ACETYLCHOLINE RECEPTOR TURNOVER IN MEMBRANES OF DEVELOPING MUSCLE FIBERS

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ABSTRACT

[^{125}I]mono-iodo-α-bungarotoxin is used as a specific marker in a description of acetylcholine receptor metabolism. It is concluded that acetylcholine receptors in the surface membranes of chick and rat myotubes developing in cell cultures have a half-life of 22–24 h. α-bungarotoxin (bound to a receptor which is removed from the membrane) is degraded to monoiodotyrosine which appears in the medium. Several observations are consistent with a model in which receptors or α-bungarotoxin-receptor complexes are internalized and then degraded: (*a*) the rate of appearance of iodotyrosine does not reach its maximal rate until 90 min after α-bungarotoxin is bound to the surface receptors; (*b*) 2,4-dinitrophenol, reduced temperature, and cell disruption all inhibit the degradation process. The degradation of surface receptors is not coupled to the process by which receptors are incorporated into the membrane. Evidence suggests that receptors are incorporated into the surface membrane from a presynthesized set of receptors containing about 10% as many α-bungarotoxin binding sites as does the surface. Additionally, a third set of acetylcholine receptors is described containing about 30% as many binding sites as does the surface. These "hidden" receptors are not precursors yet are not readily accessible for binding of extracellular α-bungarotoxin. These findings are discussed in relation to both plasma membrane biosynthesis and control of chemosensitivity in developing and denervated skeletal muscle.

Research on the biosynthesis and turnover of membrane proteins has been handicapped by the absence of specific, sensitive markers for membrane components. The small polypeptide, α-bungarotoxin, is such a marker for the acetylcholine (ACh) receptor (20). ACh receptors are present in large numbers in the plasma membranes of differentiating skeletal muscle, and tissue cultures of such muscle provide a convenient experimental system in which membranes containing ACh receptors are created and destroyed. Evidence for the incorporation of ACh receptors into the membranes of myotubes, monitored by [^{125}I]α-bungarotoxin binding, has been reported by this laboratory (16). We have continued this research and now have a more complete description of ACh receptor metabolism.

This communication describes the turnover of ACh receptors, with emphasis on its degradative aspect. While the bulk of the data concerns chick skeletal muscle in culture, the rate of receptor degradation is similar for receptors in rat muscle cultured in vitro. Another aspect of this communication is the description of two functionally different pools of ACh receptors besides those mediating ACh sensitivity of the plasma membrane. One

of these pools may consist of precursors to the surface ACh receptors.

MATERIALS AND METHODS

Chemicals

α-bungarotoxin was isolated from the crude venom of *Bungarus multicinctus* (obtained from the Miami Serpentarium, Miami, Fla.) and was purified as previously described (11, 15). α-bungarotoxin was iodinated by the chloramine-T method (18), using ^{131}I or ^{125}I, and the iodinated α-bungarotoxin was purified by gel filtration and ion-exchange chromatography (15). The iodinated residues of purified [^{125}I]α-bungarotoxin were shown by exhaustive pronase digestion followed by high voltage paper electrophoresis to be 98% monoiodotyrosyl and 2% diiodotyrosyl residues. Specific activities of iodinated α-bungarotoxin were obtained by radioisotope counting at known efficiency and measuring protein concentration by the method of Lowry et al. (23), using a 10% solution of bovine serum albumin (BSA) (Schwarz/mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) to prepare standards. Specific activities ranged from 2.6 to 5.7 × 10^4 Ci/mol for both [^{125}I]α-bungarotoxin and [^{131}I]α-bungarotoxin. The labeling reaction did not change the binding activity of the α-bungarotoxin as judged by serial dilution experiments with unlabeled α-bungarotoxin.

Monoiodotyrosine, labeled with ^{125}I or ^{131}I, was prepared by the chloramine-T method, using a very large molar excess of tyrosine in the reaction mixture so that monoiodotyrosine would be formed, and was purified by column chromatography on Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) (5).

Diiodotyrosine and iodohistidine were synthesized and purified by Ms. Sandra Biroc. Pepstatin (Banya Pharmaceutical Co., Tokyo) was a gift from Dr. Stephen Max. Cytochalasin B was a gift from Drs. Howard Holtzer and John Sanger. All other chemicals were purchased from commercial sources in the United States.

Radioisotope Counting

Radioisotope counting was usually done in a Packard Tricarb Scintillation Spectrometer (Packard Instrument Co., Inc. Downers Grove, Ill.). Aqueous samples were counted in scintillation cocktail that was one part Triton X-100: two parts toluene fluor (16 g PPO (2,5-diphenyloxazole), 0.2 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) in 1 gallon toluene). The ratio of sample to cocktail was 3:20. Counting efficiency for ^{125}I was 66% and for ^{131}I was 20%.

Tissue Culture

Thigh muscle was dissected from 11-day-old White Leghorn chick embryos. A single cell suspension was obtained from minced tissue either by trypsinization (0.05%, 15 min) or mechanical disruption. Cell suspensions were filtered through gauze and then silk filters. When cells were trypsinized, they were then plated overnight to remove red blood cells. The next day, cells were trypsinized from the plate and myoblasts were selected from fibroblasts by differential adhesion; that is, cells were preplated for 60 min and the cells which did not adhere were collected for final plating. This less adhesive fraction is enriched for myoblasts (38). Mechanical disruption was done in a 40-ml conical centrifuge tube with 0.5 ml medium per thigh. The suspension was agitated on a vortex mixer for 60 s. Large particles were allowed to settle and the supernate was decanted and filtered to remove aggregates and debris. Cells obtained by both methods were plated on Falcon Plastic petri dishes coated with rat tail collagen. Plating densities were 5×10^4–5×10^5 cells for 35-mm dishes and 5×10^5–5×10^6 for 100-mm dishes. The newer method of cell preparation, mechanical disruption, yields about 10 times as many cells in one-fourth the time. Also, the cultures contained fewer "fibroblasts" than the trypsin-dissociated muscle.

Basic culture medium (F12) was modified Ham's F12 (21). F12 was bicarbonate buffered and gassed with 5% CO_2 in air; it contained 15% horse serum, 0.5% BSA, and 2% embryo extract. For short-term experiments several other media were used. F11 was like F12 but lacked phenol red indicator dye and was buffered at pH 7.2 with 18 mM Na *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) instead of 18 mM bicarbonate. UnF11 was like F11 but contained no horse serum or embryo extract. Wash medium was HEPES-buffered Hanks' balanced salt solution with 0.5% BSA. Wash medium was often buffered with tricine (18 mM) instead of HEPES. At times, when quantification was not necessary, cultures were washed in tricine-buffered 0.9% NaCl. Wash media were used to rinse unbound α-bungarotoxin from cultures.

Column Chromatography

BioGel P-60 and P-2 columns were used to analyze detergent extracts and samples of medium. P-60 columns were always developed with 1% Triton X-100, 10 mM Tris buffer, pH 7.8, and radioactivity was quantitatively recovered. P-2 columns were developed with 0.05 M ammonium acetate buffer, pH 5.0 or 7.5. Recovery ranged from 60% to 70% of the applied radioactivity. In some experiments carrier iodotyrosine and iodide were added to samples before chromatography on BioGel P-2. In these experiments recovery of radioactivity was about 70%.

Saturation of Surface ACh Receptors with α-Bungarotoxin and Removal of Unbound Toxin

ACh receptors exposed on the surface of cultured myotubes were routinely saturated with 0.1–0.5 μg/ml α-bungarotoxin for 30 min in F12, F11, or UnF11 at 37°C (16). After this treatment, there is no detectable

ACh sensitivity as measured by intracellular recording and iontophoretic application of ACh (16). Autoradiographs of such cultures demonstrate that the vast majority of α-bungarotoxin binding sites are on myotubes, and in young chick muscle cultures such as those used in almost all of the present studies the sites are distributed over the entire myotube surface without obvious clumping (16, 35). Cultures of fibroblasts or of dividing myogenic cells before cell fusion bind very little α-bungarotoxin (16, 29, 30, 35, and see Fig. 10). Unbound α-bungarotoxin was removed by immersing the cultures in a large (about 2 liters) bath of wash medium at or below room temperature. The wash medium was changed two times at about 10-min intervals; the filled culture dishes were removed from the bath during each change. This method of washing is quite efficient in removing unbound α-bungarotoxin. In one experiment, 98.7% of the counts remaining on the culture after this procedure were excluded during column chromatography on Bio-Gel P-60 after solubilization in 1% Triton X-100, indicating that there was only a very small amount of free α-bungarotoxin. If, after removal of unbound α-bungarotoxin, the cultures were immediately rechallenged with another saturating dose, the additional α-bungarotoxin bound was less than 3% of that bound during the initial saturation. In early experiments, cultures were washed eight times with 1 ml medium per 35-mm culture dish during a 30-min period while cultures were on a rotary table. This method did not provide superior rinsing and, more important, resulted in a small (but, for some experiments, unacceptable) loss of myotubes from the culture plates. We devised the bath washing method to minimize cell loss.

Extraction of ACh Receptors and α-Bungarotoxin-Receptor Complexes from Myogenic Cell Cultures

All of the specific α-bungarotoxin binding sites were readily extracted from myogenic cell cultures into detergent solution. Routinely, the culture medium was removed and 1 ml of 1% Triton X-100 in 10 mM Tris-HCl buffer pH 7.8 was added. After a few minutes the culture dish was swirled and the solution removed. The culture was re-extracted with 0.5 ml of the solution with vigorous pipetting. The combined extracts contained all of the ACh receptors present in or on the cells or all bungarotoxin-receptor complexes formed by preincubation of cultures with α-bungarotoxin.

More than 95% of the specific α-bungarotoxin binding sites in the extract remained in solution when the extract was centrifuged for 30 min at 7,000 *g* to remove insoluble material.

Filter Assay for ACh Receptors

A DEAE-cellulose filter binding assay for quantitative measurement of solubilized ACh receptors has been previously described by Klett et al. (19) and by Schmidt and Raftery (34). The assay allows reproducible recovery of α-bungarotoxin-receptor complexes. We have modified the assay somewhat and thereby lowered the background. A Whatman CM-82 filter, (diameter 2.5 cm) the function of which is to remove much (~80%) of the unbound α-bungarotoxin from the sample, was placed over the Whatman DE-81 filter (diameter 2.5 cm). The two-filter stack was then equilibrated with 20 mM sodium phosphate buffer at pH 5.7 and placed on two thicknesses of blotter paper. The sample (100–300 μl) was then applied to the filter stack. After 30 s, the sample was washed into the filters with about 0.3 ml buffer. The buffer was slowly aspirated through capillary action provided by the blotter paper. After another 30 s the filters were separated, the upper (CM-82) filter was discarded, and the lower (DE-81) filter was washed in 50 ml buffer for more than 6 h, placed in a scintillation vial with 1 ml 1% Triton and 10 ml scintillation cocktail and counted after 24 h (counts increase during the 24-hour wait). Retention on the DEAE-cellulose filters is 55% ± 10% of the bungarotoxin-receptor complexes and 0.05%–0.15% of the free α-bungarotoxin in the sample.

Some samples were prepared by detergent solubilization of [^{125}I]α-bungarotoxin-receptor complexes from myogenic cell cultures. Other samples were detergent extracts of myogenic cell cultures, which were subsequently incubated at room temperature with [^{125}I]α-bungarotoxin. Various incubation parameters have been studied, including temperature, α-bungarotoxin concentration, and time. A typical saturation curve for specific α-bungarotoxin binding sites is illustrated in Fig. 1. Portions of a clarified detergent extract were incubated for 1 h at room temperature with various concentrations of [^{125}I]α-bungarotoxin. The amount of [^{125}I]α-bungarotoxin-receptor complex formed was determined by the filter assay. As illustrated in Fig. 1, the specific sites in these samples are saturated at 0.01 μg/ml α-bungarotoxin (1.25×10^{-9} M) whereas the "nonspecific" sites are not saturated at the highest concentration. We have used 0.01 μg/ml [^{125}I]α-bungarotoxin for 1 h at room temperature as standard conditions in our assays of solubilized ACh receptors.

The nonspecific sites have been partially characterized in that they run together with most of the cell protein (Fig. 12) to the 2S–6S region of a sucrose gradient during velocity sedimentation in which α-bungarotoxin-receptor complexes are about 10S (4, 25, 26, 32). Binding of α-bungarotoxin to this material does not saturate in 3 h with 1 μg/ml α-bungarotoxin, and the complexes have an extremely broad thermal dissociation profile, indicating inhomogeneity. Approximately 40% of these complexes are not destroyed by incubation in 1% sodium dodecyl sulfate for 60 min at 37°C, indicating that the association may be in part covalent. The nonspecific component binds to DEAE-cellulose filters with an efficiency of about 30%. The nonspecific binding is distributed among the various cell fractions in nearly the same manner as the total cell protein. Under the standard binding conditions defined above, a consistent amount of nonspecific

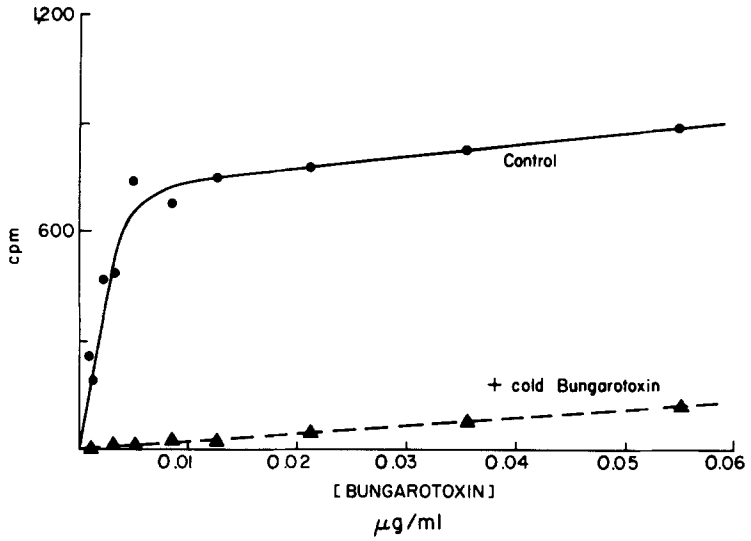

FIGURE 1 Saturation of solubilized ACh receptors with α-bungarotoxin. Approximately 20 large (100-mm) cultures were sequentially extracted into 8 ml of 1% Triton X-100, 10 mM Tris, pH 7.8. The extract was centrifuged for 30 min at 20,000 *g*. 300-μl portions of the supernate were incubated with the indicated concentrations of [^{125}I]α-bungarotoxin for 1 h at room temperature. Three 100-μl portions at each concentration were assayed by filter as described in Materials and Methods, and the mean is plotted. For nonspecific sites, the aliquots were preincubated for 1 h with 1 μg/ml unlabeled bungarotoxin. Total binding (—●—); nonspecific binding (—▲—). The specific activity of the [^{125}I]α-bungarotoxin was 5.5 × 10^4 C/mol. The experiment was repeated two times.

binding is encountered in each type of experiment described in this report. Expressed as a percent of the specific surface sites, typical values for nonspecific binding are: to intact 5-day-old myogenic cells (1–3%), to 1% Triton X-100 extracts of cell cultures (15–20%), and to 1% Triton X-100 extracts of crude cell membrane preparations (5%). These values were determined from numerous sucrose gradient centrifugations where the radioactivity sedimenting in the 10S region of the gradient was considered to be toxin-receptor complexes (illustrated in Fig. 12). Thus, nonspecific binding to intact cells is a minor problem and is aggravated by exposing the rest of the cell protein. It can be seen that under standard filter assay binding conditions the amount of nonspecific binding is small (Fig. 1). However, in cases where the number of receptors is small relative to the total amount of protein in the sample (Fig. 15), we have abandoned the filter assay and considered the 10S region on sucrose gradients as specific binding.

Polyacrylamide Gel Electrophoresis

1% Triton X-100 extracts containing labeled α-bungarotoxin-receptor complexes were analyzed by electrophoresis on polyacrylamide gels. An acrylamide-methylene bis acrylamide mixture (3.75% and 0.10%, respectively) was polymerized in 0.37 M Tris-HCl buffer pH 8.9, 0.5% wt/vol Triton X-100, 0.03% vol/vol *N,N,N′,N′*-tetramethylethylenediamine with 0.7 mg ammonium persulfate per ml of solution. Gels 0.6 × 10 cm were overlaid with H_2O during the 2 h polymerization time. Buffer compartments were then filled with pH 8.3 buffer containing 3 g Tris, 14.4 g glycine, and 5 g Triton X-100 per liter. 75–150-μl samples (containing sucrose) were layered on the gels and electrophoresis was carried out at room temperature at constant current 1 mA/gel for 30 min followed by 2 mA/gel for 4 h, gels were sliced in 2-mm sections on a Gilson Aliquogel Fractionator (Gilson Medical Electronics, Inc., Middleton, Wis.), and radioactivity was counted by scintillation spectrometry.

OPERATIONAL DEFINITIONS

ACh Receptors

α-bungarotoxin interacts specifically and in an essentially irreversible manner with ACh receptors. The complex formed between toxin and receptor moves as 10S material in sucrose gradients, and, as indicated above, all reference to ACh receptors or specific α-bungarotoxin binding sites in this report is in relation to types of experiments in which the nature of the toxin binding sites has been examined on several occasions by sucrose gradient velocity sedimentation. An evaluation of

the problem of nonspecific binding is presented above. [^{125}I]α-bungarotoxin-receptor complexes extracted from chick myogenic cell cultures and incubated with 10,000-fold excess α-bungarotoxin at 37°C dissociate with first order kinetics with a half time of 9–15 days. (We do not know whether this slow rate of dissociation is a measure of the reversibility of the toxin-receptor interaction or just a measure of slow spontaneous denaturation of the receptor or the toxin.) Toxin-receptor complexes formed by incubation of solubilized receptors with [^{125}I]α-bungarotoxin and purified by chromatography on Bio-Gel P-60 are similarly stable. Bungarotoxin and homologous snake toxins have been used by various investigators to determine the number of ACh receptor sites (2, 6, 7, 15, 16, 20, 22, 27, 31). The number of α-bungarotoxin binding sites is proportional to (perhaps equal to) the number of ACh receptors, and the terms are therefore used interchangeably in this report.

Degradation of ACh receptors

Since degradation cannot be measured directly, the release of radioactivity into the culture medium after [^{125}I]α-bungarotoxin has bound to ACh receptors of the myotubes is monitored. Evidence that such release indicates concomitant disappearance of ACh receptors is presented below. To measure the rate of release, a large number of identical myogenic cell cultures were saturated with [^{125}I]α-bungarotoxin, the unbound toxin was removed by washing as described in Materials and Methods, and the cultures were incubated under appropriate experimental conditions, usually in HEPES-buffered F11 or UnF11 in a 37°C room. At subsequent times, sets of four or more culture dishes were taken for analysis. The medium from each culture dish was removed, centrifuged for 5 min at 1,000 *g* to sediment any cell debris, and a portion of the supernate was counted by scintillation spectrometry. The radioactivity remaining associated with the myotubes in each culture was extracted in 1% Triton X-100 and counted. The fractional amount of degradation was calculated as cpm in medium/(cpm in medium + cpm bound to cells). Variation from the mean value for a set of four cultures was usually less than ± 10% of the mean. Linear plots of degradation vs. time up to 6 or 8 h yield straight lines the slopes of which approximate the rate constant for degradation of ACh receptors. We have used this approximation throughout the Results section, expressing degradation as a fraction of initial α-bungarotoxin binding sites destroyed per hour.

Incorporation

As will be shown below, there is a steady turnover of ACh receptors. Therefore, the rate at which the total number of receptors per culture is increasing is slower than the rate at which new receptors are appearing. Thus, it is necessary to distinguish between incorporation of ACh receptors and accumulation of receptors. Incorporation of ACh receptors into the plasma membrane has been described previously (16) and is defined as the appearance of new α-bungarotoxin binding sites on the myotube surface. In order to measure the rate of incorporation separately from that of degradation or removal, all of the existing bindings sites on the surface were blocked with unlabeled α-bungarotoxin and therefore most of the sites that subsequently were degraded were already inactivated. Identical cultures were divided into two sets. One set (four cultures) was saturated with [^{125}I]α-bungarotoxin, the unbound toxin was washed away, and the α-bungarotoxin-receptor complexes in each culture plate were extracted and counted by scintillation spectrometry. Thus, we determined the total number of α-bungarotoxin binding sites per culture dish at the start of the experiment (T = 0). At the same time, the remaining set of cultures (a large number) was saturated with unlabeled α-bungarotoxin, unbound α-bungarotoxin was removed, and the cultures were returned to the appropriate medium at the appropriate temperature. This time was considered T = 0. The number of ACh receptors appearing on the cultures at subsequent times was then determined by measuring the number of new α-bungarotoxin binding sites, using [^{125}I]α-bungarotoxin. Appearance of new sites measured in this way is a linear function of time for at least 15 h. The rate of incorporation is the slope of this line. Under steady-state conditions, incorporation rate and synthesis rate should be identical or at least proportional to each other. However, as shown below, incorporation can occur in the absence of new receptor biosynthesis.

Accumulation

The rate of ACh receptor accumulation in myogenic cell cultures should be the difference

between the rate of incorporation and the rate of degradation (see Table VII). To measure accumulation of ACh receptors, sets of cultures having no prior exposure to α-bungarotoxin were assayed for receptors at given time intervals. The ACh receptors on cultured myotubes were saturated with [^{125}I]α-bungarotoxin, unbound [^{125}I]α-bungarotoxin was removed, and the [^{125}I]α-bungarotoxin-receptor complexes were extracted and counted by scintillation spectrometry. Accumulation is the difference between cpm bound at a given time and cpm bound at the start of the experiment. During the period of muscle differentiation in vitro, accumulation is a linear function of time for several days (see Fig. 10).

RESULTS

Degradation of [^{125}*I*]α-*Bungarotoxin Bound to ACh Receptors*

There is a slow release of radioactivity into the medium after [^{125}I]α-bungarotoxin has specifically bound to surface receptors. [^{125}I]α-bungarotoxin-ACh receptor complexes are extremely stable (10) and the release of radioactivity into the medium, we will show below, is an energy-requiring, proteolytic process and parallels the disappearance of ACh receptors from the myotube surface. Fig. 2 shows the kinetics of release of radioactivity into the medium during incubation of chick myogenic cell cultures at 37°C. As shown in Fig. 2, degradation is a first-order process. This implies that the rate of degradation is proportional to the number of toxin-receptor complexes in the membrane. The first-order kinetics also suggest a mechanism that degrades at random, not discriminating α-bungarotoxin bound to "old" or "new" receptors. The rate constant for degradation is the slope of the line in Fig. 2 and is about 0.03/h. The "half-life" of [^{125}I]α-bungarotoxin bound to receptors in the membrane is about 22 h under our standard culture conditions (see also Table VI). We have also measured the rate of degradation in cultured embryonic rat myotubes (Fig. 2). The rate constant in rat myotubes is also about 0.03/h.

The radioactivity appearing in the culture medium after [^{125}I]α-bungarotoxin has bound to surface receptors can be separated into three components by chromatography on Bio-Gel P-60 columns: (*a*) a high molecular weight excluded peak, (*b*) partially included free α-bungarotoxin, and (*c*) a completely included low molecular weight component. Fig. 3 shows P-60 chromatographic profiles of radioactivity in the medium collected at several of the time points shown in Fig. 2. Free α-bungarotoxin is detectable only during the first 8-h period and represents from 5% to 15% of the radioactivity released during this period (in 10 experiments) or 1%–3% of the total [^{125}I]α-bungarotoxin initially bound. The free [^{125}I]α-bungarotoxin released probably represents small amounts initially trapped or nonspecifically bound to cells, plastic, and collagen (see Materials and Methods: Saturation of Surface ACh Receptors with α-Bungarotoxin and Removal of Unbound Toxin), while the other two peaks are continually produced. The other two components maintain approximately their relative sizes. Most (≥70%) of the material is the low molecular weight component. The culture medium containing released radioactivity has often been analyzed on Bio-Gel P-2 columns which had been calibrated with BSA, ^{125}I, tyrosine, and [^{131}I]monoiodotyrosine. Generally, more than 70% of the radioactivity eluted in

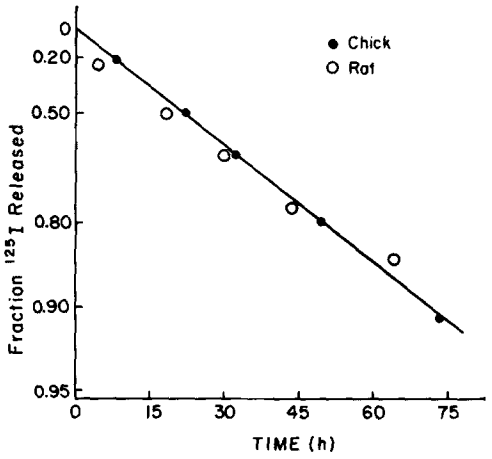

FIGURE 2 First-order kinetics of ACh receptor degradation. Eight 6-day-old cultures grown on 35-mm dishes, were labeled with [^{125}I]α-bungarotoxin, washed, and returned to 2 ml of F12 medium as described in Operational Definitions (under Degradation). All operations were carried out sterilely. At each of the indicated times, the entire 2 ml of F12 was removed from each dish and was replaced by an additional 2 ml of fresh F12 medium. After the medium was centrifuged to remove cellular debris, 1 ml of the supernate was counted and the other 1 ml was reserved for the experiment described in Fig. 3. After the medium had been removed for the last time point, the myotubes were extracted with 1% triton to determine the number of remaining [^{125}I]α-bungarotoxin-ACh-receptor complexes. Rat, —O—; chick —●—. Standard deviations for chick data are between ±0.01 and ±0.006.

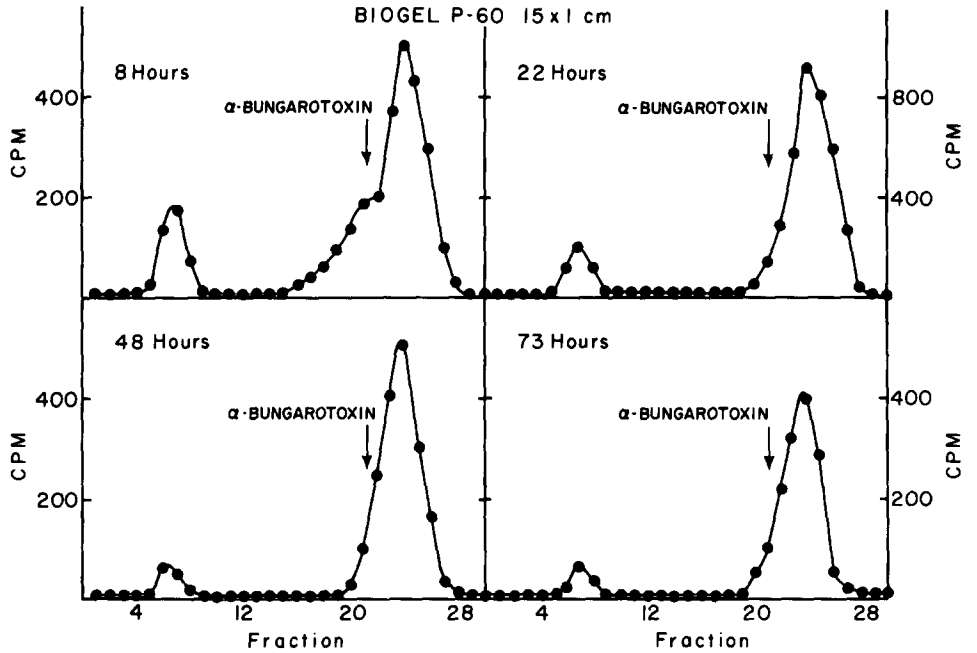

FIGURE 3 Bio-Gel P-60 chromatography of the degraded [^{125}I]α-bungarotoxin. F12 medium was collected during each of the intervals: 0–8 h, 8–22 h, 22–48 h, 48–73 h after chick muscle ACh receptors were labeled with [^{125}I]α-bungarotoxin (see legend to Fig. 2). Each 1-ml sample was layered on a 15 × 1-cm Bio-Gel P-60 column. The elution buffer was 1% Triton X-100, 100 mm Tris-HCl pH 7.8. 0.5-ml fractions were collected at a flow rate of 6 ml/h. Fractions were collected directly into scintillation vials and counted after the addition of 3.5 ml Triton-toluene fluor. Recovery of radioactivity from such columns was always quantitative.

the position of monoiodotyrosine. The elution profile from a preparative run is shown in Fig. 4, and the details of a large scale sample preparation are given in the legend to Fig. 4. Chromatographic profiles of samples of culture medium are quantitatively similar except that the excluded peak is slightly larger. The breakdown product was chromatographed on silica gel thin-layer plates (Eastman Kodak Co., Rochester, N. Y.) with *n*-butanol:acetic acid:H_2O 4:1:1, as solvent and visualized by autoradiography. The low molecular weight component co-chromatographs with both synthetic iodotyrosine and with the labeled moiety of pronase-digested [^{125}I]α-bungarotoxin (R_f = 0.53). The high molecular weight radioactive moiety released into the medium has been only partially characterized. After centrifugation of medium containing this component for 2 h at 100,000 *g*, the size of the excluded peak on subsequent Bio-Gel P-60 chromatography was reduced by about 98% compared to a portion which was not centrifuged. There was no change in the size of the other peaks.

As exemplified in Fig. 2, no lag is evident before the onset of release of radioactivity into the culture medium after [^{125}I]α-bungarotoxin has specifically bound to surface receptors. However, in such experiments the cells were exposed to [^{125}I]α-bungarotoxin 1 h before T = 0. The kinetics of release of radioactivity were examined on a much finer time-scale in order to determine if such a lag exists. Advantage was taken of the fact that degradation is random (first-order kinetics) and thus the rate constant does not depend upon the fraction of receptors existing as toxin-receptor complexes. Thus, cells could be exposed to [^{125}I]α-bungarotoxin for a brief period, rinsed in warm medium to remove unbound [^{125}I]α-bungarotoxin, and then the released radioactivity could be collected at various time intervals thereafter and the material analyzed. Data from one such experiment are presented in Fig. 5, and the experiment is detailed in the legend to Fig. 5. The release of iodotyrosine accelerates to the maximal rate over a period of 60–90 min and extrapolation back from long time points intercepts zero on the ordinate at 40–50 min in three separate experiments. The inhibition of iodotyrosine release by 2,4-dinitro-

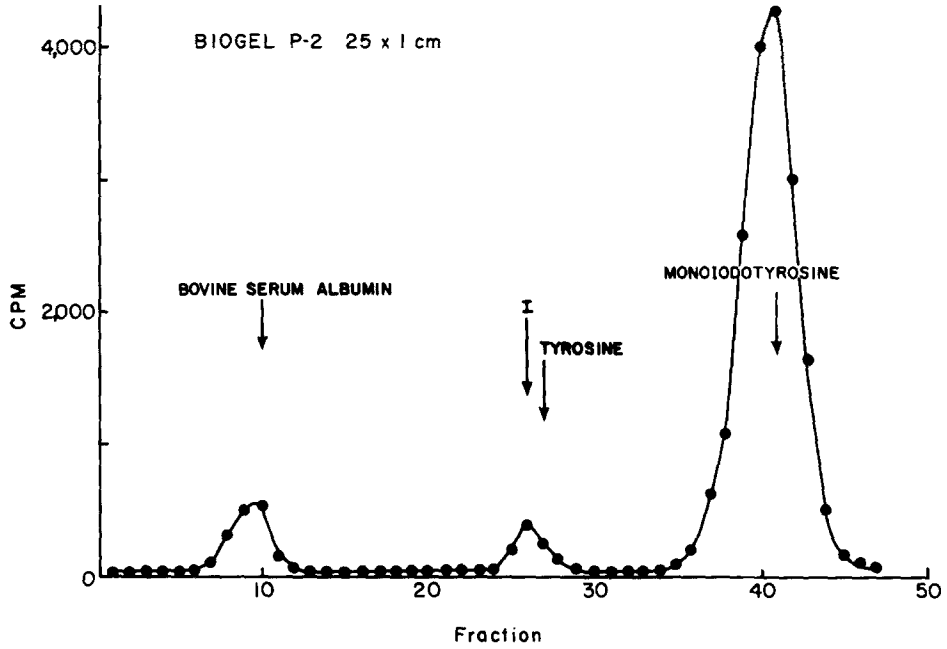

FIGURE 4 Chromatography on Bio-Gel P-2 of radioactive material released from chick myotubes after [^{125}I]α-bungarotoxin was bound to ACh receptors. 15 chick muscle cultures were plated at 10^6 cells/100-mm dish and cultured in complete medium for 5 days. After incubation for 30 min with 0.1 μg [^{125}I]α-bungarotoxin/ml and extensive rinsing to remove unbound toxin, the cultures were incubated at 37°C for 16 h in HEPES-buffered, balanced salt solution. The medium was removed, centrifuged at low speed to sediment cell debris, and the supernate was lyophilized, dissolved in 4 ml of distilled water, applied to a Bio-Gel P-2 column 1.0 × 30 cm and eluted with 0.05 M ammonium acetate, pH 5.0. The column was calibrated with markers as indicated. 1-ml fractions were collected. Recovery of radioactivity from such columns was between 65 and 70%.

phenol (DNP), also exemplified in Fig. 5, is discussed below.

Evidence that [^{125}I]α-bungarotoxin Degradation is a Measure of ACh Receptor Degradation

The experiments described immediately below demonstrate the identity between release of radioactivity from myotubes after [^{125}I]α-bungarotoxin binding and the degradation or inactivation of ACh receptors and show that α-bungarotoxin does not markedly affect the rate of receptor degradation. In our culture system, incorporation of new receptors into the surface stops 2–3 h after the inhibition of protein synthesis[1] by puromycin (Fig. 6). Although puromycin does cause slight inhibition of degradation from the outset (see Table V), degradation continues independently for at least 6 h after incorporation has stopped. To test whether receptors were inactivated at the same rate at which bungarotoxin was degraded, we examined the time period from 3 h to 8 h in puromycin, where the rate of incorporation of new receptors (measured as described in Operational Definitions) is zero. We measured the rate of degradation (i.e., the loss of radioactivity from myotubes after [^{125}I]α-bungarotoxin binding) and, in cultures which were not pretreated with [^{125}I]α-bungarotoxin, the rate at which the number of surface receptors decreased. Since we know that there is no incorporation during this period, the rate of decrease in the total number of surface receptors should be equal to the rate of degradation (provided the α-bungarotoxin does not alter the rate of degradation). The legend to Fig. 7 describes the experimental procedures in more detail. The slope of the degradation curve in Fig. 7 is −810 cpm/h and

[1] The effect of preincubation with 100 μg/ml puromycin on the incorporation of [^{3}H]leucine into hot TCA-precipitable material during a 10-min pulse was investigated. 98% inhibition of incorporation was observed if length of preincubation was greater than one minute.

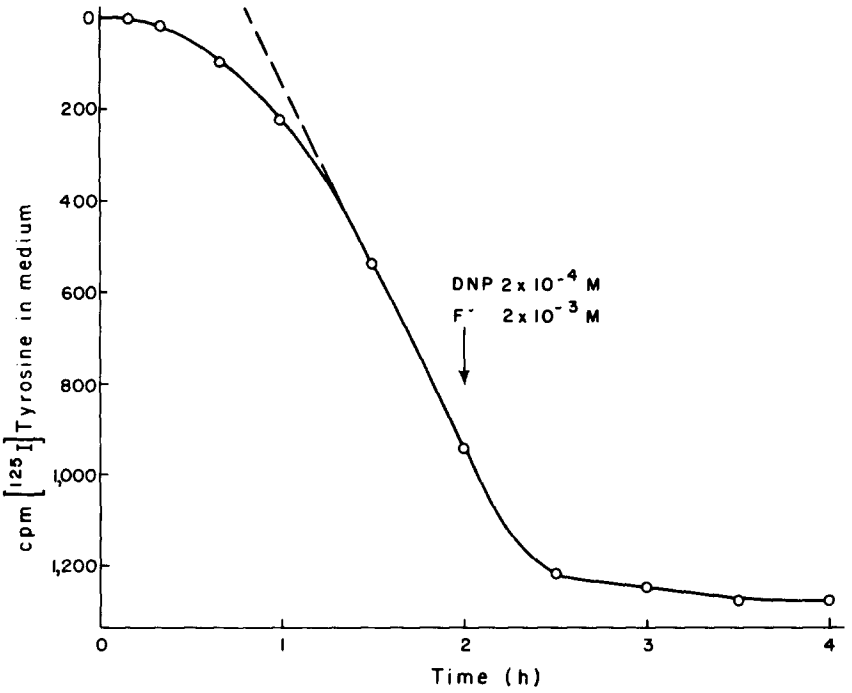

FIGURE 5 Time-course of [^{125}I]tyrosine release into the medium after brief exposure of myotubes to [^{125}I]α-bungarotoxin. Large plates (100 mm diameter) of 5-day-old chick muscle cultures were transferred to HEPES-buffered medium and incubated for several hours in a 37°C room. Then (T = 0) warm medium containing 0.5 μg/ml [^{125}I]α-bungarotoxin was placed on the cells for 5 min. The unbound [^{125}I]α-bungarotoxin was removed by six 30-s rinses with warm medium. The medium was then replaced at the indicated times and the used medium (3 ml/dish) was centrifuged briefly to remove cell debris, and then carrier iodotyrosine was added and a portion was fractionated on Bio-Gel P-2 and the fractions were assayed for radioactivity by scintillation spectrometry. The radioactivity in the iodotyrosine peak was calculated and the cumulative total iodotyrosine released was plotted as a function of time. After the maximal rate of iodotyrosine release was obtained (as judged from other experiments of this type), medium containing DNP and fluoride replaced the normal medium. Time zero is the time of initial contact of [^{125}I]α-bungarotoxin and myotubes.

the slope of the net decrease curve is −847 cpm/h. The differences in intercepts and slopes of the two curves are not significant, and the rates are identical within experimental error. It is also clear that under these conditions bungarotoxin neither stabilizes receptors nor accelerates their degradation. If it had, the degradation rate, as measured by release of radioactivity, would have been less (stabilization) or greater (acceleration) than the net decrease in number of receptors.

α-bungarotoxin-receptor complexes are not degraded by a mechanism which degrades the bungarotoxin and reactivates receptors. Thus, after 3 h in the presence of puromycin, the incorporation rate is zero, although toxin degradation and receptor disappearance continue.

These observations establish the identity between release of radioactivity into the medium and loss of α-bungarotoxin binding sites from the surface membrane in the presence of puromycin. That is, the appearance of a molecule of iodotyrosine in the medium represents the removal of an α-bungarotoxin binding site from the membrane. It is conceivable that in other conditions the [^{125}I]α-bungarotoxin associated with ACh receptors might be degraded to yield [^{125}I]tyrosine while the receptors were spared and became α-bungarotoxin binding sites again. In this case, one must imagine that puromycin couples the α-bungarotoxin degradation and receptor degradation processes. Unless puromycin does have such an effect, the identity between release of radioactivity and loss of α-bungarotoxin binding sites holds for various experimental conditions. In presenting

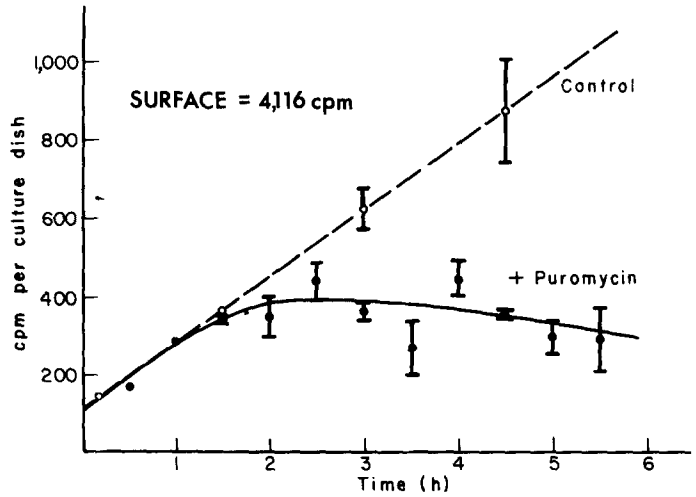

FIGURE 6 Inhibition of incorporation by puromycin. Two large sets of cultures were prepared for incorporation measurements as described in Operational Definitions. One set was incubated in F11 medium and the other in F11 medium containing 20 μg puromycin/ml. Before each of the indicated time points, five cultures from each set were incubated with [^{125}I]α-bungarotoxin for 30 min to saturate the new ACh receptors. Each point is the mean value for five cultures. Control (—O—); 20 μg/ml puromycin (—●—). Error bars indicate standard deviation from the mean. This experiment was repeated twice.

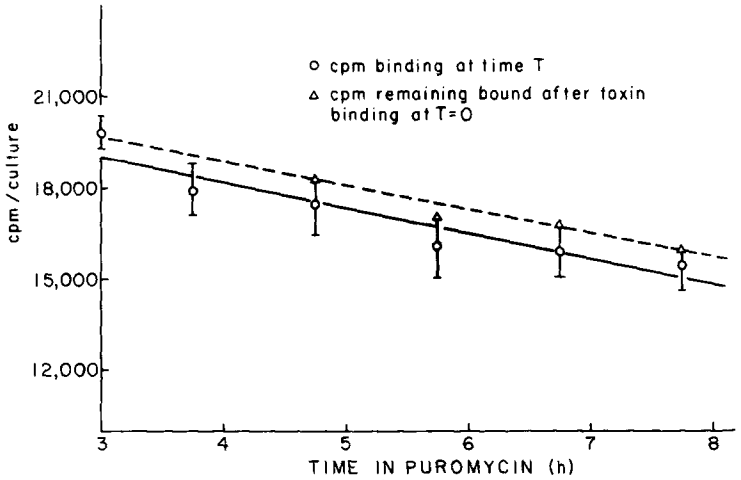

FIGURE 7 Equivalence of degradation and loss of receptors. Two sets (30 cultures and 24 cultures) of identical 6-day-old cultures, grown on 35-mm dishes, were pretreated with 20 μg/ml puromycin for 3 h. One set of 30 cultures (—Δ—) was saturated with [^{125}I]α-bungarotoxin for the last 30 min of the 3-h pretreatment. All of the cultures were then washed in tricine-buffered Hanks-BSA medium. The cultures were then returned to the incubator in F12 medium containing 20 μg/ml puromycin, except for six labeled cultures which were immediately extracted as the 3-h time point. Before each of the indicated time points, six cultures from the set which had not been initially saturated with [^{125}I]α-bungarotoxin (—O—) were labeled for 30 min. At each of the time points, these six cultures and six from the set which had been initially labeled were washed and then extracted and counted. The lines drawn were fit to the data by least-squares linear regression; the error bars indicate standard deviation from the mean. (Error bars are omitted from the degradation curve, for ease of illustration.)

further observations on [^{125}I]α-bungarotoxin degradation and the appearance of radioactivity in the medium, we will refer to this process simply as "degradation" both to indicate that it is actually the breakdown of [^{125}I]α-bungarotoxin associated with ACh receptors which is being studied and to

indicate that the concurrent breakdown of ACh receptors is being hypothesized.

TABLE I

Effect of DNP and Fluoride on ACh Receptor Degradation

Additives	Medium	% of control rate (mean ± SEM)	n*
2×10^{-4} M DNP, 10^{-3} M F^-	F11	45 ± 2	12
2×10^{-4} M DNP, 10^{-3} M F^-	UnF11	16 ± 2	12
5×10^{-4} M DNP	UnF11	6 ± 1	8
10^{-3} M F^-	UnF11	104 ± 2	4
10^{-2} M F^-	UnF11	63 ± 13	4

Chick muscle cultures were plated at 1×10^5 cells/35-mm dish and cultured in F12 medium at 36°C for 5 or 6 days. Degradation was measured as the release of radioactivity into the medium after [^{125}I]α-bungarotoxin was bound to ACh receptors. Degradation was measured over a 6- or 8-h period at 37°C. In each experiment, four or five cultures were used as controls for four others containing the additives.

* Number of cultures tested.

Factors Affecting Degradation

We have examined the effects of a variety of medium additives and experimental conditions on the rate of degradation. Many of these conditions are tabulated in Tables I-V. It must be borne in mind that many of the inhibitors we use may have side effects which influence degradation. What is perhaps most revealing is that few of these substances have major effects on degradation.

(*a*) Degradation is sensitive to interference with ATP synthesis (Table I). Inhibition of degradation by DNP is consistently greater than 50% in F12 or F11, and in our simpler medium, UnF11, greater than 80%. The onset of inhibition, illustrated in Fig. 5, occurs in about 30 min at 37°C. The effect of cyanide is similar to that of DNP. Interestingly, fluoride is a poor inhibitor even at 10^{-2} M, although at this concentration it produces obvious adverse effects on the morphology of myotubes.

(*b*) Degradation is greatly diminished by cell disruption. Experiments with crude membrane preparations and cell homogenates are described in Table II. The ability of these preparations to degrade bungarotoxin-receptor complexes to pro-

TABLE II

Degradation of α-Bungarotoxin-Receptor Complexes in Cell Homogenates and Isolated Membranes

Method of preparation	Incubation conditions	Iodotyrosine produced	I-tyrosine production rate constant
		%	h^{-1}
(1)* Homogenization in 300 mM sucrose, 5 mM KCN, pH 7.4			
(*a*) Supernate from 1,000 *g*, 10-min centrifugation	300 mM sucrose, 5 mM KCN, pH 7.4, 37°C 24 hr	0	0
(*b*) Same as 1 *a* except particulates were washed three times in F11 by centrifugation at 50,000 *g*, 15 min	F11 plus 5 mM ATP	<0.6	<0.0008
(2)‡ Nitrogen cavitation 800 psi in 250 mM sucrose, 2 mM $MgSO_4$, 5 mM Tris pH 7.4, 2 mM NaN_3. Pellet from 20,000 *g*, 15 min centrifugation after addition of 1 mM EDTA	F11 37°C, 4½ h 37°C, 16 h	0 <1	0 <0.0005
(3)* Homogenization in F11 (Dounce homogenizer, "A" Pestle)	37°C, 7 h	1.4	0.002

In (1), membranes were prepared according to the method of Reporter and Raveed (33). In (2), membranes were prepared as described by Wallach and Kamat (36).

* Before homogenization, chick muscle cultures were incubated with [^{125}I]bungarotoxin and washed to remove unbound [^{125}I]bungarotoxin.

‡ The vesicles produced by nitrogen cavitation were labeled with [^{125}I]α-bungarotoxin in suspension, and unbound bungarotoxin was removed by repeated centrifugation.

In every case the entire incubation medium was made 1% in Triton X-100 and analyzed for iodotyrosine by Bio-Gel P-60 chromatography. Long columns were used to achieve complete separation of [^{125}I]α-bungarotoxin and [^{125}I]tyrosine. % I-tyrosine was calculated as the proportion of radioactivity eluting in the included fractions.

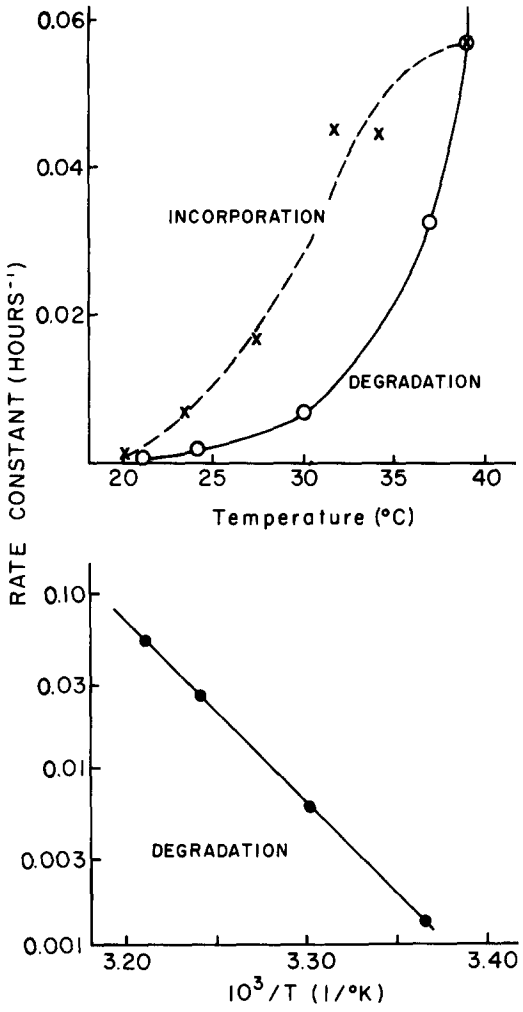

FIGURE 8 Temperature dependence of incorporation and degradation. Degradation curve: A large set of cultures (25) was saturated with [^{125}I]α-bungarotoxin, and unbound [^{125}I]α-bungarotoxin was removed. Five cultures were then incubated in 2 ml of F11 at each of the indicated temperatures for 6 h. Cultures were then assayed and the rate constant for degradation at each temperature was calculated as described in Operational Definitions (under Degradation). Cultures were maintained at these given temperatures by floating the culture dishes in water baths. Temperatures reported are those of the water bath but agreed well with measurements made by a probe immersed directly in the culture medium (—O—). Incorporation curve: A large set of cultures (24) was saturated with unlabeled bungarotoxin, and the unbound bungarotoxin was removed. Four cultures were then incubated at each of the indicated temperatures in 2 ml of F11 for 8 h. All of the cultures were then equilibrated at 22°C and saturated with [^{125}I]α-bungarotoxin for 45 min, and the incorporation (cpm/h) was divided by the total number of surface sites (cpm). The ordinate in this case is fraction surface sites per hour (- - × - -). Since accumulation was found to be zero at about 39–40°C, data on incorporation were normalized so that the value at 39°C equaled that of degradation, in order that incorporation and degradation curves determined on separate culture sets at different times could be compared. In the lower figure, degradation is plotted as log rate constant vs. 1/Temperature. The slope of the straight line through these points is −10,800° which corresponds to an activation energy of 50.3 kcal (—●—). The experiment was repeated two times.

duce iodotyrosine is small. Thus, it appears that intact cell structure is required for degradation.

(*c*) Degradation is a strong function of temperature. In Fig. 8 the temperature dependence of the rate of degradation is shown. The logarithm of degradation rate is a linear function of temperature (Fig. 8), and the Q_{10} of the process is about eight.

(*d*) Degradation is resistant to several protease inhibitors we have tested, the majority of which are probably excluded from cells (Table III). The single exception was Trypan Blue, a dye which has been shown in other systems to be internalized by cells and to inhibit the action of lysosomal enzymes (17). Inhibition of degradation was largest when cells were pre-incubated with the dye.

(*e*) The rate of degradation is an inverse function of the concentration of extracellular protein (Table IV). In serum-free medium containing about 5% BSA the rate of degradation is only half that in protein-free medium. It should be noted that protein-free medium will not sustain cultured cells for periods of time even as short as 1 day, so the high rate of degradation observed in this condition cannot be considered within the normal range. BSA extracted with chloroform-methanol and denatured in 8 M urea is as effective as native BSA.

(*f*) Degradation rate is optimum at about pH 7 and drops off steeply on the acid side and slowly on the basic side of this optimum (Table III).

(*g*) Conditioned medium has no capacity to degrade [^{125}I]α-bungarotoxin. F12 medium was conditioned by 48-h exposure to chick myogenic cell cultures 5–7 days old. [^{125}I]α-bungarotoxin (0.2 μg/ml) was added to the medium and the mixture was incubated for 55 h at 37°C. Then [^{131}I]α-bungarotoxin was added as a marker, and the medium was analyzed by chromatography on

TABLE III

Effects of Inhibitors of Proteolytic Enzymes and of pH on ACh Receptor Degradation

Treatment	% of control rate (mean ± SEM)
Phenyl methane sulfonyl fluoride	102 ± 1
N-α-tosyl-L-phenylalanyl-chloromethane (sat. solution)	103 ± 2
N-α-tosyl-L-arginine-methylester 4×10^{-4} M	99 ± 5
Soybean trypsin inhibitor 400 μg/ml	103 ± 1
Pepstatin 10^{-5} M	90 ± 3
Trypan blue 4.2×10^{-4} M	
Given at T = 0	59 ± 3
Given 16 h before T = 0	26 ± 3
pH 5.0	35 ± 1
pH 6.0	54 ± 2
pH 7.2	100 ± 2
pH 8.0	90 ± 3
pH 9.0	78 ± 1

Chick muscle cultures were plated at 1×10^5 cells/35-mm dish and cultured in F12 medium for 5 or 6 days at 36°C. Degradation was measured as the release of radioactivity into the medium after [^{125}I]α-bungarotoxin was bound to ACh receptors. Degradation was measured over a 6- or 8-h period at 37°C in UnF11 medium. In each experiment four or five cultures were used as controls and four others contained the additive. The pepstatin experiments were done at pH 5, 6, and 7 against controls at these same pH's, since pepstatin binding to cathepsins is enhanced at lower pH. The pH of UnF11 was titrated to 5.0 or 6.0 with HCl, and to 8.0 and 9.0 with NaOH. Quench corrections were made for counting radioactivity by scintillation spectrometry in the presence of Trypan blue.

TABLE IV

Effects of BSA on ACh Receptor Degradation

Concentration of BSA in the medium	Rate of degradation (mean ± SEM)
(*g/100 ml*)	h^{-1}
0	0.045 ± 0.002*
0.1	0.047 ± 0.004*
0.3	0.044 ± 0.004*
0.5	0.037 ± 0.001*
1.0	0.045 ± 0.005*
1.5	0.034 ± 0.001*
2.0	0.033 ± 0.003*
3.0	0.031 ± 0.002*
5.0	0.027 ± 0.002*
0	0.052 ± 0.002‡
2.0	0.033 ± 0.001‡
2.0 (lipid extracted, urea denatured)§	0.031 ± 0.002‡

Chick muscle cultures were plated at 1×10^5 cells/35-mm dish and cultured in F12 medium at 36°C for 5 or 6 days. Degradation was measured as the release of radioactivity into the medium after [^{125}I]α-bungarotoxin was bound to ACh receptors. Degradation was measured over a 6-h period at 37°C. BSA was dissolved in UnF11 medium lacking BSA.

* $n = 4$.

‡ $n = 5$.

§ BSA was stirred overnight in chloroform:methanol (3:1) to extract any associated lipids, then denatured in 8 M urea. After dialysis and lyophilization, the "denatured" BSA was dissolved in UnF11 lacking BSA. This solution was slightly turbid, in contrast to solutions of native BSA.

long Bio-Gel P-60 columns where native [^{125}I]α-bungarotoxin and [^{125}I]tyrosine were completely resolved. The ^{131}I and ^{125}I chromatographic profiles were identical, indicating that no breakdown ($<<1\%$) of [^{125}I]α-bungarotoxin had occurred.

(*h*) The effects of a number of other compounds on ACh receptor degradation are listed in Table V. These include inhibitors of protein synthesis, potential inhibitors of endocytotic processes, choline, *d*-Tubocurarine, and a concentration of Triton X-100 which causes desensitization of myotubes to ACh (10).

In the discussion we will consider the question of whether degradation might involve internalization followed by proteolytic digestion and diffusion of digestion products out into the medium. Such a model predicts that cells must be permeable to iodotyrosine. To establish that cells are permeable to iodotyrosine, myotubes were removed from petri plates by minimal trypsinization, washed in complete medium to remove trypsin, and suspended in medium containing [^{125}I]tyrosine (1 μM) and [^{3}H]inulin. After a few minutes of incubation the suspension was centrifuged to pellet the myotubes, and the supernate was removed. The ratios of ^{125}I to ^{3}H in the supernate and the pellet were assayed. Normalizing the ^{125}I to ^{3}H ratio in the supernate to 1.0, the normalized ratios in the pellets in three experiments were 1.3, 2.0, and 1.4. Such pellets of myotubes contain almost equal volumes of extracellular and intracellular water.[2] Thus, a ratio of 2.0 would indicate complete

[2] Aileen Ritchie, personal communication.

TABLE V

Effects of Miscellaneous Agents on ACh Receptor Degradation

Additive	Medium	% of control rate (mean ± SEM)	n*
Cycloheximide 100 μg/ml	F11	80 ± 4	18
Puromycin 50 or 100 μg/ml	UnF11	83 ± 2	24
p-fluorophenylalanine 0.1–1.0 mg/ml	UnF11	101 ± 4	12
Colchicine 100 μg/ml	F11 and UnF11	73 ± 2	20
Cytochalasin B 2.5 μg/ml in 1% DMSO	UnF11	85 ± 1	6
Choline 5×10^{-3} M	UnF11	119 ± 13	4
d-Tubocurarine	UnF11	103 ± 1	8
Triton X-100 0.01% (wt/vol)	F11	104 ± 6	4

Experiments were performed as described in Materials and Methods and the legend of Table I.
* Number of cultures tested.

equilibration of iodotyrosine with intracellular water while a ratio of 1.0 would indicate that both isotopes were confined to extracellular space.

Very little iodotyrosine is still associated with cells which had been degrading bound [^{125}I]α-bungarotoxin for 6 h. The radioactivity remaining bound to such myotubes was extracted with 1% Triton X-100, chromatographed on Bio-Gel P-60, and found to be 99% bungarotoxin-receptor complexes and 1% iodotyrosine. To test for incorporation of iodotyrosine into cell proteins, we incubated cells in medium containing 2,500,000 cpm (approximately 0.5 μM) [^{125}I]tyrosine for 2 h at 37°C and then measured the radioactivity precipitable by 10% TCA. There was 500 cpm in the TCA precipitate. Thus, the degradation product, iodotyrosine, does not accumulate to any significant extent inside myotubes, and its incorporation into proteins is negligible or nonexistent.

Degradation is not Tightly Coupled to Synthesis of Incorporation

The first example of uncoupled incorporation and degradation has already been mentioned in reference to Fig. 6. After 3 h in puromycin, incorporation is completely blocked. Degradation continues at a constant rate although from the outset (time of addition of puromycin) it shows slight inhibition (17%) (Table V). In one experiment degradation continued at a constant rate for 16 h in the presence of 10 μg/ml puromycin.

Another condition whereby one can demonstrate that incorporation and degradation are not tightly coupled is temperature alteration. This becomes obvious when, by referring to Fig. 8, one mentally constructs a difference curve between incorporation and degradation rates. This difference, the net rate of receptor accumulation, is optimum at some temperature and lower on either side of that temperature. We have also directly measured the accumulation rate (see Operational Definitions) as a function of temperature over an 8-h period. Fig. 9 shows that there is an optimum temperature range around 35°C for accumulation of receptors on myotube surfaces. At lower temperatures, incorporation drops off more steeply than degradation, and at higher temperatures degradation increases much more rapidly than does incorporation. Through nearly the entire range studied, incorporation was greater than degradation, so that there was a net increase in ACh receptors with time.

Degradation Rate as a Function of Developmental Stage

In myogenic cell cultures the rate of accumulation of ACh receptors is rapid for several days after the onset of fusion (Fig. 10), but in older cultures the number of ACh receptors ceases to increase rapidly and may even decrease. Surprisingly, the rate constant for degradation of ACh receptors does not change with the age of the myotubes (Table VI). Whatever the mechanisms are by which the density of ACh receptors in the plasma membranes is regulated (see Discussion), it does not appear that modulation of the degradative pathway is involved.

Accumulation Equals Incorporation Minus Degradation

The data in Figs. 8 and 9 suggest that the rate of accumulation of receptors (Fig. 10) is simply the

TABLE VI

Rate of ACh Receptor Degradation as a Function of Culture Age

Age of Culture	Rate of degradation (mean ± SEM)	Half-life	n*
(*days*)	h^{-1}	(*h*)	
3	0.039 ± 0.002	16	4
4	0.023 ± 0.001	28	5
5	0.023 ± 0.001	28	4
6	0.035 ± 0.001	18	10
7	0.034 ± 0.001	18	7
8	0.032 ± 0.001	19	4
9	0.028 ± 0.001	22	4
10	0.030 ± 0.001	21	4
13	0.023 ± 0.001	28	4
22	0.026 ± 0.002	24	4

Chick muscle cultures were plated at 1 × 10^5 cells/35-mm dish and cultured in F12 at 36°C. Degradation was measured as the fraction of radioactivity released into the medium per h after [^{125}I]α-bungarotoxin was bound to ACh receptors in chick muscle cultures. Degradation was measured over a 6- or 8-h period at 37°C in F11 medium. Half-lives were determined graphically by extrapolation. Mean half-life for all data was 22 h.

* Number of cultures tested.

result of the difference between the rates of incorporation and degradation. If our operational definitions of degradation, incorporation, and accumulation are valid, then this relationship should hold quantitatively. In Table VII we have tabu-

TABLE VII

Comparison of Rate of Accumulation, Incorporation, and Degradation

Age	Incorporation	Degradation	Accumulation	1-D*
(*days*)	*% change in total receptor number per h*			
3–5	5.1 ± 0.9	2.8 ± 0.2	2.1 ± 0.2	2.3
	(3.6–6.6)	(2.3–3.9)	(1.72–2.65)	
5–7	4.3 ± 0.2	3.1 ± 0.1	1.12 ± 0.1	1.2
	(3.8–5.0)	(2.3–3.5)	(0.94–1.26)	

Incorporation and degradation measurements were carried out as described in Operational Definitions. Each value listed is the mean and SE of at least three such measurements from independently initiated culture sets. The lowest and highest values are listed in brackets. Accumulation was measured as described in Operational Definitions. Rates of accumulation were calculated for 4- and 6-day cultures from three curves such as that shown in Fig. 10.

* Incorporation rate minus degradation rate.

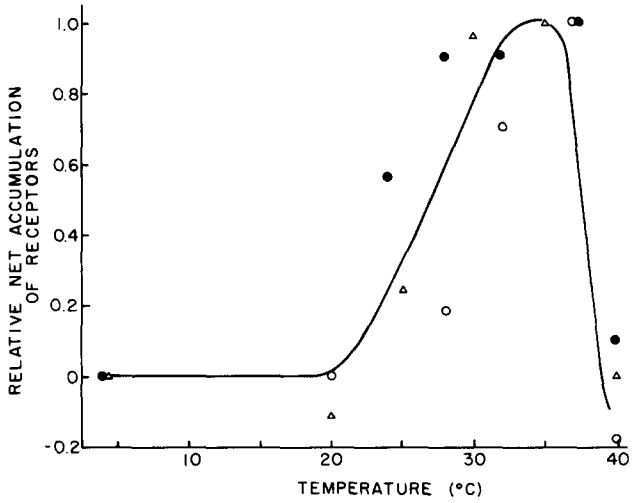

FIGURE 9 Relative rate of ACh receptor accumulation in chick muscle cultures at different temperatures. Chick muscle cultures plated at 1 × 10^5 cells/35-mm dish were grown for 5 days in F12 medium at 36°C. Then groups of five cultures were incubated in F11 medium at each of the indicated temperatures for 6 h by floating the culture dishes in covered waterbaths. Cultures were subsequently incubated at 20°C with [^{125}I]α-bungarotoxin for 45 min, unbound toxin was removed, and the toxin-receptor complexes were extracted and counted by scintillation spectrometry. Data from three experiments are depicted with different symbols. Data from each experiment were normalized so that the largest accumulation in each experiment was equal to 1.0. Accumulation at the lowest temperature was equal to zero. The solid line is the difference curve between incorporation and degradation curves of Fig. 8.

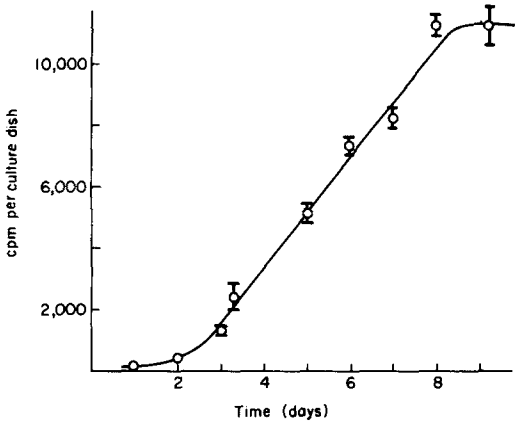

FIGURE 10 Accumulation of ACh receptors as a function of culture age. A large set of chick muscle cultures, prepared by mechanical disruption, was plated at 5×10^6 on 35-mm petri dishes and cultured in F12 at 36°C. At each of the indicated times, four of the cultures were assayed for surface ACh receptors as described in Operational Definitions (under Accumulation). Error bars indicate standard deviation from the mean. The experiment was repeated three times.

lated our data on rates of incorporation and degradation (taken from experiments on cultures from 3 to 7 days after plating) and rates of accumulation (taken from three experiments similar to that of Fig. 10). These data show that within experimental error the rate of accumulation is the difference between the rates of incorporation and degradation.

Characterization of an ACh Receptor "Pool"

The kinetics of inhibition of new receptor incorporation into chick myotube plasma membranes by cycloheximide[3] are shown in Fig. 11. (Similar data for inhibition by puromycin are shown in Fig. 6).[4] The absence of inhibition during the first 2 h suggests that there exist presynthesized binding sites not readily accessible to extracellular labeling by α-bungarotoxin. Additional α-bungarotoxin binding sites have been found in myotube homogenates, and the number of these binding sites decreased, during exposure to cycloheximide, coordinately with an equal increase of new binding sites on the surface (12).

When all the surface α-bungarotoxin binding sites are first saturated with unlabeled α-bungarotoxin and then a 1% Triton X-100 extract of the myotubes is prepared, a new set of α-bungarotoxin binding sites can be detected. This new set of binding sites, labeled in solution with [^{125}I]α-bungarotoxin and freed of unbound toxin by chromatography on Bio-Gel P-60, has been examined by several physical criteria, using [^{131}I]α-bungarotoxin-surface receptor complexes as an internal marker. During velocity sedimentation in sucrose gradients, the majority of the new sites are indistinguishable from surface receptors. A 5%–20% linear sucrose gradient centrifugation experiment is shown in Fig. 12. Both types of α-bungarotoxin binding sites have an S value of 10. Also shown is the distribution of cell protein. A

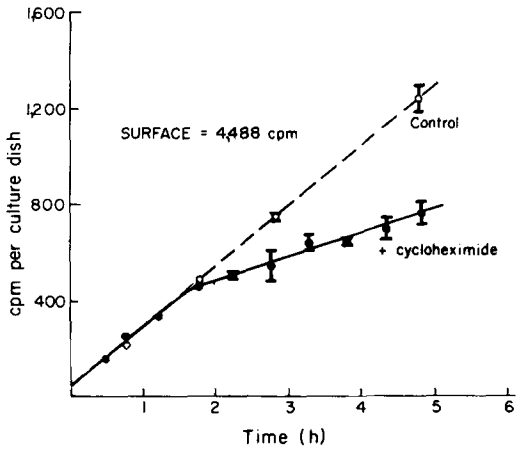

FIGURE 11 Inhibition of incorporation by cycloheximide. Two large sets of cultures were prepared for incorporation measurements as described in Operational Definitions (under Incorporation). One set (—○—) was incubated in F11 and the other (- -●- -) in F11 containing 100 μg/ml cycloheximide. Before each of the indicated time points, five cultures from each set were saturated with [^{125}I]α-bungarotoxin for 30 min to determine the number of new ACh receptors. Plotted are the means of values obtained from five cultures. Error bars indicate standard deviation from the mean. The experiment was repeated seven times.

[3] When 100 g/ml cycloheximide was added to chick muscle cultures in F12 medium at 37°C, the incorporation of [^{3}H]leucine into hot TCA-precipitable material was inhibited by 92%. This level of inhibition developed within 10 min of the addition of cycloheximide.

[4] We have observed that the point of inflection in the incorporation curve was insensitive to the choice of inhibitor. That is, the inflection was observed at about 2 h whether puromycin or cyclohexmide was used. However, as can be seen by comparison of Figs. 6 and 11, a fraction of the incorporation (about 20%) is insensitive to cycloheximide inhibition but is sensitive to inhibition by puromycin.

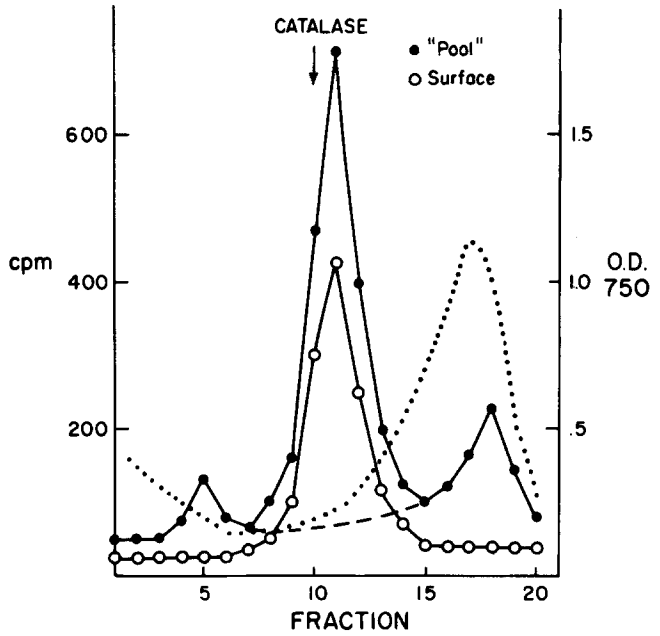

FIGURE 12 Velocity sedimentation comparison of surface and "pool" receptors. Approximately 10 large (100-mm) cultures were saturated with [^{131}I]α-bungarotoxin, and unbound bungarotoxin was removed. The cultures were then extracted into about 5 ml 1% Triton. The extract was centrifuged and the supernate was concentrated to about 1 ml by Diaflow ultrafiltration (filter size UM-10). The concentrated extract was then labeled for 1 h with 0.01 μg/ml [^{125}I]α-bungarotoxin, and free [^{125}I]α-bungarotoxin was removed by Bio-Gel P-60 chromatography. All of the ^{131}I-containing fractions (a single, excluded peak) from the column were pooled and a portion was taken for velocity sedimentation. 200 μl were layered in a thin band over a 5–20% linear sucrose gradient (5 ml) containing 1% Triton. Centrifugation was carried out in a Beckman SW-65 rotor at 62,000 rpm for four h (20°C). The protein distribution was determined on a separate run. ^{131}I (—O—); ^{125}I (—●—); Lowry protein analysis O.D. 750 (· · · ·). The experiment was repeated three times.

minor (20%) bungarotoxin binding component runs to the 4S region of the gradient (see Materials and Methods). As indicated by the gradient profile, binding of bungarotoxin to the 10S component is completely blocked by 10^{-3} M *d*-tubocurarine, while the formation of the 4S component is not. These *d*-tubocurarine-protectable binding sites of the "pool" are indistinguishable from surface receptors by electrophoresis in polyacrylamide gels and by column chromatography on Bio-Gel P-60 and P-300.

The number of specific α-bungarotoxin binding sites in this "pool" was titrated, using the filter assay (Fig. 13). Cultures were divided into two sets. One set was saturated with unlabeled bungarotoxin, and both sets were washed and extracted. The extracts were incubated with increasing concentrations of [^{125}I]α-bungarotoxin, and toxin-receptor complexes were assayed by the filter assay. The set of cultures which was not treated with unlabeled α-bungarotoxin yields the total number of surface receptors plus extra sites revealed by 1% Triton X-100 solubilization. The set in which the surface was saturated with unlabeled α-bungarotoxin before extraction yields the number of receptors that were not labeled externally. By subtraction, one obtains the number of surface receptors. By this analysis, Triton X-100 solubilization reveals a "pool" which contains 40% as many α-bungarotoxin binding sites as does the surface. From similar titration curves done on 1% Nonidet P-40 (Shell Chemicals, London) extracts of cultures, a "pool" size equal to 33% of the surface sites was found. We have determined the "pool" size in seven independently initiated sets of primary chick myogenic cultures at day 4 or 5 after plating at 1×10^6 cells/100 mm petri dish. These values range from 24% to 66% as many binding sites as were present on the cell surfaces, with a mean value of 44%.

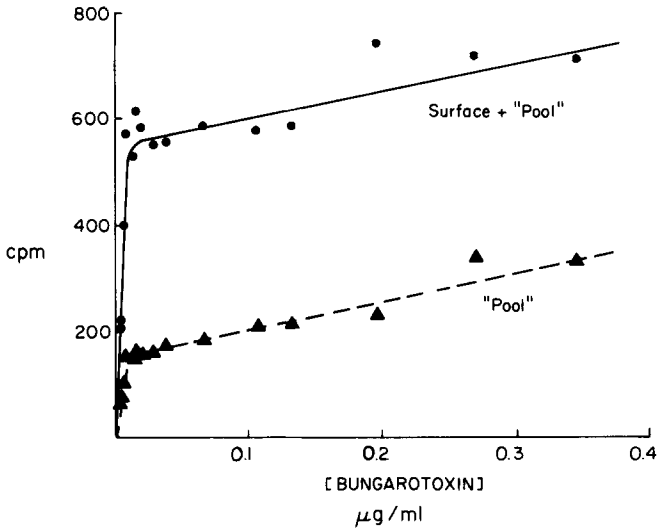

FIGURE 13 Pool size measurement. Saturation curves were done like that in Fig. 1. For one of the saturation curves, "Pool," the surface receptors were blocked with unlabeled bungarotoxin before the extract was made. For the other, Surface + "Pool," the cultures had no exposure to bungarotoxin before the extract was made. Surface + "Pool" (—●—); "Pool" (—▲—). The experiment was repeated two times.

"Hidden" and "Precursor" Components of the Pool

Incubation of myotubes in 0.1 μg/ml α-bungarotoxin for 30 min saturates the major population of surface ACh receptors and renders the myotubes physiologically insensitive to iontophoretically applied ACh (16). After such an incubation, the pool of α-bungarotoxin binding sites, as demonstrated above, contains about 40% as many sites as were originally saturated. However, if cultures are incubated overnight in medium containing α-bungarotoxin, the remaining pool of α-bungarotoxin binding sites contains only about 10% as many receptors as there were easily saturable surface receptors.

We have directly measured the size of the remaining pool as a fraction of the surface on three independent culture sets (twice by sucrose gradient centrifugation, once by filter assay). In these instances, the length of block by unlabeled bungarotoxin was 10 h, 16 h, or 3 days, and the pool sizes were 10%, 5.5%, 12.1%, respectively, with a mean of 9.2%. Despite the large reduction in pool size resulting from overnight incubation in α-bungarotoxin, the cells maintain the capacity to insert new ACh receptors into their surface membrane for 2–3 h in the presence of cycloheximide (Fig. 14). (We do not know the reason for the slightly smaller initial slope in this experiment compared to Fig. 11.) Thus, those ACh receptors in the "pool" which form complexes with α-bungarotoxin during overnight incubation are not precursors for surface receptors, and we term these sites "hidden."

The other set of α-bungarotoxin binding sites in the "pool" is approximately equal in number to

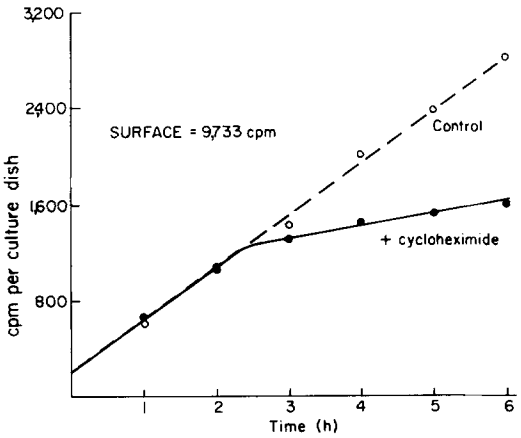

FIGURE 14 Cycloheximide inhibition of incorporation after overnight treatment with bungarotoxin. Experiment is identical in design to that in Fig. 11 except that the cultures were saturated with unlabeled bungarotoxin (0.2 μg/ml) overnight (12 h) instead of for 30 min. Standard deviations (not shown) are like those in Fig. 11. The experiment was repeated two times.

the sites which can appear on the surface in the presence of cycloheximide or puromycin. This observation suggested that the set of α-bungarotoxin binding sites which do not interact with α-bungarotoxin in the medium might be precursors to surface receptors. Therefore, we sought additional circumstantial evidence related to this possibility. We measured the kinetics of disappearance of these sites and the simultaneous appearance of new sites in the plasma membrane after addition of puromycin to the culture medium. As shown in Fig. 15, the pool size goes to zero as incorporation of new receptors into the surface membrane ceases. In myotubes saturated with α-bungarotoxin for 30 min instead of overnight, the total pool decreases after inhibition of protein synthesis, but this total pool has a much longer half-life. Thus there is still a large population of hidden sites after incorporation has ceased.

Properties of Hidden and Precursor Pools

Since it appeared that hidden sites were not the major precursor for surface sites, we wondered if surface sites could supply the hidden pool. Cultures in which surface sites were saturated with unlabeled α-bungarotoxin were incubated overnight in α-bungarotoxin-free medium. On the following day, the pool in these cultures bound 4,715 ± 71 cpm whereas the pool in unpretreated controls bound 8,133 ± 117 cpm [^{125}I]α-bungarotoxin. Therefore, a fraction of the hidden sites can be labeled with α-bungarotoxin via the surface sites. Continuous incubation with α-

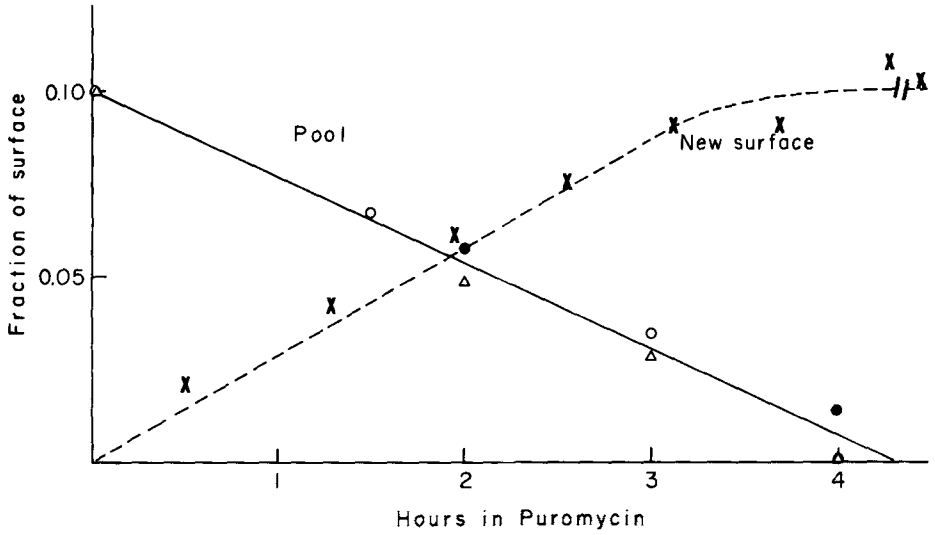

FIGURE 15 Kinetics of pool disappearance and new receptor appearance. Decrease of "precursor" in presence of puromycin. All cultures were incubated overnight with 0.5–1.0 μg/ml unlabeled α-bungarotoxin. The next day, sets of five to six cultures were pretreated with 20 μg/ml puromycin for the indicated times. All cultures were then washed to remove unbound α-bungarotoxin. In one experiment (—O—), sets were immediately extracted in 1% Triton X-100. In the other two experiments (—●—, —Δ—) the cells were scraped from the dish, homogenized by 25 strokes of a Dounce (pestle B) homogenizer (in 10 mM Tris at 0°C), and centrifuged at 50,000 *g* × 30′. The pellets were extracted with 1% Triton X-100, 10 mM Tris, pH 7.8. Each of the extracts was incubated with 0.01 μg/ml [^{125}I]α-bungarotoxin for 1 h at room temperature. The complexes formed were freed of unbound α-bungarotoxin by Bio-Gel P-60 chromatography. The fractions in the excluded peak were pooled, and a portion was taken for analysis by velocity sedimentation as described in the legend to Fig. 12. In all cases, the area under the 10S peak in the gradient was measured. For each experiment, all values were normalized such that the control value equals 0.10 of the surface (see Results). Incorporation was measured as described in Fig. 6 except that cells were first saturated with unlabeled α-bungarotoxin for about 15 h instead of 30 min. The actual plateau level reached in this experiment was about 8% of the total surface, but it has been normalized to 0.10 for comparison with the pool decrease. The long time point after the indicated break is at 6 h 20 min (--×--). Errors in this experiment are comparable to those in Figs. 6, 11, and 14.

bungarotoxin is required only to saturate the hidden sites.

The rate of degradation of surface α-bungarotoxin-receptor complexes was compared to the rate of degradation of hidden α-bungarotoxin-receptor complexes. Surface receptors were labeled by a 30-min exposure to [^{125}I]α-bungarotoxin whereas surface plus hidden sites were labeled by overnight exposure to [^{125}I]α-bungarotoxin. The ratio of surface plus hidden to surface sites in this experiment was 1.43. For each set, degradation was measured during a 48-h period, and the calculated rate constants were indistinguishable. The half-life of both sets of α-bungarotoxin complexes was 22 h. This experiment was sufficiently sensitive to detect a difference of 2 h between the half-lives of the surface and hidden sites.

If the "hidden" portion of the "pool" were actually on the plasma membrane, then it might be possible to expose these sites by treatments which do not rupture the cell membrane. In an effort to expose the hidden sites (make them readily available for α-bungarotoxin binding) we have unsuccessfully tried treating myotubes with 0.001% trypsin, 20 μg/ml collagenase, hypotonic shock (20 and 75 mosM), 0.01% Triton X-100, and 2 M NaCl.

All of the hidden and precursor sites in the pool are present on particulate matter larger than microsomes, as judged by sedimentation experiments with muscle homogenates. We do not know the subcellular localization of the pool components, but preliminary experiments indicate that the pool and surface receptors can be partially separated by subcellular fractionation.

DISCUSSION

Degradation of Surface Receptors

The release of [^{125}I]tyrosine into the culture medium after [^{125}I]α-bungarotoxin has specifically bound to ACh receptors represents an energy-requiring, proteolytic breakdown of the [^{125}I]α-bungarotoxin. The rate of release of radioactivity parallels the normal rate of loss of ACh receptors from the membrane. This identity is established in the experiment illustrated in Fig. 7. This experiment also shows that the formation of a bungarotoxin receptor complex does not markedly affect receptor degradation, for the slope of the "cpm remaining bound" line which represents the rate of loss of radioactivity is the same as the rate of loss of free receptors. The unaltered rate of receptor catabolism in the presence of α-bungarotoxin is in contrast to the effect of antibodies and lectins on catabolism of membrane antigens and glycoproteins. For example, in polymorphonuclear leukocytes, binding of concanavalin A and *Rincus communis* agglutinin induces the internalization of their respective lectin binding sites (28). In such cases, it appears that cross-linking of membrane proteins to form patches and the capping of these patches may lead to internalization. α-bungarotoxin is much smaller than antibodies and lectins, is monovalent, and does not cause patch formation (35).

An important constraint on our further studies of degradation is that we have only proved that degradation of [^{125}I]α-bungarotoxin *quantitatively* parallels the loss of ACh receptors in the one case: that is, in the absence of protein synthesis and incorporation of new ACh receptors into the surface membrane. One can argue that under normal conditions the degradation of [^{125}I]α-bungarotoxin which had been associated with ACh receptors might sometimes result in reactivation of the receptor but that puromycin somehow prevents this reactivation. This premise leads to complicated models of puromycin action and of receptor turnover which we feel are unlikely to be correct. Furthermore, we have shown that the difference between incorporation and degradation rates at various temperatures (Fig. 8) is consistent with data on accumulation of ACh receptors over this temperature range (Fig. 9), and we have shown (Table VII) that the large difference between incorporation and accumulation rates in cultures maintained under normal culture conditions is quantitatively accounted for by the rate of degradation. These data are not consistent with a model in which α-bungarotoxin selectively stimulates receptor degradation nor with a model in which α-bungarotoxin is degraded without concomitant degradation of receptors. All of our data on degradation are compatible with a model in which α-bungarotoxin-receptor complexes are internalized by the myotube, both toxin and receptor are proteolytically destroyed and the [^{125}I]tyrosine generated from proteolysis of [^{125}I]α-bungarotoxin diffuses out of the myotube into the culture medium. In subsequent discussion of degradation we adopt the hypothesis that release of iodotyrosine into the culture medium is a valid measure of receptor degradation.

The loss of radioactivity from muscle cultures following incubation with [^{125}I]α-bungarotoxin was found to be much greater than previously

reported from this laboratory (16); we have no explanation for this. It was also reported previously (16) that in one experiment incorporation and accumulation were similar in very young cultures. This similarity should hold only for the very initial phase of accumulation and does not hold (Table VII) for cultures during the major period of receptor accumulation.

The first-order kinetics of receptor degradation (Fig. 2) suggest that receptors are degraded randomly, "old" and "new" ACh receptors being degraded at the same rate. The half-life of receptors in the plasma membrane of either chick or rat myotubes under normal culture conditions is about 22–24 h (range 16–28 h with mean of 22 h for all data presented in Table VI). The random character of degradation suggests that the first step, which may be endocytosis, occurs all over the myotube surface, since receptors themselves are distributed in this way. However, it is possible that the degradation process involves specialized locations on the membrane (for example, special sites of endocytosis) and that some other mechanism, for example, translational mobility of receptors in a fluid plasma membrane (9), serves to randomize the process.

Several of our results, taken together, suggest that degradation involves internalization of α-bungarotoxin-receptor complexes. Isolated membranes and even cell homogenates have greatly reduced capacity to degrade their α-bungarotoxin-receptor complexes. The degradation process is energy-requiring, being inhibited by DNP and by cyanide. The temperature sensitivity of degradation may be another indication of this. The rate of release of degradation product, iodotyrosine, accelerates for more than 1 h at 37°C before reaching maximal rate. The absence of any such phenomenon would have been strong evidence against intermediate steps (such as internalization) in the degradation process. Finally, studies involving the protease inhibitors and pH changes are consistent with internalization before proteolysis. The only protease inhibitor which had a marked effect upon degradation rate was one which is thought to be taken up by cells and to inactivate lysosomal enzymes (17). These observations are consistent with internalization before proteolysis; they do not constitute proof. However, let us briefly examine the major alternative hypothesis, that degradation occurs extracellularly by cell-surface or secreted proteases. To maintain this hypothesis, one must suppose that extracellular proteolysis is mediated by energy-requiring proteases with neutral pH optima and with resistance to the protease inhibitors employed in this study. One must devise a scheme such that the degradation process requires a very long time to reach "steady-state" and toxin-receptor complexes are attacked in such a way that [^{125}I]α-bungarotoxin is digested to yield [^{125}I]tyrosine (involving, at a minimum, two endoproteolytic cleavages) before it is released from the degrading site. If secreted proteases are hypothesized, they must be short-lived, since conditioned medium does not have significant proteolytic activity against α-bungarotoxin. If cell-surface proteases are envisaged, then the energy requirement is unexpected. In sum, the hypothesis of extracellular proteolysis is tenable if the above assumptions are made.

Control of Receptor Metabolism

It has been reported that cultures of nongrowing L cells turn over membrane proteins rapidly whereas in growing L cells, membrane protein is very stable (37). The implication is that the point of control lies with degradation. In contrast, during the early stages after fusion of myoblasts into multinucleated myotubes, there is a rapid increase in myotube surface area and also an increase in density of ACh receptors (16), whereas at late stages in culture (the plateau region of curve in Fig. 10 or even later), the number of ACh receptors remains constant or even decreases and the surface area of the myotubes apparently does not change. Yet, the rate constant for degradation is always about 0.03 h^{-1} (Table VI). Therefore, the adjustment in rates of incorporation and degradation cannot be due to an increase in the rate constant for degradation. Either the rate of incorporation slows down or the rate of degradation (i.e., total sites degraded per unit time, *not the rate constant*) reaches a level equal to that of incorporation. These possibilities will be investigated.

Several studies have reported that synthesis of membrane proteins is coupled to their degradation (14, 37). That is, inhibition of protein synthesis or amino acid starvation not only stops synthesis but also degradation. Mechanisms of coupling for membrane biosynthesis and degradation might involve maintenance of a constant or steadily increasing surface area. Our data show that inhibition of receptor biosynthesis and incorporation does not greatly influence the rate of receptor degradation (Table III). Also, the rates of incorporation and degradation have different temperature dependences (Fig. 8). There are basic similarities

shared by both synthetic and degradative mechanisms, such as inhibition by DNP and a requirement for intact cell structure. These conditions, however, are very general requirements and cannot be considered as a basis for coupling.

Characterization of an Additional Pool of ACh Receptors Revealed by Solubilization of Myotubes in 1% Triton X-100

There appear to be two classes of ACh receptors in chick myotubes which are not readily available to α-bungarotoxin in the medium and which do not seem to contribute to the chemosensitivity of myotubes measured by iontophoresis and intracellular recording (16).

One class of sites, containing approximately 10% as many sites as there are surface sites, cannot interact with extracellular α-bungarotoxin. The kinetics of disappearance of this class is rapid and nearly matches the appearance of new sites on the myotube surface. These observations suggest that this class contains precursors to surface sites. The other class we have termed "hidden" to indicate that receptors of this class do not readily interact with extracellular α-bungarotoxin, yet these receptors do not seem to be the precursors for readily available surface sites. During a puromycin block, the kinetics of decrease of this class of sites is quite slow and, thus, differs from that of the precursor sites.

We have not yet devised methods for completely independent measurement of either class of sites. Thus, solubilization following saturation of the surface sites reveals both precursor sites and hidden sites. Prolonged incubation with α-bungarotoxin saturates most of the hidden sites without labeling the precursor sites. It is reasonable, however, that a fraction of hidden sites will not be labeled even by continuous incubation with α-bungarotoxin unless the hidden sites are produced at a very slow rate. This fraction would be expected to contaminate measurements of precursors.

All of these receptors behave like surface receptors when examined as α-bungarotoxin-receptor complexes by sucrose gradient velocity sedimentation and by electrophoresis in polyacrylamide gels, and all can be protected from α-bungarotoxin by a high concentration of *d*-tubocurarine. The interactions of α-bungarotoxin with solubilized receptors from the surface or with those from the pool appear to be identical. Using the filter assay for α-bungarotoxin-receptor complexes, we have estimated a forward rate constant for the formation of α-bungarotoxin-receptor complexes to be 2-4 $\times$ 10^5 liters/mol-s, which agrees with published measurements for α-bungarotoxin (13). α-bungarotoxin-receptor complexes formed either by the exposure of cells to α-bungarotoxin followed by solubilization of the complexes, or by the interaction of α-bungarotoxin with receptors in 1% Triton X-100 solution are, for practical purposes, "irreversible". In both cases the dissociation half-times at 37°C in the presence of 10,000-fold excess unlabeled α-bungarotoxin are in the range of 9–15 days.

Receptor pools of the sort described in this report have not been reported for *Electrophorus* or *Torpedo* electroplax, and we have not found any ACh receptor pool in innervated adult rat diaphragms.[5] It appears that ACh receptors not readily available for α-bungarotoxin binding occur in embryonic muscle cultures and in denervated adult muscle[5] where there is substantial synthesis of new receptors.

Fig. 16 schematically illustrates the operationally defined classes of receptors, the arrows indicating the interrelationships we find consistent with our data. This diagram constitutes a conceptual framework for much of the data presented in this report and also emphasizes some as yet unresolved questions concerning interrelationships. Among these questions are the following. Do hidden sites arise directly from precursor sites? In what manner are surface receptors and hidden sites related? Are both surface and hidden sites directly degraded? There is also the important question: what are the cellular localizations of precursor and hidden sites?

Turnover of ACh Receptors in Skeletal Muscle

There has been a report of release of iodotyrosine from denervated rat diaphragm muscle in organ culture after [^{125}I]α-bungarotoxin binding (3). In that report the release followed a lag time amounting to about 6–8 h from the initial contact of [^{125}I]α-bungarotoxin with the muscle, and the half-times for release were 7.8 and 8.4 h in two experiments. Cycloheximide markedly altered the half-life (about 24 h), and inhibitors of oxidative phosphlorylation even more drastically reduced release. Our results for chick muscle in tissue

[5] Unpublished observations.

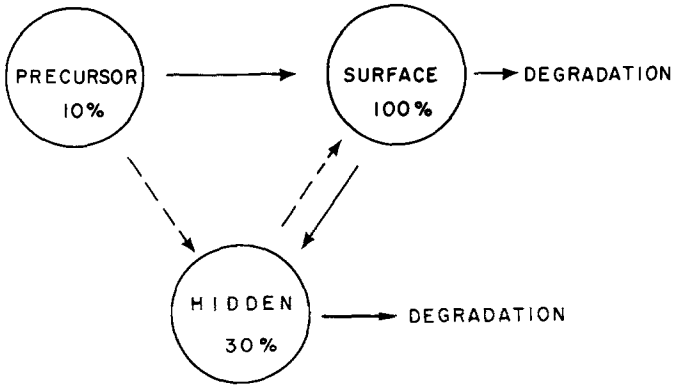

FIGURE 16 Hypothetical interrelationship of receptor classes.

culture support the identification of the radioactive moiety released from the muscle as predominantly iodotyrosine. We likewise find that the process follows first-order kinetics and is sensitive to inhibitors of oxidative phosphorylation. However, we find a much longer half-time for release, only slight inhibition of degradation by cycloheximide and puromycin, and see that there is rapid approach to the maximal rate of release (Fig. 5).

The goals of our studies on ACh receptor metabolism are to elucidate the mechanisms involved in membrane biosynthesis, and to more clearly define the control mechanisms which operate in regulation of chemosensitivity in skeletal muscle. The mechamisms involved in membrane construction and destruction are almost surely qualitatively the same for cells in tissue culture and for cells in vivo. We know the approximate time-course of changes in extrajunctional chemosensitivity during maturation of neuromuscular connections and after denervation of adult muscles (1, 8, 15, 24). Both instances involve large changes in the number of ACh receptors per unit area of muscle fiber surface membrane. The mechanisms which are involved in these changes certainly include the basic mechanisms of ACh receptor biosynthesis, incorporation into plasma membrane, and degradation.

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