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Synthesis of Acetylcholine Receptor by Denervated Rat Diaphragm Muscle

(α-bungarotoxin/affinity chromatography/organ culture)

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ABSTRACT **Acetylcholine receptor was purified by affinity chromatography from denervated rat hemidiaphragms that had been incubated in organ culture for 24 hr in medium containing [^{35}S]methionine. Radioactive acetylcholine receptor was identified in purified preparations by zone sedimentation in a sucrose gradient, by isoelectric focusing, and by precipitation with an antiserum to the acetylcholine receptor from electric eel. When innervated and denervated hemidiaphragms were incubated with [^{35}S]methionine in organ culture, and the acetylcholine receptors from each were purified separately, only the preparation from denervated muscles contained radioactive receptor as determined by zone sedimentation. We conclude that newly synthesized receptor is accumulated as a result of muscle denervation.**

During neuromuscular transmission in vertebrate skeletal muscle, acetylcholine (AcCh) that is released from motor nerve terminals binds to specific receptors on the muscle cell, causing a change in permeability of the muscle membrane to sodium and potassium ions. In adult muscle the AcCh receptors occur in high concentration in the postsynaptic membrane at the neuromuscular junction and few, if any, are present in extrajunctional membrane. Denervation of the muscle, however, produces a large increase in the number of extrajunctional receptors (1–6). Although the functional significance of this increase is not known, the striking correlation between high levels of extrajunctional receptors and the ability of muscles to establish new synapses (7) suggests that extrajunctional receptors may play a role in synapse formation.

The increase in extrajunctional AcCh receptors caused by denervation could occur either because new receptors are synthesized or because a latent form of receptors in the extrajunctional membrane is unmasked. Inhibitors of protein and RNA synthesis have been shown to block the appearance of new receptors after denervation (8–10), indicating that synthesis of either new receptor or a protein required for expression of preexisting receptor occurs after denervation. To determine directly whether new receptors are made, we have incubated muscles in [^{35}S]methionine and isolated the AcCh receptor. Our results demonstrate that new AcCh receptors are synthesized after denervation.

MATERIALS AND METHODS

Incubation of Muscles with [^{35}S]Methionine. Rats (Sprague–Dawley, 50–80 g) were denervated by transection of the left phrenic nerve in the thorax. Three days later the denervated hemidiaphragms were removed and incubated in organ culture as described previously (11). Each dish contained two hemidiaphragms in 12 ml of a modified Trowell's medium containing 0.2 mM glutamine, 0.5 mM each of leucine, isoleucine, and valine, 2 μM methionine, 50 U/ml of penicillin, 50 U/ml of streptomycin, and 10 U/ml of mycostatin. After 1–2 hr in culture fresh medium was added. In some experiments, 1 mCi of [^{35}S]methionine (295–340 Ci/mmol, New England Nuclear), which had been lyophilized with 5 μg of cysteine and taken up in a small volume of incubation medium, was added at this time to each dish. In other experiments, the muscles were cultured for 18–24 hr before addition of the isotope, since preliminary experiments indicated that the net increase in receptor was larger during the second day in culture.

Purification of the AcCh Receptor. Muscles were rinsed briefly in chilled saline and the ribs, central tendon and borders of the muscles were cut away. The remaining tissue was added at a concentration of 50 mg of wet weight per ml to a solution of 50 mM NaCl, 50 mM Tris·HCl, pH 7.4, 1 mM [ethylenebis(oxyethylenenitrilo)]tetraacetate (EGTA), 0.4 mM phenylmethylsulfonyl fluoride and 0.2 μg/ml of Pepstatin (21) and homogenized in a Duall-Kontes ground glass homogenizer at 0°. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the pellet was resuspended in Triton buffer (50 mM NaCl, 50 mM Tris·HCl, pH 7.4, 1% Triton X-100, 0.5 mM EGTA) at about half the original volume of homogenate. This suspension was centrifuged at 100,000 × *g* for 60 min. The supernatant (Triton extract) was then applied to an 0.3 ml cobratoxin-Sepharose column (see below) in a plastic tuberculin syringe at 0°. The Triton extract was passed through the column three times over a period of 2–3 hr, resulting in retention of over 80% of the toxin-binding activity in the extract. The column was then washed successively with 1.5–2 ml of Triton buffer, 1.5–2 ml of a solution containing 1 M NaCl, 50 mM Tris·HCl, pH 7.4, 1% Triton X-100, 0.5 mM EGTA, and 2 ml of Triton buffer. α-Bungarotoxin (α-BuTx) binding activity was then eluted with 1 ml fractions of 1 M carbamylcholine chloride in Triton buffer. Fractions containing radioactivity were dialyzed exhaustively against Triton buffer at 4° and stored in sterile plastic tubes at 0°. Recovery of toxin-binding activity from the cobratoxin-Sepharose ranged from 25 to 40%. A summary of one purification is given in Table 1.

AcCh Receptor Assay. Samples were incubated with 10 nM α-^{125}I-BuTx for 45 min at 35° and the toxin–receptor complex collected on a DEAE filter as described previously (22). Toxin-binding activity in homogenates was measured in a supernatant fraction prepared by addition of Triton X-100 (final

Abbreviations: α-BuTx, α-bungarotoxin; AcCh, acetylcholine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetate.

TABLE 1. *Purification of AcCh receptor from muscles cultured in [^{35}S]methionine*

Fraction	Total ^{35}S (10^8 cpm)	Acid-insoluble ^{35}S (10^8 cpm)	α-^{125}I-BuTx binding (pmol)
Homogenate	12.6	8.2	7.1
Triton extract	2.0	1.6	6.2
Cobratoxin-Sepharose fractions	0.0013	—	2.1

Left hemidiaphragms from 10 rats (50–60 g) that had been denervated 3 days earlier were cultured as described in *Methods*. After 26 hr in culture, medium containing 5 mCi of [^{35}S]methionine was added, and the muscles were incubated 24 hr longer. The AcCh receptor was then purified as described in *Methods*. The total weight of the diaphragm tissue was 600 mg.

concentration, 1%), extraction at 0° for 30 min, and centrifugation at 25,000 × *g* for 30 min. The radioactivity on the filters was measured in a well-type gamma counter (Packard Instrument Co.) to avoid interference from the ^{35}S present.

Zone Sedimentation. Zone sedimentation was performed as previously described (23) except that ^{125}I was detected by analyzing the fractions directly in a gamma counter.

Isoelectric Focusing. The receptor fractions were incubated with α-BuTx or α-^{125}I-BuTx as detailed in the *text*. The reaction volume in each case was adjusted to 85 μl with Triton buffer and mixed with 15 μl of 20% sucrose, 2% Triton X-100, 4% Ampholine (pH 3–10), 0.005% Evan's Blue. Isoelectric focusing in cylindrical polyacrylamide gels was performed as described previously (23) except that the gels were 2% Ampholine (pH 3–10). The gels were sectioned and the ^{125}I radioactivity was measured in a gamma counter, or the ^{35}S was determined after overnight incubation with Protosol (0.4 ml, Packard Instrument Co.) at 55° followed by addition of Aquasol (Packard Instrument Co.).

Immunoprecipitation. Each reaction (130 μl) contained in Triton buffer 30 μl of normal serum or of an antiserum to the acetylcholine receptor from electric eel (13), 0.03% sodium azide and either ^{35}S-labeled affinity-purified protein (1.9×10^3 cpm, 54 fmol of receptor) or ^{35}S-labeled protein (5.1×10^4 cpm) from the flow-through of the affinity column. After incubation of the reaction for 3 hr at room temperature, goat antiserum to rabbit immunoglobulin G (100 μl) was added and the incubation was continued for 12 hr at 4°. The immune precipitates were collected by centrifugation (top speed for 5 min in a Misco microcentrifuge) and the supernatants were removed for assay of AcCh receptor. The pellets were washed by three cycles of resuspension in Triton buffer (0.4 ml) followed by centrifugation as above. The final pellet was dissolved in 1% acetic acid (0.2 ml) and radioactivity was measured in Aquasol.

Materials. Unlabeled acetylcholine receptor was purified from extrajunctional regions of denervated rat diaphragm muscle as described previously (22). α-^{125}I-BuTx was prepared as described earlier (22). The preparation used in these experiments had an initial specific activity of 750 Ci/mmol. The preparation of cobratoxin-Sepharose has also been described

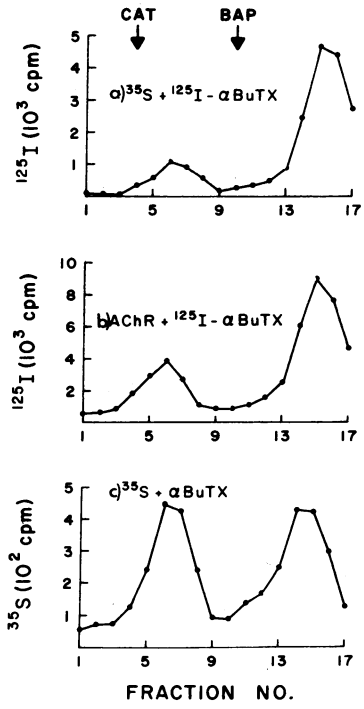

FIG. 1. Zone sedimentation of purified fractions of AcCh receptor. Fractions containing AcCh receptor, purified from labeled and unlabeled muscles, were incubated with α-BuTx for 15 min at 35° as described below and analyzed by zone sedimentation as described earlier (23). The volume of each reaction was adjusted to 160 μl with Triton buffer before layering on the gradient. (a) The reaction (volume 125 μl) contained affinity column fraction from the experiment described in Table 1 (1.05 nM receptor), 0.55 nM α-^{125}I-BuTx, and 1% Triton X-100. (b) The reaction (volume 50 μl) contained 2.5 nM purified extrajunctional receptor and 2 nM α-^{125}I-BuTx. (c) The reaction (volume 160 μl) contained affinity column fraction as above (0.72 nM receptor), 170 nM α-BuTx, and 1% Triton X-100. The recovery of radioactivity was 79%. The gradients of Figs. 1b and 1c were run in parallel with a third tube containing the sedimentation markers, catalase (CAT, 11.3 S) and bacterial alkaline phosphatase (BAP, 6.1 S) Sedimentation was from right to left in all cases. The gradient of (a) was run in a separate experiment with a repeat of (c) which gave an essentially similar result.

(22), except that for these experiments the Sepharose was treated with epichlorhydrin and desulfated (12) before the toxin (*Naja naja siamensis*) was coupled to it.

Triton X-100 (scintillation grade) was obtained from Packard Instrument Co., carbamylcholine chloride and phenylmethyl sulfonyl fluoride from Sigma Chemical Co., and Pepstatin (21) was the gift of Dr. A. L. Goldberg. Alkaline phosphatase and catalase were obtained from Worthington Biochemical Corp. Rabbit antiserum against the AcCh receptor from *Electrophorus electricus* and goat antiserum to rabbit immunoglobulin G were generously given by Dr. J. Patrick.

RESULTS

Hemidiaphragms that had been denervated for 3–4 days were incubated in organ culture with medium containing [^{35}S]-methionine for 20–24 hr. In some cases, the muscles were allowed to remain in culture for a day before the isotope was added. After incubation, the muscles were homogenized, a crude membrane fraction was prepared, and the fraction was

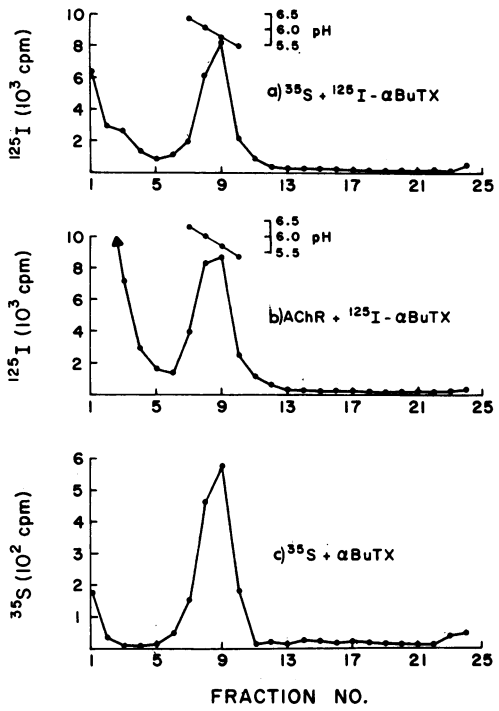

FIG. 2. Isoelectric focusing of purified fractions of AcCh receptor. Purified fractions of AcCh receptor were incubated with α-BuTx for 30 min at 35° as described below and analyzed in parallel by isoelectric focusing as described in *Methods*. The volume of each reaction was adjusted to 85 μl with Triton buffer before layering on the gel. (a) The reaction (volume 45 μl) contained affinity column fraction (0.95 nM receptor) from the experiment described in Table 1, 10 nM α-^{125}I-BuTx, and 1% Triton X-100. (b) The reaction (volume 15 μl) contained 4.6 nM purified extrajunctional receptor and 6.5 nM α-^{125}I-BuTx. (c) The reaction (volume 85 μl) contained affinity column fraction (0.96 nM receptor), 300 nM α-BuTx, and 1% Triton X-100. The values of the isoelectric point in this experiment are slightly higher than those determined previously (23) for reasons that are unclear, but may be related to the different pH range of the gels. When α-^{125}I-BuTx was run on this system there was some radioactivity at the top of the gel but no significant radioactivity in the position of the peak of toxin–receptor complex.

extracted with a solution containing 1% Triton X-100. The AcCh receptor was adsorbed from the Triton extract onto Sepharose 4B to which cobratoxin had been covalently attached, and was eluted with 1 M carbamylcholine. The results of one such preparation are shown in Table 1. The purified fractions contained 29% of the AcCh receptor and 0.016% of the radioactivity precipitable by trichloroacetic acid in the original homogenate (Table 1).

To determine if ^{35}S radioactivity in the purified fractions corresponded to the AcCh receptor, preparations were analyzed by sucrose gradient zone sedimentation, by isoelectric focusing, and by reaction with an antiserum to the AcCh receptor from electric eel. In the first two cases, the behavior of the ^{35}S-labeled preparations after incubation with labeled and unlabeled α-BuTx was compared to that of α-^{125}I-BuTx reacted with an unlabeled preparation of AcCh receptor from extrajunctional regions of denervated muscle (22).

When the ^{35}S fraction was reacted with α-^{125}I-BuTx and analyzed by zone sedimentation, two peaks of ^{125}I were detected (Fig. 1a): one at a position characteristic of free toxin and one at approximately 9 S. A similar pattern of ^{125}I radioactivity was shown by a mixture of ^{125}I-toxin and unlabeled receptor purified from denervated muscle (Fig. 1b). When the ^{35}S fraction was reacted with unlabeled toxin and analyzed, the ^{35}S radioactivity was recovered (Fig. 1c) in two major peaks of radioactivity corresponding to sedimentation constants of about 3 S and 9 S. In the experiment shown in Fig. 1c, the 9S peak accounted for 47% of the total radioactivity in the gradient.

TABLE 2. *Immunoprecipitation of ^{35}S-receptor*

Material	Percent ^{35}S cpm precipitated (mean ± SD)		Percent α-^{125}I-BuTx binding activity lost from supernatant (mean ± SD)	
	Control serum	Anti-eel AcChR serum	Control serum	Anti-eel AcChR serum
Affinity purified	7 ± 4 (4)	33 ± 3 (4)	24 ± 4 (4)	82 ± 6 (4)
Affinity flow-through	5 ± 1 (4)	5 ± 1 (4)		

Material passing through the cobratoxin-Sepharose column after application of the Triton extract in Table 1 (affinity flow-through) and material eluted from the column with 1 M carbamylcholine (affinity purified) were incubated with sera from normal rabbits and from rabbits immunized with eel AcCh receptor as described in *Methods*. The number in parentheses represents the number of separate determinations.

In a similar set of experiments, the toxin–receptor complexes were detected after isoelectric focusing in polyacrylamide gels. When the ^{35}S-labeled fraction was reacted with α-^{125}I-BuTx, the ^{125}I radioactivity focused in a peak (Fig. 2a) at approximately pH 5.75, as did complex formed between purified unlabeled receptor and α-^{125}I-BuTx that was analyzed in parallel (Fig. 2b). The ^{35}S fraction was also reacted with unlabeled α-BuTx and analyzed on a third gel. The radioactivity focused in a single peak (Fig. 2c) at an identical position. In the experiment of Fig. 2c, the total ^{35}S radioactivity recovered on the gel was 48%.

A third test for the presence of radioactive AcCh receptor in the purified preparations was reaction with an antiserum raised against the AcCh receptor from eel (13). Affinity-purified material was incubated with normal or with immune rabbit serum and, subsequently, with goat anti-rabbit IgG. Both the amount of radioactivity (^{35}S) precipitated and the amount of ^{125}I-toxin binding activity remaining in the supernatant were measured. The immune serum specifically precipitated (immune serum minus control serum values) 58% of the binding activity and 26% of the radioactivity (Table 2), indicating that 47% of the total radioactivity was associated with the receptor. When normal and immune sera were incubated with detergent extract that had been passed through the affinity column, each serum precipitated the same amount of radioactivity (5% of the total, Table 2). These results are consistent with earlier experiments (23) in which unlabeled AcCh receptor from rat diaphragm was precipitated by rabbit antiserum to the eel receptor.

All three tests indicate that for the preparation of Table 1 about 50% of the radioactivity in the purified fractions oc-

curred in receptor. We conclude, therefore, that incubation of denervated muscles in [^{35}S]methionine results in the appearance of radioactively labeled AcCh receptor. The remainder of the radioactivity was associated with one or more components, sedimenting at 3 S and presumably focusing outside the pH range of the isoelectric gels. The relation of this radioactivity to the receptor, if any, is unknown.

We have investigated whether the accumulation of radioactive receptor is related specifically to denervation by incubating innervated and denervated hemidiaphragms in parallel. Although the "innervated" hemidiaphragms are denervated at the time of culture, they should provide an effective control, since extrajunctional receptors do not appear during the first 48 hr after denervation (5). Accordingly, the left hemidiaphragms of five rats were denervated and 3 days later both left and right hemidiaphragms were cultured together for 20 hr in medium containing [^{35}S]methionine. Denervated and control hemidiaphragms were then separately pooled and homogenized and the AcCh receptor was purified from each homogenate, as above. The denervated hemidiaphragms incorporated about 40% more radioactivity per g of wet weight into trichloroacetic-acid-insoluble material than did the innervated hemidiaphragms (Table 3). It should be noted that the size of the methionine pool in innervated and denervated rat hemidiaphragms is approximately the same from 1 to 7 days after nerve section (14).

Both detergent extracts were applied to affinity columns and eluted with carbamylcholine. Recovery of AcCh receptor activity was 22% and 16% in denervated and innervated muscles, respectively, while the purified fractions from denervated muscle contained about 50% more radioactivity than the fractions from innervated muscle. When the purified material was analyzed by sucrose gradient zone sedimentation, there was a clear difference between the two preparations (Fig. 3). The preparation purified from denervated muscle contained major peaks of radioactivity at 9 S and 3 S, while the preparation from innervated muscle contained a peak at 3 S and little or no radioactivity sedimenting at 9 S. Thus, only the hemidiaphragms denervated several days before organ culture accumulated substantial amounts of radioactive AcCh receptor.

DISCUSSION

Two types of AcCh receptors, with distinct pharmacological and physical properties [for references, see (23)] occur in vertebrate skeletal muscle. Junctional receptors are associated with the muscle membrane underlying the nerve terminal, whereas extrajunctional receptors are found in muscle membrane outside the specialized region of the nerve-muscle synapse. The two types of receptor are apparently controlled in different ways. The number of junctional receptors in adult muscle appears to be relatively constant (5, 15,*), while the amounts of extrajunctional receptor undergo changes of up to two orders of magnitude during development (16) and after denervation and reinnervation (1–6). Several recent experiments suggest that one important factor that regulates the amount of extrajunctional receptor is muscle activity (15, 17–20); the biochemical basis of changes in the level of extrajunctional receptor has, however, remained unknown.

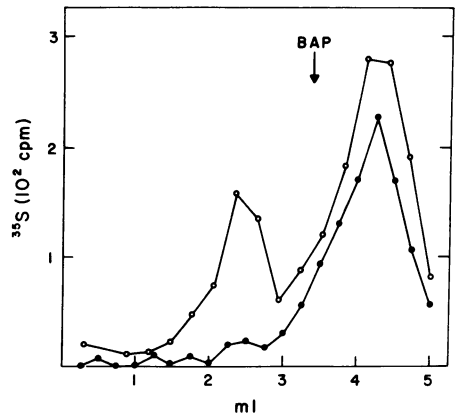

FIG. 3. Zone sedimentation of purified fractions of AcCh receptor from denervated and innervated muscles incubated in [^{35}S]methionine. Affinity column fractions (0.2 ml) from the experiment described in Table 3 were analyzed by zone sedimentation as described (23). Alkaline phosphatase (50 μg) was added to each sample as an internal standard. Recovery of radioactivity from the gradient was 97% for the denervated sample (○) and 80% for the innervated sample (●).

The experiments reported here demonstrate that several days after denervation, when levels of extrajunctional receptor are dramatically increased, newly synthesized AcCh receptor is accumulated by muscles in organ culture. In contrast, innervated muscles do not accumulate detectable amounts of newly synthesized receptor. Because of the long incubation times with isotope and the lack of information about rates of receptor degradation, the present results do not provide quantitative information about relative rates of receptor synthesis in normal and denervated muscle. Nevertheless, they are consistent with the idea that levels of extrajunctional receptor are regulated by changing the rate of receptor synthesis. In addition, the demonstration that amino-acid incorporation into the receptor is detectable should make it possible to test directly whether muscle activity affects recep-

TABLE 3. *Purification of AcCh receptor from "innervated" and denervated muscles after incubation with [^{35}S]methionine in organ culture*

	Denervated muscle		"Innervated" muscle	
	Acid-insoluble radioactivity (10^6 cpm)	α-^{125}I-BuTx binding (fmol)	Acid-insoluble radioactivity (10^6 cpm)	α-^{125}I-BuTx binding (fmol)
Homogenate	470	2080	340	690
Triton extract	66	1450	46	450
Cobratoxin-Sepharose fractions	0.018	450	0.012	110

The left hemidiaphragms of five rats (70–80 g) were denervated, and 3 days later both left and right hemidiaphragms from each animal were cultured in the same dish. After 2 hr in culture, medium containing [^{35}S]methionine (1 mCi per dish) was added and the incubation was continued for 21 hr. Left and right hemidiaphragms were then pooled, and AcCh receptor was purified from each as described in *Methods*. The combined left hemidiaphragms weighed 510 mg and the right hemidiaphragms 520 mg.

* E. Frank, K. Gatuvik, and H. Sommerschild, "Cholinergic receptors at denervated mammalian motor end-plates," manuscript submitted.

tor synthesis, and whether extrajunctional receptors turn over rapidly, as has recently been suggested (11).

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