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Synthesis of acetylcholine receptor during differentiation of cultured embryonic muscle cells

(α-neurotoxins/affinity chromatography/immunological crossreaction)

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ABSTRACT **Acetylcholine receptor, a component of the specialized muscle membrane, appears during the differentiation of embryonic myogenic cells in tissue culture. Demonstration of incorporation of the radioactive precursor L-[^{35}S]methionine into purified receptor polypeptides is presented as evidence for its synthesis *de novo*. The identity of the purified radioactive species is established by cosedimentation of the [^{35}S]receptor with [^{3}H]α-toxin binding activity on sucrose gradients and by crossreaction with antiserum to purified acetylcholine receptor of *Electrophorus electricus*.**

Myogenic cells in tissue culture undergo morphological, functional, and biochemical alterations analogous to those observed in differentiating muscle tissue *in situ* (1). Mononucleate replicating cells derived from fetal skeletal muscle, when maintained in tissue culture, withdraw from the cell cycle, align, and subsequently fuse to form multinucleate fibers exhibiting cross-striations and spontaneous or induced contractions. The myogenic process is accompanied by accumulation of muscle specific isoenzymes, increases in the rates of synthesis of contractile proteins, and development of a specialized plasma membrane manifest by its chemical and electrical excitability. As in the case of contractile elements and specific enzymes, the specialized components of the membrane are elaborated during the period of cell fusion. One of the components of this membrane system is the acetylcholine (ACh) receptor. Until now, study of the accumulation and turnover of the receptor in cultured muscle cells has been limited to indirect methods using radioactively labeled α-neurotoxins as tags *in vivo* (2–5).

The ACh receptor from fish electric tissue has been the subject of extensive biochemical, pharmacological, and immunological investigation (6) which has resulted in a technology applicable to the direct study of the metabolic activity of this protein in cultured cells. In this paper we present some of the characteristic properties of the ACh receptor from cultures of fetal calf skeletal muscle, as well as the biochemical and immunological methods for its identification. Demonstration of incorporation of the radioactive precursor [^{35}S]methionine into purified receptor polypeptides is presented as evidence for its synthesis *de novo*.

METHODS

Cell Culture. Primary cultures of skeletal myogenic cells of fetal calf were established as described by Buckingham *et al.* (7). All cultures were prepared by plating 2–4 million cells per 100-mm tissue culture dish (Falcon Plastics, Oxnard, Calif.).

Labeling with Radioactive Amino Acids. Cultures to be labeled with radioactive amino acids were first washed twice with phosphate-buffered saline (PBS). For the experiments in which the rate of actomyosin synthesis was to be measured, one or more plates were pulse labeled for 4 hr with 10 μCi/ml of L-[^{14}C]aminoacid mixture (100 mCi/mmol, C.E.A., France) in a volume of 2 ml per plate. Labeling was done in the standard medium used for cell culture (4:1 Dulbecco's modified Eagle's medium and medium 199) containing 10% undialyzed fetal calf serum (Gibco, Grand Island, N.Y.). Cells to be labeled with L-[^{35}S]methionine (200 Ci/mmol, C.E.A., France) were washed and transferred to modified Eagle's medium plus 2% dialyzed fetal calf serum containing 5 μM L-methionine rather than the normal 100 μM. Labeling was done in 4 ml of medium with up to 50 μCi per plate. Uptake of label, as measured by the loss of radioactivity from the supernatant culture fluid, was continuous throughout the 24-hr labeling period.

Preparation and Solubilization of Receptor Containing Membrane Fraction from ^{35}S-Labeled Cells. Cells were removed from the culture dishes and suspended in PBS (line 1, Table 2). Whole cells, nuclei, and membrane fragments were collected by low-speed centrifugation at 1000 × *g* for 5 min. The pellet was homogenized in 8.5% sucrose 0.05 M Tris·HCl (pH 7.6) and the unbroken nuclei removed by low-speed centrifugation. The nuclear pellet was treated in the same manner twice more, and the pooled supernatants containing membrane fragments (line 2, Table 2) were adjusted to 0.5 M KCl to aid in the removal of contractile proteins. Membrane fragments were collected by centrifugation at 100,000 × *g* for 1 hr. The pellet was extracted with 0.1 M Tris·HCl, 0.1 M NaCl, 1.0% Triton X-100, pH 8.0 for 1 hr at 4°. The extract was subsequently cleared by centrifugation at 100,000 × *g* for 1 hr, followed by filtration of the supernatant through a plug of glass wool (line 3, Table 2).

Toxin Affinity Columns. Erabutoxin B was coupled to cyanogen bromide activated Sepharose 4B by the method of Ratner (8). Toxin was coupled to activated washed beads at a concentration of 100 g/ml.

Before incubation with receptor extracts, the toxin columns were washed with 0.1 M Tris·HCl, 0.1 M NaCl, 1.0% Triton X-100 (pH 8.0). Adsorption was done at room temperature by recycling for 2.5 hr. The average binding capacity was 500 pmol of toxin binding sites per ml of beads. The charged column was washed at 4° alternately with 20 volumes of 0.1 M Tris·HCl, 1.0 M NaCl, 1% Triton X-100 (pH 9.0) and 0.05 M PO_4, 1.0 M NaCl, pH 6.0 until a stable base line of ^{35}S radioactivity was detected in the effluent. Decamethonium 0.1 M in 0.01 M Tris·HCl, 0.01 M NaCl, 1% Triton, 0.1% BSA pH 8.0 was applied and the column was stoppered for 36 hr at 4 °. The decamethonium eluate, containing 0.001% of the original ^{35}S counts was diluted and applied to a 0.2 ml column of Whatman DE-52 in 0.01 M Tris·HCl, 0.01 M NaCl, 1% Triton, pH 8.0 and washed extensive-

Abbreviations: PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; ACh, acetylcholine. Tris/NaCl/Triton, 0.1 M Tris·HCl, 0.1 M NaCl, 1% Triton X-100, pH 8.0.

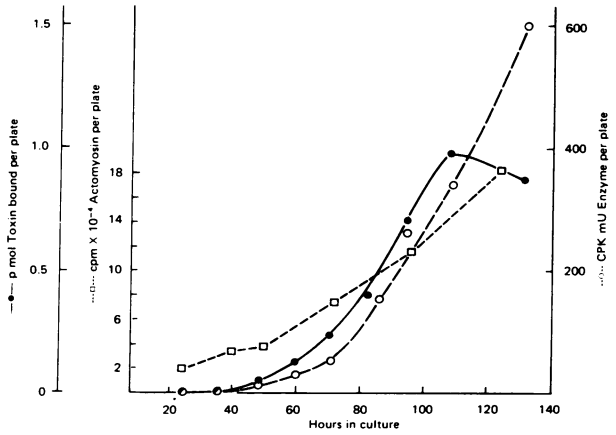

FIG. 1. Time course of increase of toxin binding sites, creatine kinase (CPK) activity, and actomyosin synthesis. At the indicated times duplicate samples were washed and frozen at −80°. Cells from each plate were suspended in 2 ml of 0.1 M glycylglycine (pH 6.75) and sonicated twice for 10 sec with a Bronson sonifier (20 W). Duplicate assays of toxin binding by the Millipore method and creatine kinase by the method of Shainberg *et al.*(15) varied by less than 10%. [^{14}C]Aminoacid incorporation into actomyosin was determined as described in *Methods*.

ly with the same buffer to remove the decamethonium. The DE-52 column was eluted with 0.1 M Tris·HCl, 1.0 M NaCl, 1% Triton X-100, pH 9.0 and assayed for receptor activity.

Toxins and Toxin Binding. Experiments with [^{3}H]toxin were done using α_1-isotoxin purified from crude venom of *Naja nigricollis* which was tritiated by the method of Menez *et al.* (9). The specific activity was 22 Ci/mmol. The ^{125}I-labeled toxin was made from purified erabutoxin B, a gift from Dr. Menez, by the chloramine T method (10). Iodinated toxin was separated from free iodide on a column of Bio-Gel P6 (Biorad, Richmond, Calif.) and further fractionated on a column of carboxymethyl cellulose (Whatman, Maidstone, Kent) to separate iodinated from noniodinated toxin species. The specific activity of the final preparation was approximately 40 Ci/mmol, as determined by titration with *Torpedo marmarata* membrane fragments (11). ^{125}I-labeled toxin was identical with [^{3}H]toxin with respect to its behavior in the Millipore assay (12) and sucrose gradient centrifugation (see *Results*).

Immunoprecipitation. A Triton X-100 extract of myotube membranes was incubated with normal serum or rabbit serum against *E. electricus* ACh receptor at 4° overnight. The total volume of the incubation was 25 μl. After preincubation, 0.2 ml of sheep antiserum against rabbit Ig (made 1% in Triton X-100) or 0.2 ml of PBS, 1% Triton X-100 was added, and the mixture was incubated 3 hr at room temperature. The samples were centrifuged in a Beckman minifuge, and 0.200 ml of the supernatant was assayed for toxin binding activity by the Millipore method.

Sucrose Gradient Analysis. Receptor–toxin complexes formed in toxin excess by preincubation at room temperature for 2.5 hr were analyzed on exponential convex gradients (13) of 10–40% sucrose (w/v) in 0.1 M Tris·HCl, 0.1 M NaCl, 1% Triton X-100 pH 8.0. Gradients for centrifugation in Beckman SW-41 rotor were prepared by starting with 12 ml of the 10% sucrose solution in the mixing chamber. Samples were centrifuged at 40,000 rpm for 24 hr at 4°. Fractions of 20 or 30 drops were collected from the bottom.

Measurement of Actomyosin Synthesis and Creatine Kinase Activity. Actomyosin was prepared from cells labeled with a L-[^{14}C]aminoacid mixture by precipitation at low ionic strength and redissolving in high salt (14). After recentrifugation of the final extract, the material exhibited only two major components by sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis: the myosin heavy subunit and actin. Total radioactivity in the extract was determined by scintillation counting (Figure 1). Total creatine kinase (EC 2.7.3.2; ATP:creatine *N*-phosphotransferase) activity in unfractionated cell extracts was assayed according to Shainberg *et al.* (15), with the exception that adenosine monophosphate was not included in the assay mixture.

Electrophoresis. $NaDodSO_4$-polyacrylamide gel electrophoresis was performed in gels of 7.5% acrylamide (16). Electrophoresis of receptor–toxin complex was performed in gels of 5% acrylamide as described (17), with 0.5% Triton X-100 included in all buffers.

Isotope Determination. ^{125}I was determined by gamma counting. Radioactivity of gradient fractions containing sucrose was determined in Noll cocktail (18). All other scintillation counting was done in toluene-based scintillation fluid. For double isotope counting, the relative ratio of ^{35}S to ^{3}H was low enough so that crossover of ^{35}S into ^{3}H was insignificant. Crossover of ^{3}H into ^{35}S was less than 0.3%.

RESULTS

Time course of toxin binding site accumulation

Binding of *Naja nigricollis* [^{3}H]α-toxin (7) was measured in sonicated extracts of cultured myogenic cells derived from embryonic calf skeletal muscle. The number of toxin binding sites in primary cultured cells increases between the third and sixth day (Fig. 1), attaining a final specific activity of approximately 1 nmol/g of protein. The increase in the amount of toxin binding material coincides with the course of cell fusion (data not shown), as well as the accumulation of creatine kinase activity and the increase in rate of actomyosin synthesis (Fig. 1).

Specificity and identity of toxin binding material

Others have shown that toxin binding to intact muscle cells in culture corresponded to functional blocking of the ACh receptor (2, 4). Our primary concern for the purpose of this report was to determine what fraction of the toxin binding material had the pharmacological and biochemical characteristics of ACh receptor.

Table 1 shows the effect of some cholinergic agents on the rate of [^{3}H]toxin binding to sonicated extracts of embryonic calf myotubes. Considering the variation in assay conditions and the method of preparation of receptor containing membranes, these data are in good agreement with previously reported pharmacological properties of a nicotinic ACh receptor (2, 3, 6). Low values were obtained with *d*-tubocurarine. Decamethonium, 1 mM, was found to completely abolish toxin binding in both Millipore and sucrose gradient assays.

A second criterion of specificity and identity of toxin binding sites is the behavior of Triton X-100 soluble material on sucrose gradients during velocity sedimentation. Whereas all toxin binding material from broken cells may be pelleted by centrifugation at 100,000 × *g* for 1 hr, this material is quantitatively extracted in 0.1 M Tris·HCl, 0.1 M NaCl, 1.0% Triton X-100 into a form which sediments as a single homogeneous peak with a relative S-value of about 9. Representative data are shown in Fig. 3B (dashed line). Although this figure shows a sucrose gradient analysis of purified material, the pattern with crude extracts is identical. Longer

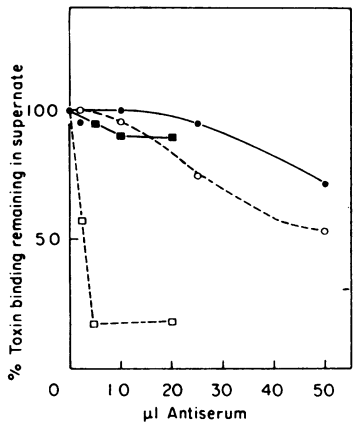

FIG. 2. Immunoprecipitation of toxin binding activity. Toxin binding remaining in the supernatant was determined by the Millipore method after incubation with normal serum and rabbit antiserum against *E. electricus* ACh receptor (● and ○, respectively) or after subsequent incubation with sheep antiserum against rabbit Ig (■ and □, respectively). 100% corresponds to 0.1 pmol.

times of centrifugation under conditions in which a majority of the toxin was bound have not revealed peaks other than those of the 9S species and the free toxin.

The sedimentation pattern of bound toxin seemed to be invariable with the age and spontaneous contractile activity of the cultures from which material was extracted. Electrophoresis on native polyacrylamide gels also resolves only a single peak of toxin binding material (data not shown).

Third, we have examined the immunological crossreaction of a rabbit antiserum raised against purified ACh receptor from *E. electricus* (19) with toxin binding material extracted from our myoblast cultures with Triton X-100. The antiserum used in this study has been shown to give a single arc in immunoelectrophoresis with purified preparations of

Table 1. Inhibition of toxin binding by cholinergic ligands

Ligand	Concentration (μM)	[^{3}H] Toxin bound (pmol)	% Rate of control
Control		0.242	100
Hexamethonium	33		99
Atropine	3.3	0.248	102
	33	0.188	78
Carbamylcholine	0.33	0.123	51
	3.3	0.056	23
	33	0.025	10
Decamethonium	0.33	0.071	30
	3.3	0.025	10
	33	0.008	3
Gallamine	0.33	0.180	75
	3.3	0.090	38
	33	0.008	3
d-Tubocurarine	0.033	0.068	28
	0.33	0.006	3

The effect of several concentrations of the listed compounds was tested on the rate of toxin binding to sonicated myotubes. The binding assays were done by the Millipore method in a total volume of 0.6 ml containing 0.041 pmol of toxin binding sites at a toxin concentration of 1.6 nM. The initial rate in the control assay was linear until 2.5 hr. Duplicate assays for each concentration assayed at 1.75 hr agreed within 10%.

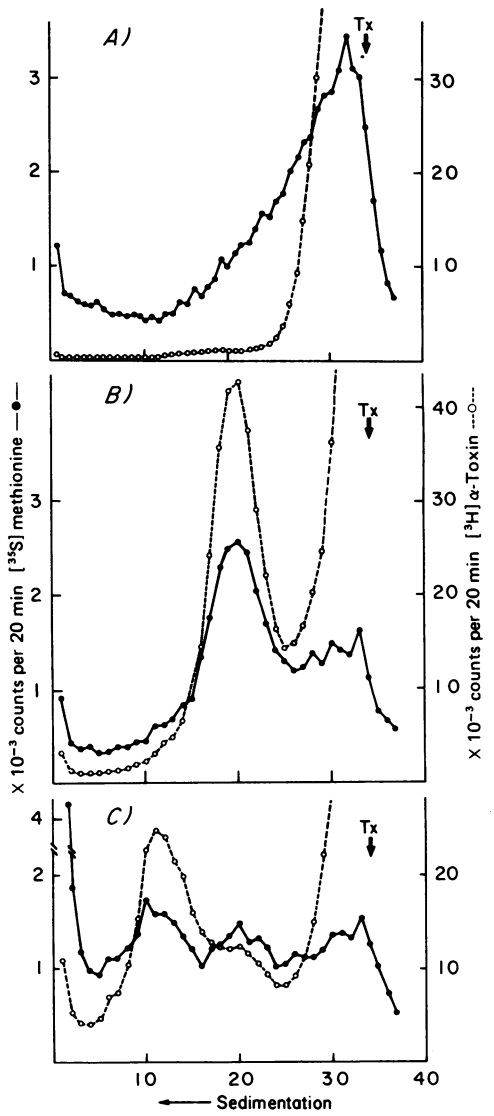

FIG. 3. Sucrose gradient analysis of ^{35}S-labeled receptor. (A) Profile of [^{35}S]- and [^{3}H]toxin binding of material adsorbed and eluted from a bovine serum albumin-Sepharose column. This experiment serves as a control for the specificity of the toxin column. (B) Profile of [^{35}S]- and [^{3}H]toxin binding of material purified by affinity chromatography. The high salt effluent from the DE-52 column, containing approximately 1.7 pmol of toxin binding sites, was preincubated overnight with 4 pmol of [^{3}H]toxin and 0.15 ml of normal rabbit serum and at room temperature for 2.5 hr before gradient analysis. (C) As in B, except preincubation was done with antiserum against *E. electricus* ACh receptor. Identical amounts of ^{35}S counts were applied to each of the three gradients. All of the ^{3}H and ^{35}S applied to the gradients could be accounted for, but only 50% of the receptor activity was recovered in both B and C. ^{35}S, ●; ^{3}H, ○.

E. electricus ACh receptor (Sobel, unpublished). Crossreaction with toxin binding material from cultured myotubes has been demonstrated in two ways. Fig. 2 shows the effect of preincubation of rabbit antiserum against *E. electricus* ACh receptor and normal rabbit serum with Triton X-100 extracts of myotubes grown *in vitro*. Toxin binding material remaining in the supernatant after centrifugation was measured by the Millipore assay method. The data from the single antiserum incubations indicate a low titer of specific crossreaction, at relatively high serum concentrations. However, incubations in which extracts preincubated with antiserum against *E. electricus* ACh receptor were subsequently

Table 2. Purification of ^{35}S-labeled ACh receptor

	^{125}I-Labeled toxin binding (pmol)	^{125}I-Labeled toxin binding (% recovery)	^{35}S cpm	^{35}S/pmol*
1. Total	6.3	100	1.45×10^9	2.3×10^8
2. Membrane	5.1	81	3.42×10^8	6.7×10^7
3. TNT extract†	3.3	52	1.90×10^8	5.8×10^7
4. Toxin column	2.5	40	1.38×10^4	—
5. DE-52	0.86	14	1.08×10^4	1.26×10^4

As described in the *text*, excess unlabeled receptor was bound to the column after the adsorption of the labeled extract. All toxin binding assays in the table are corrected to the 100% value of the original crude ^{35}S-labeled extract (6.3 pmol). Total activity bound to the column (labeled plus unlabeled extracts) equaled 21 pmol. Total eluted equaled 7 pmol.

* An inverse specific activity; reduction of this ratio indicates enrichment of receptor relative to bulk protein.

† 0.1 M Tris·HCl, 0.1 M NaCl, 1.0% Triton X-100 (pH 8.0).

incubated with sheep antiserum against rabbit Ig demonstrate a precipitation of toxin binding material. This indicates that, in fact, a specific crossreaction occurs, but that in the absence of the second antiserum it is only mildly precipitating and/or only slightly inhibitory to toxin binding.

The second technique used to assess crossreactivity was centrifugation of the receptor toxin complex after preincubation with normal nonimmune or specific sera. Fig. 3B and C shows the type of data obtained. The dashed lines show the position of toxin binding material. When receptor–toxin complex was preincubated with antiserum against *E. electricus* ACh receptor, a more rapidly sedimenting peak of bound toxin appeared. It has been found that all of the toxin binding material can be shifted to the more rapidly sedimenting position by increasing the amount of antiserum. The ratio of antiserum to toxin sites required for the shift is approximately the same as required for precipitation by the sandwich technique. The data presented in Fig. 3 also demonstrate that reaction with specific antiserum results in no inhibition of toxin binding (total counts sedimenting as > 9 S is the same with specific and normal sera) and no precipitation at the relatively low serum to receptor ratio used.

From these data and the known properties of the ACh receptor from other systems we conclude that all of the toxin binding material from cultured fetal calf myotubes represents ACh receptor.

De novo synthesis of ACh receptor

In order to determine if appearance of receptor molecules, as assayed by labeled toxin binding, was accompanied by *de novo* synthesis, we have analyzed the incorporation of [^{35}S]methionine into the receptor polypeptides. Primary cultures were normally exposed to the radioactive precursor for a 24-hr period from about 72 to 96 hr (see Fig. 1).

Table 2 is a summary of the purification of ACh receptor from a [^{35}S]methionine-labeled cell extract. The steps up to and including solubilization of the receptor activity in Triton X-100 (line 3, Table 2) generally resulted in a 3- to 5-fold purification with a recovery of 50–75% of toxin binding sites. Step 4 of the purification was affinity chromatography of the material solubilized in 0.1 M Tris·HCl, 0.1 M NaCl, 1.0% Triton X-100 (pH 8.0) on a column or erabutoxin b coupled to cyanogen bromide activated Sepharose 4 B (see *Methods*). Adsorption was done at room temperature by recycling 2 ml of this extract over a column bed volume of 0.1 ml for 2–3 hr. Receptor activity was assayed before and after adsorption to calculate the amount bound. After washing the column was saturated with receptor by recycling an unlabeled Tris·HCl, NaCl, Triton X-100 extract of a 1-week-old myotube culture. Higher yield and greater producibility of recovery was achieved when columns were saturated with receptor. The total amount of receptor bound from the unlabeled extract was also determined. After washing the column was incubated with decamethonium for elution. Decamethonium was removed by chromatography on a column of Whatman DE-52. ^{125}I-labeled toxin binding, and total ^{35}S cpm were determined. The material from the DE-52 column was judged to be 20,000-fold purified relative to the crude homogenate, as calculated from the reduction in the ^{35}S cpm per toxin binding activity.

In order to determine the degree of purity of the ^{35}S-labeled ACh receptor, the previously discussed criteria were evaluated. It was found that 50% of the ^{35}S counts in the purified material could be specifically precipitated using the conditions for the sandwich technique described in the legend to Fig. 2. However, a more convincing demonstration of the identity of the labeled material was obtained by analysis of cosedimentation of ^{35}S counts with toxin binding material upon sucrose gradient centrifugation. Fig. 3A shows such an analysis with material eluted from a control affinity column of bovine serum albumin (Calbiochem, Berkeley) coupled to Sepharose. It is clear from this experiment that no toxin binding material is eluted from such a column and that very little eluted ^{35}S-labeled material sediments in the 9S region. Assay by the Millipore method confirms the result that receptor does not bind to bovine serum albumin-Sepharose columns and that no toxin binding material is eluted.

Figure 3B shows the migration of ^{35}S-labeled material from a toxin affinity column. The column purified material used in this experiment had been preincubated with nonimmune normal rabbit serum overnight at 4°. It has been determined that such a preincubation did not alter the sedimentation rate of the receptor-toxin complex. It is estimated from this figure that approximately 75% of the ^{35}S counts cosediment with the [^3H]toxin bound to receptor.

The experiment shown in Fig. 3C is identical to that in 3B except that overnight incubation was done in the presence of antiserum against *E. electricus* ACh receptor. In this case 25–30% of the ^{35}S-labeled material is shifted to a faster sedimenting species, as is a large fraction of the receptor–toxin complex. The general pattern of sedimentation of ^{35}S counts in this experiment was reproducible; however, the significant proportion of counts found at the bottom of the tube was not. In a duplicate analysis the bottom two fraction contained only 80 cpm, whereas in the figure shown the same two fractions contained 300 cpm.

Analysis of column purified ^{35}S-labeled protein by Na-DodSO$_4$-polyacrylamide gel electrophoresis resolves three polypeptide species: two minor ones at 75,000 and 52,000 daltons, which account for 30–40% of the counts, and a major species at 40,000 daltons, accounting for 60–70%. Due to the uncertainty of the purity (25–75%) of the final preparation it is not possible to designate which of the polypeptide species corresponds to those of the receptor. The gel analysis does, however, indicate the relative homogeneity of the purified proteins.

DISCUSSION

The conclusions to be drawn from the present work are: (*i*) It is feasible to study directly the metabolism of the ACh receptor in cultured cells. (*ii*) Synthesis of the receptor polypeptides does occur during the time when toxin binding sites are accumulating. (*iii*) The amount of receptor synthesized, as determined by ^{35}S incorporation, is approximately that expected for a stable molecule.

This last point is based on an assumed molecular weight for a single toxin binding site of 10^5 or 5×10^4 and a content in methionine of 3.4 mole % [as determined for *E. electricus* ACh receptor (20)]. Given the specific activity of the [^{35}S]methionine (4×10^3 cpm/pmol), one calculates an expected specific activity of newly synthesized receptor of 6 to 12×10^4 cpm of ^{35}S/pmol of toxin binding sites. From the experimental results, correcting for the known quantity of added carrier receptor and the fact that only 1 pmol of the 6.3 in the labeled extract was accumulated during the labeling period, and assuming a purity of 25–75%, an experimental value for the specific activity of newly synthesized receptor of 2.4 to 7.2×10^4 cpm/pmol is calculated.

More direct measurements of synthesis and degradation rates using this approach are in progress. Furthermore, a number of unanswered questions as to the molecular homogeneity of receptor molecules can possibly be approached in the tissue culture system. Two groups have shown that receptor–toxin complexes in the extrajunctional area of denervated muscle gave markedly reduced half-lives as compared with those of the end plate region (21, 22). Others have shown differences in sensitivity to cholinergic antagonists (23, 24) and possibly even differences in physical chemical parameters such as isoelectric point (25). It is not yet clear what the physiological or structural significance, if any, of the reported differences in receptor from a single tissue might be, but it is tempting to speculate that such differences might be related to the rather unique regulatory role of muscular activity on receptor concentration, and metabolic stability could be related to such differences.

A possible indication of molecular heterogeneity from our experiments can be seen in Fig. 3. The shift of toxin binding material (which is for the most part carrier from unlabeled extracts) is greater than the shift of ^{35}S-labeled material. This could be interpreted to mean that newly synthesized receptor does not yet have the antigenic determinant recognized by our antiserum. A second interpretation is that the bulk of the ^{35}S-labeled material remaining in the 9S position is not receptor but an impurity. This is made unlikely by the control experiment in Fig. 3A, showing very little nonspecific radioactivity in that region of the gradient.

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Note. After this paper was submitted for publication, detailed publication of the work of J. P. Brockes and Z. W. Hall came to our attention. These authors have described: (*i*) the purification of ACh receptor from normal and denervated rat diaphragm [(1975) *Biochemistry* **14**, 2092–2099]; (*ii*) a comparison of junctional and extrajunctional receptor which demonstrated an isoelectric point difference in the two receptor types [(1975) *Biochemistry* **14**, 2100–2106]; (*iii*) and the *de novo* synthesis of extrajunctional ACh receptor in organ culture of denervated rat diaphragm [(1975) *Proc. Nat. Acad. Sci. USA* **72**, 1368–1372].

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