X-100, 0.1% $NaDodSO_4$, 100 mM NaCl, 10 mM sodium phosphate, 10 mM NaN_3, and one or more ^{125}I-labeled chains at 1×10^{-9} M the same as that for quantitative immune precipitation. But instead of adding anti-antibody serum the next day, we added anti-antibody conjugated to agarose. The microfuge tubes (held horizontally) were agitated at 4 °C for 2 h. Then the tubes were centrifuged for 2 min. The supernatant was aspirated, and the pellet was washed 3 times by recentrifugation with 1 mL of 0.5% Triton buffer and twice with 1 mL of water. Bound ^{125}I chains were solubilized by the addition of 100 μL/tube of 2.3% $NaDodSO_4$, 0.625 M Tris-HCl buffer, pH 6.8, and 10% glycerol. After 30-min agitation at room temperature, tubes were centrifuged for 2 min, and the liquid was removed. Then β-mercaptoethanol was added to 5%, and aliquots were applied to gels. Adding β-mercaptoethanol later prevented solubilization of anti-antibody polypeptide chains which were not covalently attached to the agarose.

Electrophoresis on slab gels [19 × 18 × 0.1 cm, using the discontinuous gel system of Laemmli (1970) in 0.1% $NaDodSO_4$] was usually at 30 mA for a period of 5 h. Autoradiography of [^{3}H]MBTA-labeled gels was performed by the method of Bonner & Laskey (1974) as follows. The gel was fixed, stained, and destained in solutions containing 7% acetic acid and 40% methanol as usual. Then the gel was dehydrated in dimethyl sulfoxide, impregnated with 2,5-diphenyloxazole by incubating 1 h in 4 volumes of a 20% solution in dimethyl sulfoxide, washed extensively, and dried. An autoradiogram was prepared by exposing the gel at −80 °C on Kodak XR5 film sensitized by a 1-ms red flash. Autoradiography of ^{125}I-labeled gels was performed by the much simpler method of exposing the dried, destained gel at room temperature to Kodak NS5T film.

Affinity columns were used to adsorb antibodies from antisera to *Torpedo* chains. The δ chain was conjugated to Ultrogel ACA 34, a mixed agarose–acrylamide gel, because antibodies to δ bound nonspecifically to Sepharose C14B, because periodate-activated conjugation to Sephadex G-75 was not effective, and because controlled pore glass gave variable results. Ultrogel (4.7 mL) was activated by using 100 mg/mL CNBr (Cuatrecasas & Anfinsen, 1971) and then agitated at room temperature with 1.9 mg of δ in 5 mL of 0.2 M sodium bicarbonate buffer, pH 9.5, and 0.5% sodium dodecyl sulfate. A maximum of 30% of the δ was bound in 4 h, giving 1.9×10^{-9} mol of δ per mL of gel. After quenching for 2 h with 0.5 M glycine, the conjugate was washed with 200 mL of 0.1% $NaDodSO_4$, followed by 100 mL of 100 mM NaCl, 10 mM sodium phosphate buffer, pH 9.5, and 10 mM NaN_3. Before using the subunit affinity columns (~3 mL) to adsorb antisubunit antibody, we prewashed them with 0.5 mL of normal rat serum and then several volumes of saline (100 mM NaCl and 10 mM NaN_3). Then antisubunit serum (1 mL) was applied, left for 30 min at room temperature, and eluted with saline. After use, bound antibodies were eluted and the column was regenerated by washing first with several column volumes of 2% $NaDodSO_4$ and 5% β-mercaptoethanol.

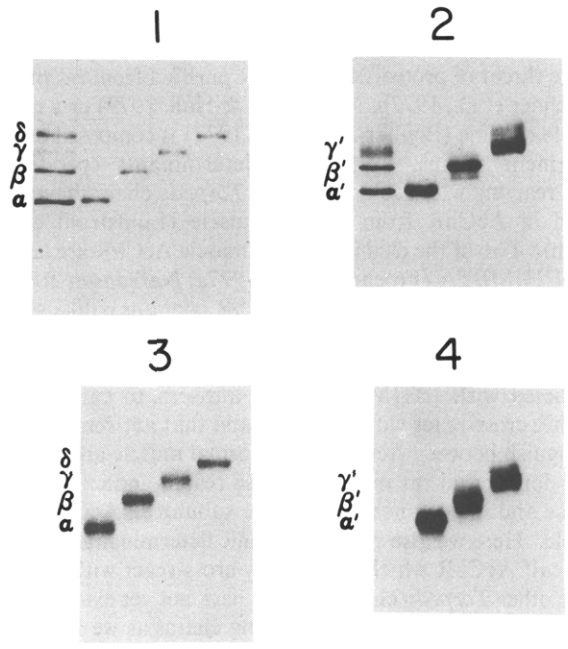

FIGURE 1: Polypeptide chains of *Torpedo* and *Electrophorus* AcChR. Samples were electrophoresed on polyacrylamide gels in $NaDodSO_4$. Part 1 shows a Coomassie brilliant blue stained gel of *Torpedo* AcChR and the four polypeptide chains α, β, γ, and δ purified from it. Part 2 shows a Coomassie brilliant blue stained gel of *Electrophorus* AcChR and the three polypeptide chains α′, β′, and γ′ purified from it. Part 3 shows an autoradiogram of the same gel shown in (1), revealing ^{125}I-labeled α, β, γ, and δ chains comigrating with their unlabeled counterparts seen in (1). Part 4 shows an autoradiogram of the same gel shown in (2), revealing ^{125}I-labeled α′, β′, and γ′ comigrating with their unlabeled counterparts seen in (2).

[^{3}H]MBTA was synthesized according to the method of Karlin et al. (1971) except that a 10-fold higher specific activity was obtained (25 Ci/nmol). AcChR's from *Torpedo* and fetal calf muscle AcChR were labeled with [^{3}H]MBTA approximately according to the method of Karlin & Cowburn (1973) as follows. Two aliquots of each AcChR (9.6 μg) in 20 μL of 0.2% cholate buffer were reduced by addition of 2 μL of 1 mM dithiothreitol for 20 min at 25 °C. Then 2.2 μL of 4.35×10^{-4} M *Naja naja siamensis* toxin III was added to one aliquot of each AcChR to protect the acetylcholine binding site. After 5 min, 4.4 μL of 0.02 mM [^{3}H]MBTA and 2 mM *N*-ethylmaleimide in acetonitrile was added to all aliquots. After 1 min, reaction was terminated by the addition of sample buffer, and the samples were electrophoresed. After staining, destaining, 2,5-diphenyloxazole impregnation, and drying, we exposed the autoradiogram for 48 h.

Results

The polypeptide chains found in Triton X-100 solubilized and affinity purified AcChR from *Torpedo* and *Electrophorus* were purified and iodinated (Figure 1). In seven preparations from *Electrophorus* an average of 60% of the protein applied to the gel was recovered in the mole ratio α′/β′/γ′ of 1.4:1:1.1. In *Torpedo* the apparent mole ratio depends on the total yield (preceding paper). Thus, at 100% yield the mole ratio in *Electrophorus* might vary significantly from these values. α′, β′, and γ′ all stain with the Schiff reagent, suggesting that they, like α, β, γ, and δ, all contain carbohydrate (data not shown).

AcChR was purified from fetal calf muscle by the methods used for AcChR from electric organs, including measures to inhibit proteolysis. The pattern of bands observed on acrylamide gel electrophoresis in $NaDodSO_4$ of preparations

Table I: Immunization of Rats with *Torpedo* AcChR Chains

group		dose (total μg)	kill day	weakness	av serum anti-AcChR antibody concn ($\times 10^{-9}$ mol/L ± SE) antinative rat AcChR	antinative *Torpedo* AcChR	anti-homologous chain	muscle AcChR (pmol/rat)	antibody-bound muscle AcChR (% of total)
A:[a]	control	0	77–89	0/3	0.06	0.06		42	0.3
	α	200–250	64–77	3/3	32.0	5000	13000	14	44.0
	β	250	77–89	0/3	0.3	1500	2700	26	1.6
	γ	250	77–89	1/3	5.9	3200	2900	20	28.0
	δ	250	69–89	1/3	29.0	4500	4100	22	36.0
B:[b]	α	300	69	2/4	9.5	3200	5200	25	22.0
	β	164	69	1/4	9.6	1400	2400	23	29.0
	γ	150	69	3/4	16.0	900	2700	32	32.0
	δ	171	69	4/4	84.0	2600	2100	23	58.0
C:[c]	control	0	47	0/4	0.00	0.00		65	0.06
	α	100	47	1/4	5.6	3200	4500	40	12.0
	β	100	47	0/4	1.6	1500	1370	44	1.8
	γ	100	47	0/4	0.00	930	1540	51	0.92
	δ	100	47	0/4	1.3	2000	900	59	8.2
	ϵ and ζ	100	47	0/4	0.05	25	394	53	0.24
D:[d]	α	150	62	8/9	21.0	6400	1300	42	35.0
	β	150	62	5/9	16.0	5800	1800	42	56.0
	γ	150	63	4/9	8.7	4000	940	40	7.0
	δ	150	63	9/9	6.6	4600	1200	33	15.0
E:[e]	α	200	72	1/12	0	2400	1300	nt[f]	nt[f]
	β	200	73	0/12	0	2500	840	nt[f]	nt[f]
	γ	200	73	0/12	0	2500	520	nt[f]	nt[f]
	δ	200	72	0/12	0	2500	840	nt[f]	nt[f]

[a] Three rat groups given five injections. [b] Four rat groups given three injections. [c] Four rat groups given two injections. [d] Nine rat groups given three injections. [e] Twelve rat groups given four injections. These rats suffered severely from chronic respiratory disease which may have had an immunosuppressive effect. [f] nt, not tested.

of AcChR from fetal calf muscle always included prominent bands approximating the molecular weight of α, β, γ, and δ but also included variable amounts of bands at higher and lower apparent molecular weights. Because the specific activity of the best preparations ranged only from 1.5 to 2.5 nmol of [^{125}I]-α-BGT bound per mg of protein, some of these bands were undoubtedly contaminants. Pure, fully active *Torpedo* AcChR is thought to have a specific activity of 8 nmol/mg (Damle & Karlin, 1978; Reynolds & Karlin, 1978). Because, even allowing for some inactivation of AcChR during purification, our preparations of fetal calf muscle AcChR were probably less than 50% pure, their appearance on gels is not shown. We did, however, demonstrate that these preparations contained a single chain which, like α and α′ of electric organ AcChR's, was specifically labeled by [^{3}H]MBTA (Figure 2).

Several groups of rats were immunized with α, β, γ, and δ chains of *Torpedo* AcChR (Table I). Some rats were immunized with extracts of unused acrylamide gels as a control. One group of rats was immunized with the ϵ and ζ chains. ϵ and ζ are characteristic of *Torpedo* membrane fractions poor in AcChR (Hucho et al., 1978) and contaminate AcChR solubilized and purified in low concentrations of cholate (preceding paper). The animals in group A have been previously described (Lindstrom et al., 1978b). Measurement of antibody concentration in successive serum samples from rats immunized with denatured α, β, γ, or δ chains showed that substantial concentrations of antibodies which cross-reacted with [^{125}I]-α-BGT-labeled native *Torpedo* AcChR developed quickly, but immunization with relatively large amounts of denatured chains over rather long periods was required to produce significant concentrations of antibodies capable of cross-reaction with muscle AcChR in vivo or in vitro. Immunization of rats with 1 μg of native AcChR produces a significant response to muscle AcChR (Lindstrom & Einarson, 1979), whereas doses totaling hundreds of micrograms were required in these experiments. This is reflected in the concentrations of antibody cross-reacting with muscle AcChR observed at sacrifice (Table I). For example, in group C, given 100 μg of each chain over 47 days, we found that concentrations of antibodies cross-reacting with rat AcChR were very low except in the case of α, whereas in group D, given 150 μg of each chain over 63 days, we found that significant concentrations were obtained with all chains. As

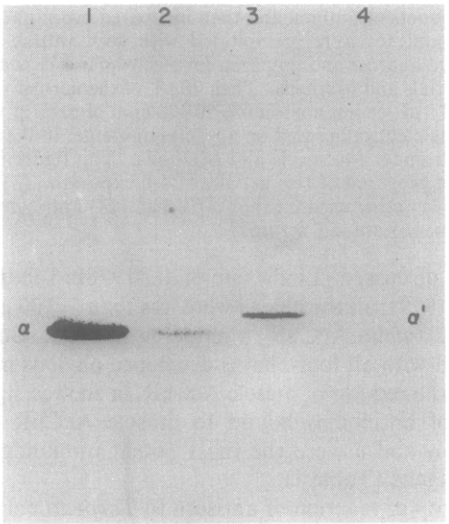

FIGURE 2: Specific [^{3}H]MBTA affinity labeling of a single polypeptide chain in AcChR from *Torpedo* electric organ and fetal calf muscle. The figure shows an autoradiogram of [^{3}H]MBTA-labeled samples electrophoresed on an acrylamide gel in $NaDodSO_4$. (1) *Torpedo* AcChR. Only α chains are labeled. (2) *Torpedo* AcChR protected from [^{3}H]MBTA by toxin. Virtually all labeling is specifically inhibited. (3) Fetal calf muscle AcChR. Only chains of 41 500 apparent molecular weight are labeled. (4) Fetal calf muscle AcChR protected from [^{3}H]MBTA by toxin.

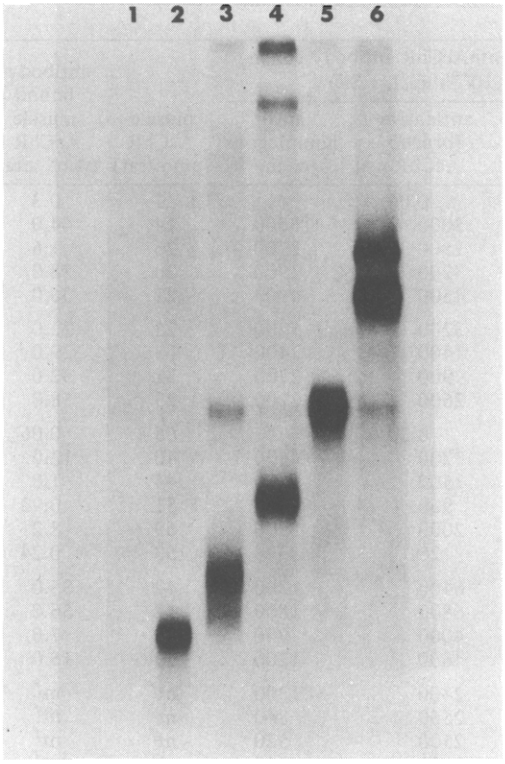

FIGURE 3: Reaction of antisera to *Torpedo* chains with a mixture of ^{125}I-labeled chains. Antigen was a mixture of ^{125}I-labeled α, β, γ, δ, and ϵ,ζ, each at 2×10^{-9} M in a total volume of 1 mL of 0.5% Triton X-100, 0.1% $NaDodSO_4$, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, and 10 mM NaN_3. Antisera (≤ 5 μL) were added to aliquots of antigen and then incubated overnight at 4 °C. Immune complexes were precipitated with goat antirat antibody conjugated to agarose and prepared for electrophoresis as described under Materials and Methods. Then 20 μL of the normal rat serum sample, or 17 μL or less containing 50 000 cpm of each of the other samples, was electrophoresed on an acrylamide gel in $NaDodSO_4$, as described under Materials and Methods. The figure is an autoradiogram prepared of this gel after 24-h exposure. (1) Normal rat serum; (2) anti-α serum; (3) anti-β serum; (4) anti-γ serum; (5) anti-δ serum; (6) anti-ϵ,ζ serum.

previously discussed (Lindstrom et al., 1978b; Lindstrom & Einarson, 1979), all the chains were less than 1/100 as potent as native *Torpedo* AcChR in inducing EAMG. Some rats immunized with all four chains developed obvious muscular weakness, showed loss of muscle AcChR, or showed significant amounts of antibodies bound to muscle AcChR in vivo. However, α and δ were the most potent immunogens and myasthenogens (Table I).

Specificity of reaction of antisera to *Torpedo* polypeptide chains was assayed by competitive immunoprecipitation using a mixture of ^{125}I-labeled chains as the antigen, followed by electrophoresis to resolve which chain was bound by each antiserum. Equal molar amounts of ^{125}I-labeled α, β, γ, and δ chains were used in order to determine how well each antichain serum could distinguish between these chains. Also included were equal molar amounts of ^{125}I-labeled ϵ and ζ chains. The test serum was added to this mixture. Then antibodies were immune precipitated along with whatever ^{125}I-labeled antigen they bound from the mix. Finally, the bound ^{125}I-labeled chain was dissociated in $NaDodSO_4$ and electrophoresed on an acrylamide gel for identification. Nonspecific precipitation by normal serum and degradation of the ^{125}I-labeled chains during this process were both negligible. Figure 3 shows that antisera from Table I, group C, to α, β, γ, δ, and ϵ,ζ quite specifically bound to their appropriate antigens. This is consistent with the competitive immunoprecipitation experiments using unlabeled chains previously reported (Lindstrom et al., 1978b) and the peptide mapping experiments reported in the preceding paper (Lindstrom et al., 1979) and elsewhere (Froehner & Rafto, 1979; Nathanson & Hall, 1979). All these experiments suggest that each *Torpedo* chain is unique and not derived from the other by overlapping proteolytic cleavage of a large peptide.

However, antisera to β and to ϵ,ζ bound small amounts of δ (Figure 3). These results are more likely to result from cross contamination of the immunogens than from antigenic similarity. The radioimmune assays used are extremely sensitive and can easily detect very small degrees of reaction. It might be expected that antisera to γ and ϵ,ζ would show traces of antibodies to δ. δ is adjacent to γ and ϵ,ζ during purification, so traces of δ might contaminate γ and ϵ,ζ immunogens. The degree of cross-reaction of antisera to γ or ϵ,ζ with δ could exceed the degree of contamination of these immunogens with δ because δ is a potent immunogen. The cross-reaction of this antiserum to β with δ was only 1.4% (it bound 2×10^{-7} mol of β per L but only 2.8×10^{-9} mol of δ per L). A curious observation is that antisera to β show cross-reaction with δ, while the reciprocal is not true. One possible explanation for the apparent cross-reaction of antisera to β with δ is that δ breaks down into fragments the size of β, of which traces contaminate β. Peptide maps of some preparations of chains are consistent with the idea of trace contamination (preceding paper). Purified β is fairly unstable and spontaneously degrades into a component with an apparent molecular weight of approximately 43 000, and aged preparations may contain nearly equal amounts of these two bands. Curiously, however, purified δ shows no tendency to spontaneously degrade.

Specificity of antisera to α, β, γ, and δ was also studied by quantitative immune precipitation (Figure 4). As observed in Figure 3, the antisera are quite specific, except for traces of cross-reaction of antisera to β and γ with δ. Most of the anti-δ antibodies in the antisera to β or γ were adsorbed by passing these antisera over an affinity column of δ (Figure 4).

Some evidence in support of the idea that ζ chains correspond to the enzymatically active subunit of Na^+/K^+-ATPase is provided by competitive immune precipitation. Purified Na^+/K^+-ATPase from *Electrophorus* electric organ (Dixon & Hokin, 1978) (a gift from George Fortes) was ^{125}I-labeled. Its high molecular weight subunit electrophoresed with the same apparent molecular weight as ζ (Figure 5). When native [^{125}I]ATPase was used as the antigen, antisera to ϵ,ζ precipitated large amounts of labeled material. However, most ^{125}I was incorporated into the lower molecular weight carbohydrate-rich chain of the ATPase. Dissociation of the ^{125}I-labeled ATPase into its component polypeptide chains before addition of antibody was necessary in order to determine which chain interacted with the antibody. Antisera to ϵ,ζ bound significantly more of the high molecular weight ATPase chain than control sera (Figure 5).

Cross-reaction of antisera to α, β, γ, and δ with chains from *Electrophorus* was assayed by competitive immunoprecipiation of ^{125}I-labeled chains from *Electrophorus* (Figure 6). Antiserum to α chains of *Torpedo* cross-reacted only with the α' chains of *Electrophorus* (hence the term α'). Antisera to β and γ chains of *Torpedo* cross-reacted only with the β' and γ' chains of *Electrophorus*, respectively. Antisera to δ of *Torpedo* also cross-reacted with the δ' chains of *Electrophorus*. In order to fully understand these results we found that several other observations were required. Figure 7 shows the reaction of anti-γ sera from the B, C, D, and E groups of rats from

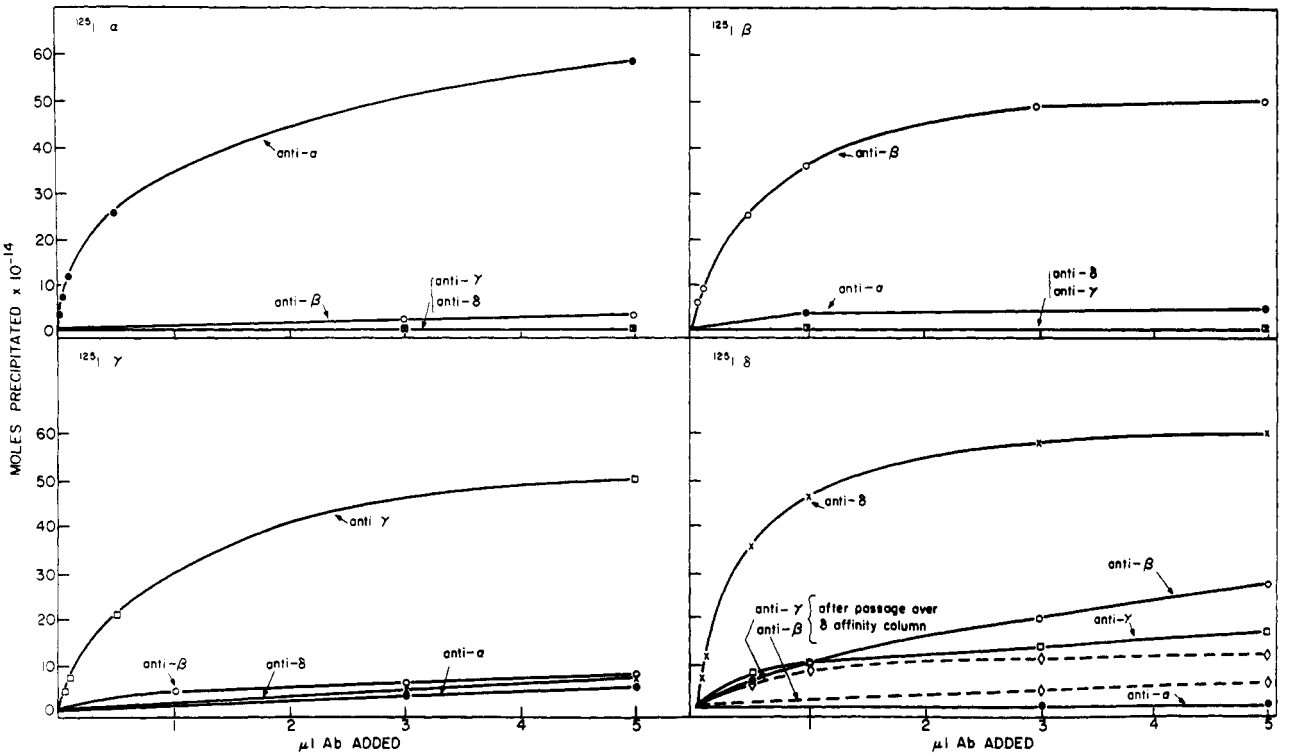

FIGURE 4: Quantitative immune precipitation of ^{125}I-labeled chains of *Torpedo* AcChR by antisera to these chains. Each ^{125}I-labeled chain (1×10^{-9} M; 1 mL in triplicate) was incubated overnight with the indicated amounts of antiserum plus normal serum to total 5 μL. Goat antirat IgG was added, the immune precipitates were collected and washed by centrifugation, and ^{125}I in the pellet was measured as previously described (Lindstrom et al., 1978b). Antibody titers (measured in moles of chain bound per liter of serum) are the slopes of the curves in the initial linear portion. The curves plateau when all available antigen is precipitated.

Table I with mixtures of ^{125}I-labeled *Torpedo* chains and with mixtures of ^{125}I-labeled *Electrophorus* chains. All anti-γ sera react with ^{125}I-labeled γ, and all but group C cross-react with ^{125}I-labeled γ′. The C group of rats was sacrificed much earlier in the course of immunization than the others and was the only set of anti-γ sera which did not show cross-reaction with ^{125}I-labeled δ. We now know that this cross-reaction between γ and δ which appears after prolonged immunization is at least in part due to antigenic similarity between γ and δ rather than to contamination of the immunogen with δ. The evidence for this comes from a cloned hybridoma cell line formed by fusing spleen cells from a rat immunized with δ to mouse myeloma cells (Tzartos and Lindstrom, unpublished experiments). Monoclonal antibodies produced by this cell line and all its subclones have high affinity for δ and low affinity for γ. These antibodies cross-react with γ′. Further, we have now found conditions under which *Electrophorus* AcChR can be prepared with a fourth subunit, δ′ (Lindstrom et al., unpublished experiments). δ′ cross-reacts with this monoclonal anti-δ antibody but not with anti-γ sera. Together, all these results suggest that α′, β′, and γ′ chains of *Electrophorus* AcChR are structurally similar to the α, β, and γ chains of *Torpedo* but that *Electrophorus* AcChR when purified in the normal way lacks a subunit corresponding to δ.

Cross-reaction of antisera to α, β, γ, and δ with [^{125}I]-α-BGT-labeled native *Torpedo* AcChR was studied by quantitative immune precipitation (Tables I and II). The concentration of each antiserum measured by using [^{125}I]-α-BGT-labeled *Torpedo* AcChR was within two- or threefold of its concentration measured by using an ^{125}I-labeled homologous *Torpedo* chain. This observation is consistent with the observation that antisera to *Torpedo* chains are very efficiently inhibited from cross-reaction with native *Torpedo* AcChR by unlabeled homologous chains (Lindstrom et al., 1978b). Both observations suggest that antisera to denatured chains bind very efficiently to native *Torpedo* AcChR and that few, if any, of these antibodies are directed at determinants revealed only on the denatured chains. Titers of antichain sera were greater against [^{125}I]-α-BGT-labeled *Torpedo* AcChR than against ^{125}I-labeled chains. This is expected because a single mole of antibody could bind as much as 2 mol of AcChR dimers (which bind a total of 8 mol of [^{125}I]-α-BGT), whereas a single mole of antibody could bind no more than 2 mol of ^{125}I-labeled chains.

Antisera to *Torpedo* chains also cross-reacted slightly, but detectably, with [^{125}I]-α-BGT-labeled native AcChR from several other species (Tables I and II). Cross-reaction with other species was greatest after prolonged immunization. These antisera, like antisera to native *Torpedo* AcChR (Lindstrom et al., 1978a), cross-react best with *Electrophorus* AcChR and less well with muscle AcChR. Cross-reaction was least with rat muscle AcChR, perhaps because many of these cross-reacting antibodies were adsorbed in the course of the autoimmune response in the immunized rats. Antiserum to α, unlike antiserum to native *Torpedo* or *Electrophorus* AcChR (Weinberg & Hall, 1979), could distinguish between AcChR from normal muscle and AcChR from denervated muscle. Antiserum to α, like most (Weinberg & Hall, 1979) but not all (Table III) sera from patients with MG, had a higher titer against extrajunctional AcChR from denervated muscle than against junctional AcChR from normally innervated muscle. Some pools of anti-β sera, but no pools of anti-γ or anti-δ sera tested, showed a smaller, but similar, discrimination. These results suggest that the extra antigenic determinants on extrajunctional AcChR (Weinberg & Hall, 1979) which account for increased reaction with sera from MG patients may in part be on α-like chains.

Specificity of the cross-reaction of antisera to *Torpedo* chains with [^{125}I]-α-BGT-labeled native fetal calf AcChR was tested by competitive inhibition of the antisera with unlabeled

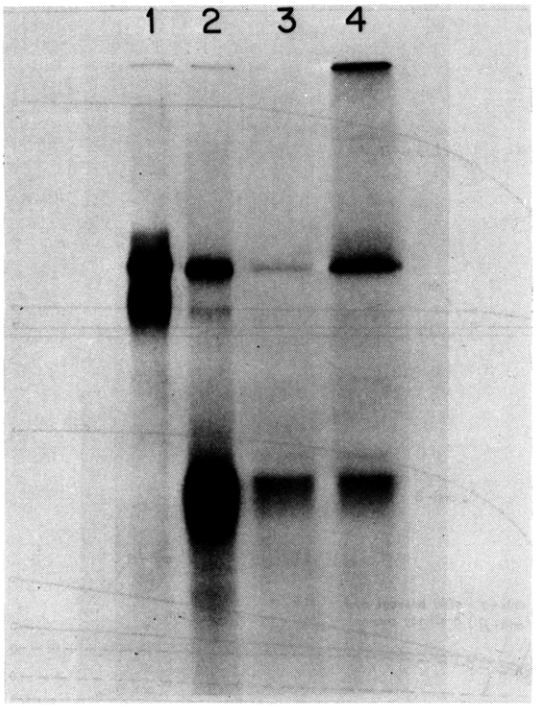

FIGURE 5: Reaction of antisera to ϵ,ζ with ^{125}I labeled chains of Na^+/K^+-dependent ATPase from *Electrophorus*. ATPase was labeled with ^{125}I (1×10^{19} cpm/mol). It was dissociated into its component chains in 2% $NaDodSO_4$ and diluted 25-fold to 2×10^{-9} M in buffer containing 0.5% Triton X-100 and 0.1% $NaDodSO_4$. 1-mL aliquots were incubated with either 5 μL of normal rat serum or 5 μL of antiserum to ϵ,ζ and then immunoprecipitated, electrophoresed, and autoradiographed, as described in Figure 3. (1) ^{125}I-Labeled ϵ,ζ; (2) ^{125}I-labeled *Electrophorus* Na^+/K^+-ATPase; (3) normal rat serum incubated with [^{125}I]ATPase; (4) antiserum to ϵ,ζ incubated with [^{125}I]ATPase.

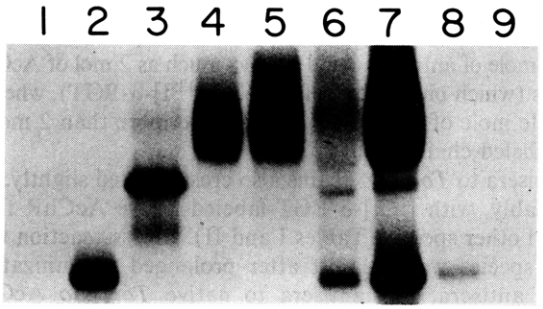

FIGURE 6: Reaction of antisera with a mixture of ^{125}I-labeled chains from *Electrophorus* AcChR. Antigen was a mixture of ^{125}I-labeled α', β', and γ', each at 2×10^{-9} M. Antisera from group E, Table I, were incubated with the antigen mix, precipitated, washed, and electrophoresed, and the dried gel was autoradiographed for 24 h as described in Figure 3. (1) Normal rat serum; (2) anti-α serum; (3) anti-β serum; (4) anti-γ serum; (5) anti-δ serum; (6) antinative *Torpedo* AcChR serum; (7) antinative *Electrophorus* AcChR serum; (8) antifetal calf AcChR serum; (9) antirat muscle receptor serum. Faint reaction with α' was visible after longer exposure of the autoradiogram.

chains (Figure 8). Antisera to α, β, and δ were inhibited only by the homologous chains. Antiserum to γ was most efficiently inhibited by γ, but δ inhibited significantly. The very low titer of anti-γ serum against fetal calf AcChR makes this experiment especially difficult, because this requires large amounts of anti-γ activity and unlabeled γ. Thus, small amounts of antibodies to δ in the serum and small amounts of δ in the γ could account for this observation. However, the antigenic similarity known to exist between γ and δ and γ' and δ' (Lindstrom et al. and Tzartos and Lindstrom, unpublished experiments) could also account for this observation.

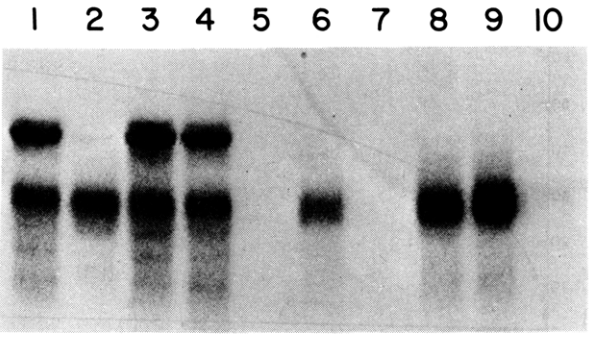

FIGURE 7: Reaction of antisera to γ from rats early and late in the course of immunization with ^{125}I-labeled chains of *Torpedo* and *Electrophorus* AcChR. Antigen for (1–5) was a mixture of ^{125}I-labeled α, β, γ, and δ, each at 2×10^{-9} M. Antigen for (6–10) was a mixture of ^{125}I-labeled α', β', and γ'. With both antigens sera were used in this order: (1 and 6) anti-γ sera from group B, Table I (bled day 69); (2 and 7) anti-γ sera from group C, Table I (bled day 47); (3 and 8) anti-γ sera from group D, Table I (bled day 63); (4 and 9) anti-γ sera from group E, Table I (bled day 73); (5 and 10) normal rat serum.

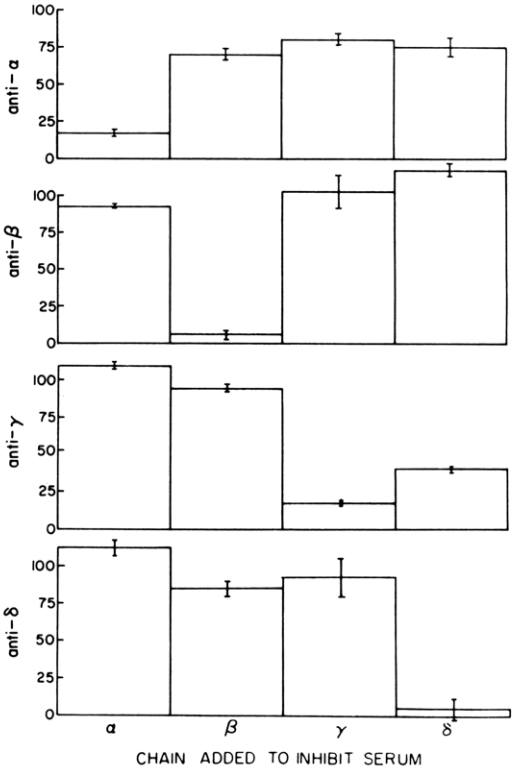

FIGURE 8: Specific inhibition of antisera to *Torpedo* chains from reaction with [^{125}I]-α-BGT-labeled fetal calf muscle AcChR by *Torpedo* chains. The method used is the same as that previously used to demonstrate that receptor from human muscle contains four sets of antigenic determinants comparable to those on the *Torpedo* subunits (Lindstrom et al., 1978b). Antisera from group D, Table I (5 μL of anti-α, -β, and -γ and 10 μL of anti-γ), were incubated overnight at 4 °C in 0.5 mL with 1.5 equiv of the moles of *Torpedo* chain bound by that amount of serum. Then 0.5 mL of fetal calf muscle AcChR at 1×10^{-9} M prelabeled with 2×10^{-9} M [^{125}I]-α-BGT was added overnight. Finally, goat antirat IgG was added to precipitate the antibody and any [^{125}I]-α-BGT labeled receptor it bound, as in a normal radioimmune assay.

This experiment with fetal calf muscle AcChR and a similar experiment with human muscle AcChR (Lindstrom et al., 1978b) suggest that muscle AcChR's contain four groups of antigenic determinants which specifically cross-react with each of the four chains of *Torpedo* AcChR. Figure 2 shows that fetal calf AcChR contains a chain comparable to α. However, we have not yet determined whether the other groups of

Table II: Titers of Representative Antisera against Several AcChR Antigens

antiserum	antibody titer against several antigens (nmol of antigen bound per L of serum)											
	[^{125}I]-α-BGT-labeled native AcChR					^{125}I-labeled denatured AcChR polypeptide chains						
						Torpedo				*Electrophorus*		
	Torpedo	*Electrophorus*	rat muscle	denervated rat muscle	fetal calf muscle	human muscle	α	β	γ	δ	α′	β′	γ′
anti-α[a]	3570	47	3.3	21.0	33	17	1600	35	10	6	37	0	0
anti-β[a]	2020	241	3.9	3.2	23	14	10	653	38	29	0	27	0
anti-γ[a]	1120	9	2.1	2.9	4.5	6.4	0	18	500	27	0	0	0
anti-δ[a]	3290	186	4.4	6.2	19	12	0	9	15	506	0	0	62
antinative *Torpedo* AcChR	9740	1038	230	270	669	191	125	83	117	222	18	3	3
antinative electrophorus AcChR	2110	4800	251	372	829	462	25	0	0	0	191	301	227
antifetal calf muscle AcChR	1094	750	776	1980	6130	1182	40	0	0	0	20	4	11
antirat muscle AcChR	2420	32	590	840	403	34	0	0	0	0	2	0	9

[a] Single rats from group C.

Table III: Titers of Sera from MG Patients Using AcChR from Human and Normal and Denervated Rat Muscle as Antigens

serum	antibody titer × 10^{-9} M [^{125}I]-α-BGT binding sites precipitated per L of serum			
	human	rat	denervated rat	(denervated titer)/(normal titer)
1	1290	7.1	23.2	3.3
2	1250	7.9	44.1	5.6
3	1240	6.8	19.1	2.8
4	936	7.1	55.6	7.8
5	697	6.6	26.3	4.0
6	557	2.1	6.3	3.0
7	505	8.2	5.9	0.7
8	432	35.6	32.0	0.9
9	298	32.2	49.2	1.5
10	239	30.4	42.0	1.4
11	216	3.1	4.3	1.4
12	100	1.9	2.7	1.4
13	99	6.3	15.3	2.4
14	57	9.8	8.6	0.9
15	56	3.7	6.9	1.9

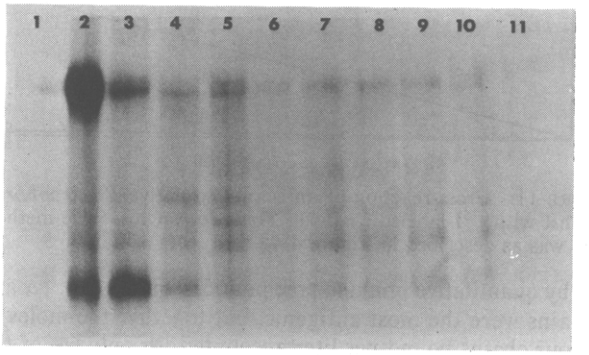

FIGURE 9: Reaction of antisera to native AcChR with a mixture of ^{125}I-labeled chains from *Torpedo* AcChR. Antigen was a mixture of ^{125}I-labeled α, β, γ, and δ, each at 2×10^{-9} M. Antisera were incubated with the antigen mix, precipitated, washed, and electrophoresed as described in Figure 3. (1) Normal rat serum; (2) antinative *Torpedo* AcChR serum; (3) antinative *Electrophorus* AcChR serum; (4) antifetal calf AcChR serum; (5) antirat muscle AcChR serum; (6) normal human serum; (7–10) sera from myasthenia gravis patients; (11) serum from a muscular dystrophy patient.

antigenic determinants, as in the case of *Electrophorus*, are distributed on separate chains.

Antisera to native electric organ or muscle AcChR cross-reacted very inefficiently with ^{125}I-labeled denatured chains (Table II and considered in subsequent paragraphs). This contrasts with the previously demonstrated efficient cross-reaction of antisera to denatured *Torpedo* AcChR chains with native *Torpedo* AcChR. This is consistent with other observations suggesting that the most immunogenic and myasthenogenic determinants on AcChR are formed by the native conformation of its component polypeptide chains (Lindstrom et al., 1976c). Further, this suggests that the antigenic determinants shared by denatured chains and native AcChR are normally not very immunogenic and are qualitatively different from the highly immunogenic determinants peculiar to native AcChR. It should be remembered that the term "native AcChR" may in this case mean native only with respect to $NaDodSO_4$ denatured polypeptide chains, because, although AcChR solubilized in cholate retains its ligand binding ability, its ion conductance channel is inactivated (unpublished experiments).

Cross-reaction between an antiserum to native *Torpedo* AcChR with all four of its ^{125}I-labeled components was detected both by competitive immune precipitation (Figure 9)

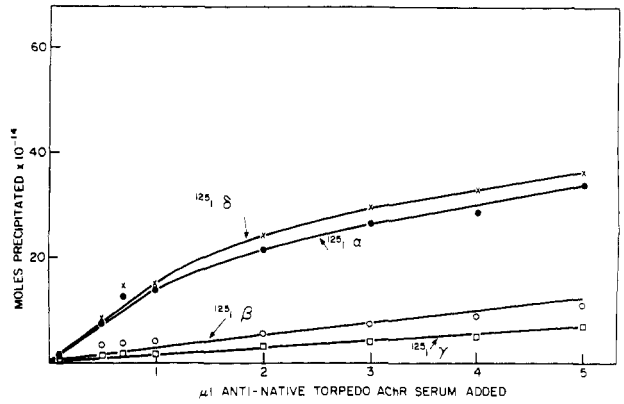

FIGURE 10: Cross-reaction of antiserum to native *Torpedo* AcChR with α, β, γ, and δ. As described in Figure 4, the indicated amounts of immune serum were incubated with triplicate 1-mL aliquots of each ^{125}I-labeled chain at 10^{-9} M. Antibody and bound ^{125}I-labeled chain were indirectly immune precipitated.

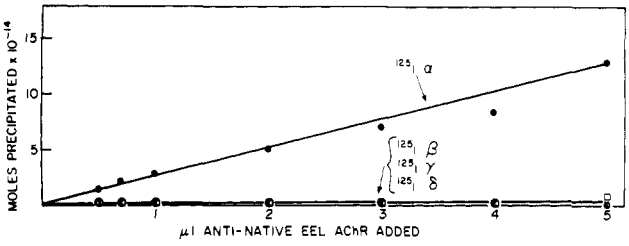

FIGURE 11: Cross-reaction of antiserum to native *Electrophorus* AcChR with ^{125}I-labeled chains of *Torpedo* AcChR. The method used was as described in Figure 4.

and by quantitative immune precipitation (Figure 10). α and δ chains were the most antigenic, but together the moles of all four chains bound per liter accounted for only 6% of the moles of [^{125}I]-α-BGT binding sites of native AcChR bound (Table II).

Cross-reaction between antiserum to native *Electrophorus* AcChR and all three of its ^{125}I-labeled components was detected by competitive immune precipitation (Figure 6) and by quantitative immune precipitation (Table II). Cross-reaction was most pronounced with the α' and γ' chains. Also, cross-reaction with denatured chains could not account for all of the titer against native AcChR, but this effect was less striking than that with antibody to native *Torpedo* AcChR.

Interspecies cross-reaction of antisera to native AcChR with ^{125}I-labeled denatured chains was most evident with α and α' (Figures 6, 9, 11, and 12). As in the case of intraspecies cross-reaction between antisera to native AcChR and denatured chains, cross-reaction with denatured chains accounted for little of the titer against native AcChR (Table II). Thus, most of the antigenic determinants responsible for interspecies cross-reaction are also conformationally dependent.

Antiserum to native *Torpedo* AcChR cross-reacted primarily with α' chains of *Electrophorus* (Figure 6). This is especially interesting since we know *Electrophorus* AcChR contains other chains whose specific cross-reaction with chains of *Torpedo* AcChR can be detected by antisera to the denatured *Torpedo* chains. Antisera to native *Electrophorus*, reciprocally, cross-reacted primarily with α (Figure 11), although some cross-reaction with δ was observed by competitive immunoprecipitation (Figure 9). As with antisera to native *Torpedo*, antisera to native *Electrophorus* cross-reacted slightly but detectably with all of its component polypeptides (Figure 6) but not with all of their analogues in *Torpedo* (Figure 9). Similarly, antisera to native fetal calf muscle AcChR (Figures 6, 9, and 12) and rat muscle AcChR (Figures

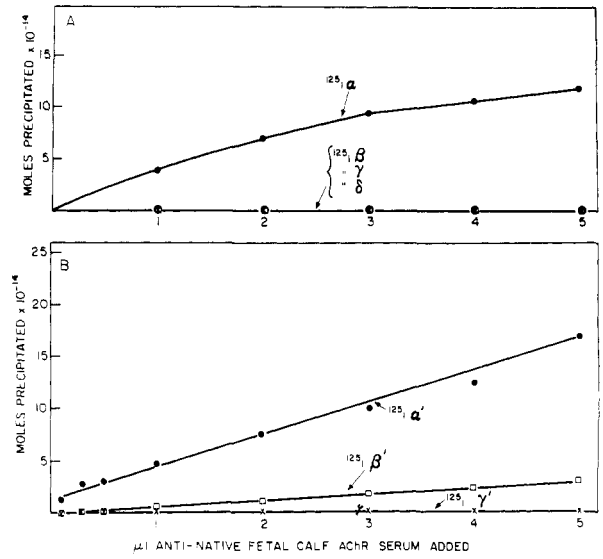

FIGURE 12: Cross-reaction of antisera to native fetal calf muscle AcChR with the ^{125}I-labeled chains composing *Torpedo* and *Electrophorus* AcChR's. (A) Reaction with ^{125}I-labeled *Torpedo* AcChR chains. (B) Reaction with ^{125}I-labeled *Electrophorus* AcChR chains.

6 and 9) cross-reacted predominantly with α or α' chains, although other evidence (Table II and Figure 8) suggests that these AcChR's, like electric organ AcChR, share analogous polypeptide chains which are not readily detected by interspecies cross-reaction of antisera to native AcChR with ^{125}I-labeled denatured polypeptide chains.

Antisera from myasthenia gravis patients with very high concentrations of antibody against AcChR from human muscle [(97–680) × 10^{-9} M] and with small but measurable cross-reaction with native *Torpedo* AcChR [(3–10) × 10^{-9} M] showed no clearly discernible cross-reaction with any *Torpedo* chain (Figure 9). Cross-reaction against chains from *Electrophorus* was not tested because there was no detectable cross-reaction with native *Electrophorus* AcChR (Lindstrom et al., 1978a). Because antisera to each of the *Torpedo* chains specifically cross-react with native human AcChR (Lindstrom et al., 1978b), it is likely that this AcChR is composed of subunits similar to those forming *Torpedo* AcChR. However, sera from myasthenia gravis patients, like antisera to native AcChR from several species, appear to be directed primarily at conformationally dependent determinants.

Discussion

The α, β, γ, and δ chains of *T. californica* AcChR are immunologically distinct from one another. They are not derived by proteolysis from two higher molecular weight chains (ϵ,ζ) prominent in electric organ membranes. These conclusions from immunochemical studies are consistent with conclusions from peptide maps of these components (Froehner & Rafto, 1979; Nathanson & Hall, 1979; Lindstrom et al., 1979). Some evidence was provided that the ζ chain may correspond to the enzymatically active subunit of Na^+/K^+-dependent ATPase. Although our results with antisera to *Torpedo* AcChR chains clearly show that these are not extensively overlapping proteolytic derivatives of a larger chain, there is a small amount of cross-reaction between β, γ, and δ chains. This might result from trace cross contamination of the immunogens, resulting in mixed populations of antibodies in the sera, and/or from single antibodies with higher affinity for one chain and low affinity for a similar but distinct antigenic determinant on another. We have constructed a number of hybridoma cell lines producing monoclonal anti-

bodies to *Torpedo* AcChR and its subunits (Tzartos and Lindstrom, unpublished experiments). A monoclonal antibody to δ also has lower affinity for γ, showing that at least part of the small cross-reaction we have observed with antichain sera is due to immunochemical similarity between chains.

AcChR purified from *Electrophorus* as normally purified is composed of three polypeptide chains corresponding to three of the four chains found in *Torpedo*. We have recently found conditions in which the fourth chain corresponding to δ', which is normally lost during solubilization and purification, can be retained in AcChR purified from *Electrophorus* (Lindstrom et al., unpublished experiments).

Several lines of evidence presented here suggest that AcChR from muscle is composed of four chains which are immunochemically similar to those in *Torpedo*. Evidence for an α-like chain is very strong. As in *Torpedo* and *Electrophorus*, a single polypeptide chain of the appropriate molecular weight was specifically labeled with the acetylcholine binding site directed affinity labeling reagent [^{3}H]MBTA. Curiously, in AcChR from denervated rat muscle, a second chain was also labeled with [^{3}H]MBTA (Froehner et al., 1977a; Nathanson & Hall, 1979). Our preparations also contained higher molecular weight chains which might correspond to β, γ, and δ but in addition contained contaminating bands. Our preparations were purified only by affinity chromatography and were not further cleaned up by lectin chromatography or sucrose gradient centrifugation. When these extra measures were taken in the purification of AcChR from denervated rat muscle, four chains were observed, reminiscent of α, β, γ, and δ as well as a higher molecular weight component reminiscent of ϵ or ζ (Froehner et al., 1977b; Nathanson & Hall, 1979). The second [^{3}H]MBTA-labeled component appeared as a shoulder on the peak of the component with a molecular weight similar to β (Froehner et al., 1977a). Development of EAMG in rats immunized with any of the four polypeptide chains of *Torpedo* suggests that rat AcChR contains four groups of antigenic determinants comparable to the *Torpedo* chains and that these determinants are exposed on the extracellular surface of the membrane and thus accessible to antibodies in vivo. Cross-reaction of antisera to native rat AcChR with α' chains of *Electrophorus*, like the [^{3}H]MBTA experiments (Froehner et al., 1977a), suggests that rat AcChR contains α-like chains. Specific cross-reaction of antisera to each of the *Torpedo* chains from human (Lindstrom et al., 1978b) and fetal calf muscle AcChR suggests that both of these contain four groups of antigenic determinants corresponding to the *Torpedo* chains. There is no direct proof, however, that the three groups corresponding to β, γ, and δ are associated with distinct polypeptide chains of comparable molecular weights. Identification of such chains in muscle AcChR should be possible using methods similar to those reported here for *Electrophorus* AcChR.

The most antigenic and myasthenogenic determinants on AcChR depend on the native conformation of the AcChR molecule and are destroyed by denaturation with $NaDodSO_4$. The antigenic determinants common to native and denatured AcChR are normally not very immunogenic. The antibodies developed to these determinants by immunization with denatured chains react very efficiently with native AcChR, whereas antibodies to native AcChR do not react efficiently with denatured chains. These two classes of antigenic determinants are qualitatively and perhaps spatially distinct on the AcChR molecule. Most interspecies cross-reaction of antibodies to native AcChR with denatured chains occurs with α-like chains; however, homologies between AcChR at other chains are detectable by antisera to denatured chains.

Antisera to *Torpedo* α chains clearly distinguish between AcChR from normal and denervated rat muscle. Some anti-β sera showed a smaller effect. The identity of the small structural difference between junctional and extrajunctional AcChR indicated by their similar subunit structure but slightly differing isoelectric point has long been a mystery (Brockes & Hall, 1975). This structural difference may be responsible for the altered pharmacological, electrophysiological, and metabolic properties of extrajunctional AcChR [enumerated in Weinberg & Hall (1979)]. The increased reaction of anti-α sera with extrajunctional AcChR suggests that the structural difference is at least in part localized on α-like chains and that it may resemble denatured more than native structure.

Specificities of antibodies to AcChR from MG patients are interesting in part because these antibodies may be viewed as fossil templates of the unknown antigen which triggers the autoimmune response in this disease. Antisera from MG patients which cross-react with native *Torpedo* AcChR recognize primarily conformationally dependent determinants of the *Torpedo* AcChR molecule. In this respect, the specificity of these antisera resembles that of other antisera to native AcChR. Extrajunctional AcChR from rat muscle contains additional antigenic determinants which account for the increased reaction of MG sera with extrajunctional over junctional AcChR (Weinberg & Hall, 1979). It is unknown whether these are the same determinants which account for the increased reaction with antisera to α.

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Spin-Label Studies of Lipid Immobilization in Dimyristoylphosphatidylcholine-Substituted Cytochrome Oxidase†

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ABSTRACT: Yeast cytochrome oxidase complexes have been prepared in which ≥99% of the endogenous lipid has been substituted by dimyristoylphosphatidylcholine and the lipid chain immobilization has been studied by spin-label spectroscopy. The ESR spectra of a C(14) phosphatidylcholine spin-label consist of both an immobilized and a fluid lipid bilayer component for all complexes, the proportion of the former increasing with increasing protein/lipid ratio. Computer difference spectroscopy has been used to obtain the two different spectral components and to determine their relative proportions. Lipid/protein titration of the complexes, in the region where the proportion of fluid lipid exceeds that of the immobilized, reveals that a constant number of lipid molecules (55 ± 5) per protein are immobilized. This is attributed to a first or boundary shell of lipids associated with the protein, which is then surrounded by fluid bilayer. Estimates of motional correlation times from the immobilized lipid difference spectrum give values of ≥50 ns, suggesting that the rate of exchange between bilayer and boundary lipids is probably at least 1 order of magnitude slower than the rate of lateral diffusion of unperturbed bilayer lipids. The variation of the spectral splittings and line widths of the fluid-component difference spectra with lipid/protein ratio shows that a second shell of lipids is perturbed by the protein, a third shell is less strongly perturbed, and a further two to three shells may be still weakly perturbed. Thus, the immobilization of the lipid may extend out to approximately six shells from the protein. These structural properties correlate with measurements of the enzyme activity which show a rapid decrease in activity with decreasing lipid/protein ratio for complexes containing less than the minimum immobilized "boundary" layer of lipid and a smaller but steady increase in activity with increasing lipid/protein ratio for complexes with from one to six shells of lipid.

Lipid–protein interactions are potentially important determinants of membrane structure and function, not only from the point of view of regulation of membrane-bound enzymes and transport proteins but also with regard to the incorporation of proteins into the lipid matrix, hence maintaining the structural integrity of the membrane envelope. Thus, it is of considerable interest to investigate the interaction between integral membrane proteins and the shells of lipid immediately surrounding the protein. It is these lipid molecules which are most likely to affect the protein conformation, to be responsible for sealing the protein into the bilayer, and to form the means of communication with the bulk bilayer phase.

Specific immobilization of a boundary layer of lipid surrounding the integral membrane protein cytochrome oxidase

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