Skeletal Muscle Acetylcholine Receptor

PURIFICATION, CHARACTERIZATION, AND TURNOVER IN MUSCLE CELL CULTURES*

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Acetylcholine receptor has been purified from embryonic skeletal muscle cells grown and allowed to differentiate in tissue culture. The polypeptide composition of purified receptor has been determined by two-dimensional electrophoresis. The purest preparations are composed of a single Mr = 41,000 class of polypeptide which exhibits some charge heterogeneity. By high resolution two-dimensional electrophoresis a spot corresponding to acetylcholine receptor was localized among total proteins of muscle membrane extracts. Synthesis of this component is shown to be developmentally regulated. Quantitative analysis of receptor synthesis and degradation has led to the conclusion that receptor is one of a class of proteins whose synthesis is tightly regulated during terminal steps of myogenesis.

The nicotinic ACh-receptor is the primary protein component of the postsynaptic membrane at the neuromuscular junction. When adult skeletal muscle fibers are surgically denervated they rapidly become supersensitive to iontophoretically applied ACh (1). The number of ACh-receptor sites determined by α-neurotoxin binding increases by more than 20-fold (2, 3). All of the new sites are localized in the extrasynaptic area of the muscle membrane and over its entire surface (4). The increase in the number of toxin binding sites following denervation can be prevented or reversed by chronic electrical stimulation of the muscle fiber (5). Since increase in number of receptor binding sites is accompanied by synthesis de novo of receptor (6) and electrical stimulation has been shown not to increase the rate of receptor degradation (7), it is probable that denervation supersensitivity arises because of an increase in the rate of ACh-receptor synthesis. Experiments in which degradation of bound radioactive toxin has been used as a measure of receptor degradation have led to the conclusion that newly synthesized, extrasynaptic receptor is degraded more rapidly than the synaptic species present initially (8, 9). Embryonic muscle fibers are analogous to denervated fibers. The entire surface is initially sensitive to ACh and after innervation extrasynaptic sensitivity (10) and toxin binding sites disappear (9).

These observations on control of synthesis and degradation of ACh-receptor in response to denervation and innervation suggest that such processes play a critical role in synaptogenesis. A model has been presented detailing possible biochemical mechanisms by which the acquisition of synaptic specificity may be coupled to regulation of synthesis and degradation of receptor polypeptides (11).

Embryonic skeletal muscle myoblasts have proven useful for the study of ACh-receptor metabolism using radioactive α-neurotoxin as a probe (12–14). We have chosen to work with primary cultures of dissociated skeletal muscle myoblasts from fetal calf embryos because of the ease in obtaining large amounts of material, the reproducibility of tissue culture, and the possibility of doing quantitative precursor labeling experiments in the defined environment of a tissue culture system.

Skeletal muscle myoblasts grow and differentiate in tissue culture. A great deal is known about the synthesis and accumulation of specific muscle proteins and acquisition of muscle phenotype during differentiation in vitro (15, 16). The ACh-receptor is a member of that class of proteins whose activity is developmentally regulated during myogenesis. This phenomenon can be observed in vitro even in the absence of nerve cells. Innervation is the final step in the process of myogenesis and as such the regulatory effects of innervation on the expression of ACh-receptor synthesis must be superimposed upon the developmental program. For these reasons we have attempted to define the molecular events which result in accumulation of ACh-receptor during myoblast differentiation.

In this paper we describe the direct precursor labeling of the AChR, its purification, and characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of its polypeptide components. By pulse-chase labeling methods we have demonstrated that synthesis of receptor polypeptides is tightly regulated during muscle differentiation.

EXPERIMENTAL PROCEDURE

Cell Culture and Labeling—Primary cultures of skeletal muscle myoblasts of fetal calf were prepared as described previously (17–19). For routine culture a 3:1 mixture of Dulbecco’s modified Eagles'...
medium and Medium 199 (Gibco, Grand Island, N.Y.) containing 1% chick embryo extract (Eurobio, Paris) and 10% selected fetal calf serum (Gibco) was used. For labeling, the cells were washed and methionine-free Eagles' minimal medium supplemented with either 5 or 20 μM L-methionine and up to 100 μCi/10-cm culture dish of [35S]methionine (200 Ci/mmol, CEA, France) in a final volume of 2 ml was added. For pulse experiments the labeling time was 4 h. For chase experiments radioactive medium was removed from the dish and the cells were washed twice with warm phosphate-buffered saline and returned to culture in myoblast-conditioned medium supplemented with 80 μM methionine. At the end of a pulse or a chase, myoblast-conditioned medium was washed with phosphate-buffered saline and immediately frozen at −80°C.

Acetylcholine Receptor Purification—The purification of receptor from [35S]methionine-labeled cells was done as described previously (20, 21). Erabutoxin b (22) was coupled to cyanogen bromide-activated Sepharose 4B* by the method of Ratner (23) except that the packed beads was found to be optimal. Higher concentrations of toxin seemed to result in greater difficulty in receptor elution. Washing with 1% Triton X-100, 0.05 M NaCl, pH 7.6 at 4°C for periods of 3 to 8 h. Shorter times (3 h) were employed when the purity and integrity of the preparation were important, longer times (8 h) when quantitation was the goal. Adsorption and washing were done in batch in 10-ml test tubes. Washing with 1% Triton X-100, 0.05 M sodium phosphate, and 0.36 M NaCl, pH 7.6 was done by adding one 10-ml aliquot to 0.1- to 0.2-ml sample of beads followed by centrifugation and aspiration of the supernatant. The process was repeated until no radioactivity could be detected in the wash, about 10 to 20 times or 1000 to 2000 column volumes.

Elution of receptor bound to toxin/agarose was done with 0.1 M decamethionin in batch at 4°C for 4 h. Affinity elution proved the most variable step in the purification (21). Eluted material was diluted 10-fold with 0.01 M Tris-Cl, 0.01 M NaCl, 0.1% Triton X-100, pH 7.6, and adsorbed to a 0.2-ml Whatman DE52-cellose column and eluted with 0.05 M sodium phosphate, 0.36 M NaCl, 0.1% Triton X-100, pH 7.6. The recovery of toxin binding sites was monitored at every step by a toxin binding assay using [3H]BuTx and Whatman DE52 filter paper discs as described (21).

Sucrose Gradient Analysis—Analysis of L35S-labeled receptor labeled with 3H-labeled Naja nigricollis α1-isotoxin was done on 10 to 40% exponential convex sucrose gradients (25) as described previously (20, 21).

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (26), on gels of 10% acrylamide. Peptides were analyzed on 20% acrylamide gels as described by G'Farrell (28). First dimension electrofocusing (horizontal, left to right, basic to acidic) was done for 16 to 20 h. The second dimension (vertical, approximate molecular weight in units x 10−3 is given) SDS-polyacrylamide gel electrophoresis was done on 1.2-mm slabs of 10% acrylamide as described by Laemmli (26). Gels were prepared for fluorography by the method of Laskey and Mills (29) and exposed to X-Omat for the times indicated. A, 35S-labeled receptor eluted from toxin/agarose with 0.1 M decamethionin. Approximately 5000 cpm were applied to the gel. The fluorogram was exposed for 244 h. B, 3H-labeled receptor eluted from the toxin/agarose column (previously eluted with decamethionin) with 9 M urea, 1% SDS, plus 5% β-mercaptoethanol. Approximately 20,000 cpm were applied to the gel. The fluorogram was exposed for 244 h. C, 35S-labeled receptor eluted with urea/SDS/β-mercaptoethanol from a toxin/agarose column. Approximately 1000 cpm were applied to the gel. The fluorogram was exposed for 2.3 months. D, 3H-labeled receptor eluted with urea/SDS/β-mercaptoethanol from a toxin/agarose column, the same as in B. These isoelectric focusing gels were dried and an autoradiogram was done. The indicated bands were cut from the dried gel and eluted for peptide mapping (see Fig. 3).

Fig. 1. Analysis of purified ACh-receptor by two-dimensional high resolution polyacrylamide gel electrophoresis. Aliquots of purified ACh-receptor labeled in culture with radioactive amino acids were analyzed by the two-dimensional electrophoretic system described by O'Farrell (28). First dimension electrophoresis (horizontal, left to right, basic to acidic) was done for 16 to 20 h. Second dimension (vertical, approximate molecular weight in units x 10−3 is given) SDS-polyacrylamide gel electrophoresis was done on 1.2-mm slabs of 10% acrylamide as described by Laemmli (26). Gels were prepared for fluorography by the method of Laskey and Mills (29) and exposed to X-Omat for the times indicated. A, 35S-labeled receptor eluted from toxin/agarose with 0.1 M decamethionin. Approximately 5000 cpm were applied to the gel. The fluorogram was exposed for 244 h. B, 3H-labeled receptor eluted from the toxin/agarose column (previously eluted with decamethionin) with 9 M urea, 1% SDS, plus 5% β-mercaptoethanol. Approximately 20,000 cpm were applied to the gel. The fluorogram was exposed for 244 h. C, 35S-labeled receptor eluted with urea/SDS/β-mercaptoethanol from a toxin/agarose column. Approximately 1000 cpm were applied to the gel. The fluorogram was exposed for 2.3 months. D, 3H-labeled receptor eluted with urea/SDS/β-mercaptoethanol from a toxin/agarose column, the same as in B. These isoelectric focusing gels were dried and an autoradiogram was done. The indicated bands were cut from the dried gel and eluted for peptide mapping (see Fig. 3).

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It has been suggested that coupling of toxin to CNBr-activated beads at high pH (9.0) results in improved desorption, J. Lindstrom, personal communication.
RESULTS

Purification

The method of α-neurotoxin/agarose affinity chromatography used here is described in detail under "Experimental Procedures." It is essentially similar to that used and described by several other laboratories (32, 33). However, it was necessary to adapt this procedure to a small scale so that receptor from one or a few culture dishes could be isolated in a rapid manner.

Our experience with toxin/agarose affinity chromatography is that the yield by specific cholinergic ligand elution is generally quite low and variable. Those factors which we have found to give better results are the following: 1) a relatively low density of toxin sites (100 μg of toxin/ml of beads), 2) saturation of binding capacity of toxin/agarose (i.e. receptor excess), 3) repeated washing with greater than 1000 column volumes by centrifugation in batch, and 4) elution times of the order of 40 h at 4°C.

Table I summarizes results of a typical purification of ACh-
receptor from fetal calf skeletal muscle cells labeled in tissue culture with [35S]methionine. A binding assay using α-[3H]-bungarotoxin was employed to monitor recovery of receptor activity. [35S]Methionine-labeled protein was monitored by scintillation counting of hot trichloroacetic acid-precipitable radioactivity. In this experiment toxin binding activity eluted from the toxin/agarose conjugate by decamethonium was 5000-fold purified relative to the crude cell homogenate. From this value it can be calculated that incorporation of [35S]methionine into receptor accounts for at most 0.02% of the total incorporation. As will be shown in a later section this value varies depending upon the exact time of labeling.

Complete and quantitative recovery of receptor polypeptides may be obtained from the affinity column. When the toxin/agarose conjugate previously eluted with decamethonium was further subjected to protein denaturants, 9 M urea, 1% SDS, plus 5% β-mercaptoethanol, a significant amount of [35S]-labeled protein was recovered. Because of denaturation, this material could not be assayed for toxin binding activity, but it was shown to be identical to ACh-receptor polypeptides by electrophoretic analysis (Fig. 1 and below). Furthermore, the quantity of [35S]-labeled receptor recovered by denaturant elution corresponded exactly to that left unaccounted for by decamethonium elution (bracketed values, Table I). In other words, all of the receptor initially adsorbed to the column remained bound throughout the washing procedure and could be eluted by urea/SDS/β-mercaptoethanol.

Characterization of Affinity Purified Receptor

Sucrose Gradient Analysis — The [35S]methionine-labeled protein recovered from the purification procedure has been identified and characterized as receptor protein by both immunological and biochemical criteria (20, 21). Analysis by sucrose gradient centrifugation has shown that greater than 80% of the [35S]radioactivity eluted from toxin/agarose column co-migrates with the peak of toxin binding activity. Furthermore, both toxin binding activity and [35S]-labeled cross-reacted with antiserum prepared against purified ACh-receptor from Electrophorus electricus (see Ref. 21). By comparison with enzyme markers of known size (34), the purified receptor-toxin complex was found to have a sedimentation coefficient of approximately 9.5 s, identical to that found for the complex in crude cell homogenates.

Electrophoretic Analysis — Analysis of the polypeptide composition of [35S]-labeled purified receptor by high resolution two-dimensional gel electrophoresis (28) demonstrates its remarkable homogeneity. The fluorograms reproduced in Fig. 1, A and B, were deliberately over-exposed in order to identify the minor components. One of these at M, = 43,000 and apparent pl 6.25 has been identified by co-electrophoresis as actin. The faint streak at M, = 200,000 is myosin heavy chain. The spot of M, = 46,000 and apparent pl 6.25 is so far unidentified. Although the fluorograms for these two dimensional gels were not quantitatively scanned, visual comparison indicated that the content of the M, = 46,000 species was much less than that found in later preparations such as those shown in Figs. 2, 5, and 8.

The remaining and by far major species all migrate at M, = 41,000 and apparent pl 6.5 to 6.7. These consist of two distinct spots and a less well defined spot on the basic pH side of the major spot. This pattern is completely reproducible and characteristic. When an autoradiogram of the electrofocusing (first dimension) gel was examined it was found that the ill defined spot derived from a relatively normal protein band (Fig. 1D).

This suggests some heterogeneity in the apparent molecular weight of this species, resulting in band spreading during the second dimension. These three species have indistinguishable apparent molecular weights on one-dimensional SDS-gel electrophoresis (Fig. 2) in which only a single symmetric band at M, = 41,000 is seen.

In order to be certain that the use of [35S]methionine did not result in our missing a methionine-free polypeptide associated with receptor or give a false idea of the relative abundance of the species which were detected, similar analyses were performed on receptor purified from cells labeled with a mixture of 150 μCi [14C]-amino-acids. The fluorogram of the two-dimensional gel analysis of [14C]-labeled receptor is shown in Fig. 1C.

These results confirm the findings from analysis of [35S]-labeled receptor. Direct analysis by SDS-polyacrylamide gel, as shown in Fig. 2, demonstrates that two-dimensional analysis of purified receptor does not miss polypeptides that might be too basic or too acidic for the Ampholines (pH 5 to 8) employed in the first dimension of two-dimensional gels.

Receptor was purified at 4°C and the remaining receptor in the supernatant was then adsorbed at room temperature so that the effects of proteolysis could be evaluated. Fig. 2 shows that receptor purified at the two temperatures is essentially similar. The increase in amount of actin (43,000) found in the sample at room temperature is partially understood. Since the extract was first adsorbed at 4°C and the remaining receptor...
Fig. 4. The resolution of ACh-receptor in unfractionated cell extracts by high resolution two-dimensional electrophoresis. \(^{35}S\)-labeled, purified ACh-receptor having an electrophoretic pattern similar to that shown in Fig. 1A was mixed in increasing proportions with \(^{35}S\)-labeled unfractionated cell extracts. By observing which labeled, purified ACh-receptor having an electrophoretic pattern extracts by high resolution two-dimensional electrophoresis, the position or expected position of the major ACh-receptor polypeptide was determined in gel patterns of (A) extracts of differentiated cells and (B) extracts of undifferentiated cells.

Table II
Receptor degradation from pulse-chase labeling
Data were obtained from the experiment described in Fig. 5. \(^{35}S\) radioactivity in total protein and in ACh-receptor at the indicated times after the pulse labeling of the cells and chase with nonradioactive methionine was determined. Values are expressed as per cent of zero time value.

<table>
<thead>
<tr>
<th>Time of chase</th>
<th>(^{35}S)-labeled protein (total/plate)</th>
<th>(^{35}S)-labeled ACh-R(^) (plate)</th>
<th>ACh-R(^)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h x 10(^{-3})cpm</td>
<td>%</td>
<td>(\times 10(^{-2}))cpm</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>3.58 (100)</td>
<td>5.46 (100)</td>
<td>100</td>
</tr>
<tr>
<td>14.5</td>
<td>2.72 (76)</td>
<td>3.20 (59)</td>
<td>69</td>
</tr>
<tr>
<td>25.0</td>
<td>2.42 (68)</td>
<td>2.10 (38)</td>
<td>47</td>
</tr>
<tr>
<td>38.5</td>
<td>1.80 (50)</td>
<td>1.80 (33)</td>
<td>42</td>
</tr>
</tbody>
</table>

* Values were obtained by considering total counts per min eluted by urea/SDS/\(\beta\)-mercaptoethanol from toxin/agarose columns.

In summary, a purification procedure has been established which results in the rapid and quantitative isolation of a protein with the characteristics of the ACh-receptor. This material has been shown to be of high purity and composed of a small number of polypeptide components of identical molecular weight and probably of similar primary structure. In the following paragraphs we demonstrate the use of purified receptor as a marker to localize the spot corresponding to ACh-receptor in high resolution two-dimensional electrophoresis of crude unfractionated 100,000 \(\times g\) extracts of \(^{35}S\)-labeled cells. By these and quantitative affinity purification techniques we demonstrate the developmental regulation of ACh-receptor synthesis in differentiating muscle cells.

Resolution of ACh-Receptor from Cell Lysates by Two-dimensional Electrophoresis—Purified \(^{35}S\) methionine labeled ACh-receptor was added to total Triton X-100 extracts of \(^{35}S\)-labeled differentiated muscle cell membranes. Fluorograms of the two-dimensional electrophorograms of such mixtures were examined for spot enhancement. The spot indicated by the arrow in Fig. 4A is the only one of several hundred identifiable spots which is enhanced in crude lysates when purified \(^{35}S\)-labeled receptor is added. Only the major species is seen in these experiments due to the intensity difference among the three and the fact that these fluorograms have not been overexposed. When the fluorogram of the gel of an extract made from cells labeled early in culture, before any
FIG. 5. SDS-polyacrylamide gel analysis of receptor purified from cells pulse-chase-labeled with \[^{35}S\]methionine. Twenty 10-cm culture dishes were labeled for several h (from 135 to 139 h of culture) with \[^{35}S\]methionine (2.5 Ci/mmol, specific activity). Cultures were washed and returned to myoblast-conditioned medium supplemented with 0.05 mM methionine. At 0, 14.5, 25, and 38.5 h (corresponding to Lanes F through I), five dishes were washed and immediately frozen at -80°C. Extracts were prepared and receptor purified from each set of dishes as described in the text. Adsorption of toxin binding activity to toxin agarose was greater than 80% in each case. All conjugates were washed and eluted with urea, SDS, and \(\beta\)-mercaptoethanol identically. Eluates were applied directly to a 10% SDS-polyacrylamide gel. The gel was dried and an autoradiogram prepared. Contamination with high molecular weight (150,000 to 200,000) material resulted from less extensive washing of affinity bound receptor. Each lane of the gel was scanned with a scanning spectrophotometer and the area under each peak integrated for quantitation. Lanes A to E represent a pulse experiment to measure rates of synthesis and are described in Fig. 8.

Adapted from [source]
Fig. 6. Receptor degradation during differentiation. Cells were labeled with $^{125}$I-BuTx (approximately 20 Ci/mmol, specific activity, at $5 \times 10^{-9}$ M for 4 h) at three different times during the culture period. Unbound toxin was washed out and the cells were returned to culture in myoblast-conditioned medium. At the indicated times the amount of cell-associated toxin was determined. For each point two or five 6-cm culture dishes were pooled. Values are recorded as counts per min per 6-cm dish on the ordinate and hours after plating on the abscissa.

Fig. 7. Synthesis and accumulation of ACh-receptor (ACh-R). ACh-receptor accumulation during myoblast differentiation was measured by the DEAE-filter assay using $^{125}$I-BuTx (10 Ci/mmol). Values recorded are total counts per min per 10-cm culture dish. The accumulation of total protein was determined by incorporation of [35S]methionine added at 0 h of culture. The rate of synthesis of total protein was determined by pulse-labeling for 4-h periods with [35S]methionine (5 $\mu$Ci/mg) and trichloroacetic acid precipitation of the unfractionated cell extract. The rate of synthesis of ACh-receptor was determined by affinity purification of labeled ACh-receptor from cells pulse-labeled for 4 h. Absorption was greater than 80% in each purification. Elution was done with urea/SDS/β-mercaptoethanol. Eluates were applied directly to a 10% polyacrylamide gel. The dried gel was exposed for autoradiography, the autoradiogram was scanned, and the area under each peak was integrated for quantitation. As in Fig. 6 the orientation of the gel is right to left, early to late times. The orientation of the scans is top to bottom, early to late.

for receptor degradation by this method was between 18 and 22 h, while that for total protein was 40 h.

In order to account for the greater than 100-fold increase in steady state receptor sites which occurs during in vitro differentiation either the rate of receptor degradation must be slower than in undifferentiated cultures or the rate of its synthesis must be more rapid. Fig. 6 shows an experiment to detect a difference in the rate of receptor degradation from the earliest time at which such an experiment is possible using toxin of the specific activity available (20 Ci/mmol) until late in the process of differentiation when maximal toxin binding activities are attained. (See Fig. 7 for the time course of accumulation of toxin binding activity.) No difference in the rate constant for receptor degradation could be observed during this period.

Receptor Synthesis — Since the rate of receptor degradation remains unchanged, it is expected that there should be a significant increase in the rate of receptor synthesis to account for the observed accumulation. Figs. 7 and 8 show the results of two experiments in which the rate of receptor synthesis was measured directly by pulse-labeling. The rate of synthesis of receptor and total protein accumulation are presented in Fig. 7A. The rate in counts per min per 10-cm culture dish per 4 h for both total protein and receptor are presented in Fig. 7B. From this experiment only an approximation of receptor synthesis is obtained because only total counts eluted from toxin/agarose are recorded. Since none of the preparations are pure receptor and since the earlier time points are likely to be less pure than later ones, these results are probably slightly skewed. When in a second experiment eluates were analyzed by SDS-gel electrophoresis (Fig. 5) and the rate of synthesis of only the Mr ~ 41,000 species was tabulated, it was found that...
the rate of receptor synthesis increased more than 60-fold (Table III, column F). When the increase in rate of total protein synthesis is taken into account (Table III, column E), the differential rate of receptor synthesis increases 17-fold (Table III, column G). The meager rates of synthesis detected in early cultures can be more than adequately accounted for by the small number of differentiated cells (detected as multinucleate fibers) (Table III, column C) present, or by the observation that occasional mononucleate bipolar cells present early in culture exhibit low levels of ACh sensitivity (39).

**DISCUSSION**

In this report we describe the characterization of the polypeptide composition of mammalian muscle ACh-receptor. The major polypeptide species of $M_r = 41,000$ occurs in at least three isoelectric forms. We have been unable to determine the origin of this charge heterogeneity and the relatively low abundance of ACh-receptor in muscle cells and the possibility of charge modification during purification make such analyses difficult. The use of muscle cell cultures might allow such evaluations to be made. However, such relationships might exist and make an evaluation of the physiological significance of such a modification difficult. The use of muscle cell cultures might allow such evaluations to be made.

The fact that some purifications have resulted in preparations almost completely lacking the higher molecular weight (43,000 and 46,000) polypeptides suggests that these may not be receptor subunits. The spot of $M_r = 43,000$ is almost certainly contaminating actin. The spot of 46,000 is unidentified; its possible identity as a receptor component is not excluded. We have previously reported the occurrence of several possibilities: 1) it is possible that species or organ differences in the polypeptide composition from ACh-receptor is labeled by trichloroacetic acid precipitation.

The rate of incorporation of [35S]methionine into receptor was determined by affinity purification of labeled extracts. Elution was done with urea/SDS/β-mercaptoethanol. Extracts were analyzed by SDS-polyacrylamide gel electrophoresis and the autoradiograms of such gels were scanned spectrophotometrically and the content of the $M_r = 41,000$ species was determined (see Figs. 5 and 6). A value in counts per min for this species was derived from the measure of $^{38}$S counts per min in the eluted sample and the fraction of the total film density of the autoradiogram which was found under the 41,000 peak.

It is not understood why our preparations of muscle receptor have a polypeptide composition somewhat different from those of E. electricus, Torpedo californica electric organ, or a mouse muscle cell line (40, 43-45). It has been reported that the receptor from electric eel contains two or three components of approximately $M_r = 40,000$, 47,000, and 53,000 (39, 42), and that of Torpedo contains four components of $M_r = 39,000$, 48,000, 58,000, and 64,000 (43, 44, 46). Recently, Sobel et al. (47) have found that the polypeptide composition of highly purified receptor from Torpedo marmorata varies from one purification step to the next, and from one preparation to another. The preparations with the highest specific activities are richer in the $M_r = 40,000$ polypeptide. Only the $M_r = 40,000$ polypeptide of both eel and Torpedo receptors is labeled with the affinity reagent 4-(N-maleimido)benzyltri(3H)methyl ammonium) (MBTA) (42).

**Table III**

<table>
<thead>
<tr>
<th>Hours</th>
<th>Nuclei per plate × 10^4</th>
<th>Fused nuclei</th>
<th>Protein/plate</th>
<th>% of total protein</th>
<th>[35S]-labeled protein (cpm/plate)</th>
<th>Relative rate ACh-R synthesis</th>
<th>Rate of incorporation of [35S]methionine into receptor was determined by affinity purification of labeled extracts. Elution was done with urea/SDS/β-mercaptoethanol. Extracts were analyzed by SDS-polyacrylamide gel electrophoresis and the autoradiograms of such gels were scanned spectrophotometrically and the content of the $M_r = 41,000$ species was determined (see Figs. 5 and 6). A value in counts per min for this species was derived from the measure of $^{38}$S counts per min in the eluted sample and the fraction of the total film density of the autoradiogram which was found under the 41,000 peak.</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>3.4</td>
<td>0.5</td>
<td>0.58</td>
<td>5.35</td>
<td>18.6</td>
<td>0.00035</td>
<td></td>
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<tr>
<td>92</td>
<td>10</td>
<td>16</td>
<td>1.48</td>
<td>18.5</td>
<td>199</td>
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<td>20.6</td>
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<td>212</td>
<td>14</td>
<td>34</td>
<td>2.31</td>
<td>19.5</td>
<td>464</td>
<td>0.00238</td>
<td></td>
</tr>
</tbody>
</table>

a Hours after plating of culture when the 4-h pulse was started (see legends to Figs. 5 and 7).

b In order to determine increase in cell number and degree of morphological differentiation parallel culture plates were fixed with methanol and stained with Giemsa. Two thousand total nuclei were counted for cells at each time. Data is expressed as total nuclei per 10-cm culture plate and the per cent of total nuclei which were found in multinucleate myotubes.

$^c$ Total cell protein was determined, using bovine serum albumin as a standard (37).

$^d$ Total [35S]methionine incorporated into protein during the 4-h pulse was determined by trichloroacetic acid precipitation.

J. P. Merlie, unpublished observation.
differences exist for ACh-receptor derived from varied sources; 2) it is possible that differences in purification procedures might result in retention of non-receptor protein or removal of loosely associated subunits; 3) as indicated above protein synthesis during purification may be an important factor in determining the final polypeptide composition.

At present it is impossible to distinguish among these possibilities and to describe a definitive polypeptide pattern for the ACh-receptor. It is however certain that the 40,000-dalton species is a true receptor component or a derivative of such a component. Whatever the subunit composition, it seems valid to base qualitative and quantitative measures of receptor turnover on those species which we have found in highly purified muscle ACh-receptor.

The qualitative demonstration by two-dimensional electrophoresis that the spot corresponding to the ACh-receptor is developmentally regulated is supported by quantitative measurements of receptor synthesis and degradation. Our studies have shown that the large increase in accumulated ACh-receptor during myogenesis in tissue culture is due exclusively to an increase in receptor synthesis. Since receptor synthesis increases 17 times more than global protein synthesis this cannot be explained simply by cell growth. Expression of ACh-receptor, like that of actin, several myosin light chains and tropomyosins (16, 19, 49) is developmentally controlled during muscle differentiation.

The steady state level of any protein is determined by both its rate of synthesis and its rate of degradation. In cultured myoblasts the steady state value of receptor sites can be measured in two ways. From toxin binding experiments we know that each 10-cm culture dish contains from 0.5 to 1 pmol of toxin-binding sites. From our determination of receptor synthesis and degradation a steady state value of toxin binding sites can be arrived at by the simple relation $[RI] = k_{s}\cdot t_{a}$, where $k_{s}$ and $t_{a}$ are rate constants for receptor synthesis and degradation respectively. A value for $k_{s}$ of 0.030 pmol/h/10-cm dish was calculated from the maximal rate of synthesis observed in the experiment described in Table III. The following assumptions were made: 1) that there is a constant in methionine of 3.4 mol%, derived from data for E. electricus receptor (39), 2) that there is one toxin-binding site per 40,000 daltons of receptor protein, the apparent mass of the major species, and 3) that the specific activity of the [35S]methionine is unaffected by cellular pools or compartmentalization. A value for $t_{a}$ of 0.04 h^{-1} was calculated from the measured half-life from the expression $t_{a} = \ln 2/k_{s}$ using the value of $t_{a} = 17$ h, an average of several determinations. Then, [R] equals 0.75 pmol of toxin binding sites/10-cm dish, a value which agrees remarkably well with that determined by toxin binding. Agreement of these values indicates that most receptor sites appearing in cultured cells do so as a result of synthesis de novo and not as a result of conversion of some preformed cell component. It cannot be excluded that receptor polypeptides pass through a relatively small rapidly turning over pool of precursor material.

Receptor protein constitutes only a small fraction of total cellular protein. Less than 0.01% of total protein synthesis is due to receptor synthesis. This represents a synthetic rate of approximately 5 molecules of receptor/min/myovube. For comparison Emerson and Beckner (48) have reported a value of 30,000 molecules/min/nucleus for the myosin heavy chain ($M_{r} = 200,000$) in cultures of quail myoblasts. Five molecules per min is the approximate production rate of a single active polyribosome of the size one would expect for the receptor subunit. This value establishes definite limits for the way in which we might think about the mechanism of regulation of receptor synthesis and for the ways in which we approach the study of this problem.

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