SYNTHESIS, INSERTION INTO THE PLASMA MEMBRANE, AND TURNOVER OF α-BUNGAROTOXIN RECEPTORS IN CHICK SYMPATHETIC NEURONS

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ABSTRACT

α-Bungarotoxin was used to identify an integral membrane protein in the plasma membrane of chick sympathetic neurons. The synthesis, insertion into the plasma membrane, and turnover of the α-bungarotoxin receptor were studied using isotopically labeled amino acids (2H, 13C, 15N) to directly label receptor molecules.

Neurons incubated in medium containing dense amino acids continued to insert unlabeled receptors from a pool of previously synthesized molecules for 2 h. Density-labeled receptors began to appear in the plasma membrane after this 2-h period. Synthesis of receptors, but not insertion into the surface, was blocked by cycloheximide (100 μg/ml). Neither colchicine (0.05 μg/ml) or actinomycin D (5 μg/ml) has any effect on α-bungarotoxin receptor synthesis or insertion.

Autoradiographic studies revealed that receptors occur on growth cones, axons, and cell bodies of single neurons and explanted ganglia. The rate of insertion of newly synthesized receptors into the plasma membrane of axons extending from explanted sympathetic ganglia was approximately the same as that into the cell body portion of the ganglion. Cytochalasin B (2 μg/ml) rapidly disrupted growth cones but had no effect on receptor insertion. These experiments suggested that the growth cone is not the sole or even the primary site for insertion of this membrane protein.

The kinetics of turnover of the α-bungarotoxin receptor were a first-order exponential with t½ = 11 h. Neurons that had their surface receptors labeled with 125I-α-bungarotoxin produced [125I]iodotyrosine. This process was inhibited by low temperature (23°C) and also by a metabolic inhibitor. This is interpreted as evidence that receptors turn over by a mechanism in which they are internalized and then proteolytically degraded.

KEY WORDS chick sympathetic neurons • α-bungarotoxin receptor • plasma membrane protein • synthesis • insertion • turnover

In culture, neurons grow long processes in a manner that appears identical to the growth of axons during development. Little is known about the mechanisms of plasma membrane synthesis or turnover during this period of rapid expansion of the cell surface. Most of our present information about membrane metabolism is extrapolated from electron microscope investigations, while bio-
Chemical studies have often been limited to neuronal cell lines that are continuously dividing. In this paper we present information on the kinetics of synthesis, insertion, and turnover of a plasma membrane protein in chick sympathetic neurons that are actively growing axons. We also present evidence that membrane proteins are inserted into the plasma membrane at multiple sites along the axon and its cell body and not only at the growth cone of growing axons.

In these experiments we have used radioactive \( \alpha \)-bungarotoxin to identify and isolate a plasma membrane protein in chick sympathetic neurons. \( \alpha \)-Bungarotoxin, a well-known ligand for acetylcholine receptors in skeletal muscle, also binds selectively to the plasma membrane of chick sympathetic neurons (5, 13, 20, 21). Chick sympathetic neurons have acetylcholine receptors in their plasma membranes (5, 7) and several aspects of \( \alpha \)-bungarotoxin binding (20, 21) suggest that it is binding to neuronal acetylcholine receptors. However, \( \alpha \)-bungarotoxin does not bind to the ligand-binding site of acetylcholine receptors. Therefore, \( \alpha \)-bungarotoxin will continue to refer to this molecule as the \( \alpha \)-bungarotoxin receptor (5).

\( \alpha \)-Bungarotoxin-receptor complexes can be extracted from membranes with non-ionic detergents and cross-linked in solution with glutaraldehyde, resulting in a covalent labeling of the receptor (5). We have used amino acids in which the usual atoms of hydrogen (\(^1\)H), carbon (\(^{12}\)C) and nitrogen (\(^{14}\)N) have been replaced by heavy isotopes (\(^2\)H, \(^{13}\)C, and \(^{15}\)N) to label \( \alpha \)-bungarotoxin receptors. \( \alpha \)-Bungarotoxin receptors synthesized with these dense amino acids are correspondingly more dense than normal receptors and can be distinguished by velocity centrifugation. Combining the covalent labeling and density-labeling techniques, we have followed the metabolism of this plasma membrane protein in neurons that are rapidly extending axons.

**MATERIALS AND METHODS**

**Cell Culture**

Primary cultures of chick sympathetic neurons were made from 11- to 14-day-old embryos after the methods of Varon and Raiborn (33). Cells were grown in Eagle's minimal essential medium (MEM) plus 2% embryo extract, 10% horse serum, 2 U/ml of nerve growth factor (Burroughs-Wellcome Co., Research Triangle Park, N. C.) and 50 mg/ml of gentamicin (Schering Corp., Kenilworth, N. J.).

**Labeling of \( \alpha \)-Bungarotoxin Receptors**

Iodinated derivatives of \( \alpha \)-bungarotoxin were prepared as previously described (9). Receptors usually were labeled by incubating intact neurons with radioactive \( \alpha \)-bungarotoxin (0.1 \( \mu \)g/ml) in complete medium at 37°. After 1 h the incubation medium was removed and the cells were thoroughly washed of unbound \( \alpha \)-bungarotoxin by submersion for 5 min into each of three small baths containing Hanks' salt solution plus 0.1% bovine serum albumin. \( \alpha \)-Bungarotoxin-receptor complexes then could be extracted from cells with 1% Triton X-100, 10 mM Tris HCl pH 7.8 and the \( \alpha \)-bungarotoxin-receptor complexes in solution stabilized with 0.1% glutaraldehyde as described previously (5). In some experiments a Triton X-100 extract of cultured neurons or sympathetic ganglia from chick embryos was incubated with radioactive \( \alpha \)-bungarotoxin (0.01 to 0.05 \( \mu \)g/ml) for 1 h at 37°, and the unbound \( \alpha \)-bungarotoxin was removed by gel chromatography, as described below.

**Filter Assay**

Binding studies were done on crude membranes prepared by homogenizing ganglia from the paravertebral sympathetic chain of 18- to 19-day-old chick embryos in 10 mM Tris HCl pH 7.8. The homogenate was centrifuged at 27,000.g for 1 h, and the resulting membrane pellet was resuspended in phosphate-buffered saline (pH 7.2) and divided into 100-\( \mu \)l aliquots in plastic test tubes. The membranes were incubated with various concentrations of \(^{125}\)I-\( \alpha \)-bungarotoxin for 2 h at 37°. Nonspecific binding was estimated by adding large excesses of unlabeled \( \alpha \)-bungarotoxin to the radioactive \( \alpha \)-bungarotoxin. The separation of bound from unbound \( \alpha \)-bungarotoxin was done by collecting the membranes on cellulose acetate filters (Millipore Corp., Bedford, Mass.) as described by Shain et al. (32). Filters were counted by scintillation spectrometry.

**Gel Chromatography**

Two types of molecular sieve gels were used: Biogel P-60 and Biogel P-2 (Biorad Laboratories, Richmond, Calif.). Biogel P-60 columns (1 \( \times \) 25 cm) were equilibrated with 1% Triton X-100, 10 mM Tris HCl, pH 7.8. Detergent extracts of chick sympathetic ganglia that had been incubated with radioactive \( \alpha \)-bungarotoxin were filtered on the column to separate unbound \( \alpha \)-bungarotoxin from \( \alpha \)-bungarotoxin-receptor complexes. Samples were eluted with 1% Triton X-100, 10 mM Tris HCl, pH 7.8.

A Biogel P-2 column (1.3 \( \times \) 20 cm) was equilibrated with 100 mM ammonium acetate pH 7.0 and calibrated by determining elution profiles for monoiodotyrosine...
Density-Labeling Experiments

Insertion of Receptors: For experiments measuring the rate of insertion of newly synthesized receptors into the plasma membrane, a set of identical cultures of chick sympathetic neurons (usually eight to twelve 35-mm plates) were prepared from paravertebral, sympathetic chains of 8-10 embryos. The neurons were allowed to grow for 1 d in normal culture medium which was then replaced (t = 0) by "dense" medium containing 3H, 14C, or N-substituted amino acids (Merck and Co., Inc., Rahway, N. J.), dialyzed embryo extract (2%), dialyzed horse serum (10%) and 2 U/ml nerve growth factor. 1 h before each time point, one or two culture plates were incubated with 125I-α-bungarotoxin (0.1 μg/ml) in dense medium for 1 h at 37°C to saturate the population of α-bungarotoxin receptors in the plasma membrane. At the end of the hour the cultures were thoroughly washed of unbound α-bungarotoxin, and the α-bungarotoxin-receptor complexes were extracted with Triton X-100 and stabilized with glutaraldehyde as described above. For each experiment it was also necessary to have light, "marker" α-bungarotoxin-receptor complexes. These were prepared from identical neuronal cultures that were grown continuously in normal medium and were incubated with 125I-α-bungarotoxin. 125I-α-bungarotoxin-receptor complexes from neurons grown in dense medium plus light, marker 125I-α-bungarotoxin-receptor complexes were layered onto 25-40% sucrose/deuterium oxide gradients and centrifuged as described below. After centrifugation, the gradients were dripped into scintillation vials and the radioactivity was counted. The sedimentation profile of light, marker receptors labeled with 125I-α-bungarotoxin was used to determine the position in the gradient of old, light receptors from neurons grown in medium containing dense amino acids. Those receptors sedimenting at a faster rate in these gradients were newly synthesized from the dense amino acids (Fig. 4).

Analysis of the number of density-labeled receptors from the sedimentation profiles of receptors in the gradients was carried out with the aid of a PDP-8 computer (Digital Equipment Corp., Maynard, Mass.). A computer program was written that could theoretically superimpose dense and light receptor peaks, each having the shape of the marker. The computer simultaneously plotted the real data and the theoretically constructed double peak. The computer operator could repeat this process each time, adjusting the theoretical proportion of dense and light peaks. The best fit was obtained by visual inspection. A more detailed description of this computer program as well as an evaluation of its accuracy has been presented elsewhere (18).

Turnover of Receptors: The turnover of α-bungarotoxin receptors in the plasma membrane was measured in a pulse-chase experiment. A set of identical cultures of chick sympathetic neurons were grown for 1 day in normal medium. The cultures were then placed in dense medium for 12 h, at the end of which 65% of the α-bungarotoxin receptors in the plasma membranes of the neurons were density-labeled. The dense medium was replaced with normal medium (t = 0). 1 h before each time point, one or two cultures were incubated with 125I-α-bungarotoxin (0.1 μg/ml) for 1 h at 37°C in normal medium. Light "marker" 125I-α-bungarotoxin-receptor complexes were also prepared. The number of density-labeled receptors remaining in the plasma membrane after transferring neurons to normal medium was determined by velocity sedimentation and computer analysis of solubilized and glutaraldehyde-stabilized α-bungarotoxin-receptor complexes as described above.

Sucrose Gradient Centrifugation

Velocity sedimentation was carried out by layering an extract containing stabilized α-bungarotoxin-receptor complexes in a thin band over a 5-20% linear sucrose gradient (5 ml) or 25-40% linear sucrose gradient in deuterium oxide (11 ml) both containing 1% Triton X-100, 1 mM phenyl methylsulfonyl fluoride (Sigma) and 1 mM EDTA. 5-20% sucrose gradients were centrifuged in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 48,000 rpm for 5-6 h at 5°C. 25-40% sucrose/deuterium oxide gradients were centrifuged in a Beckman SW 41 rotor at 38,000 rpm for 48 h at 13°C. 30-40 fractions were collected and the radioactivity was counted.

In sucrose gradients of extracts from sympathetic neurons treated with 125I-α-bungarotoxin, we regularly saw a 4-6S peak that was caused by unbound or nonspecifically bound 125I-α-bungarotoxin. This peak was not blocked by incubation of neurons in 10^{-4} M tubocurarine that completely blocked binding to the 11S peak. It is important to note that the position of this
peak does not shift in neurons grown in dense medium (Fig. 4) where it might otherwise interfere with an accurate estimation of the number of light receptors in a gradient. We were able to reduce the size of this peak by thorough washing of unbound α-bungarotoxin from cells and also by filtering α-bungarotoxin-receptor complexes in solution on Minicon A-75 filters (Amicon Corp., Lexington, Mass.) that retained ~70% of the complexes while permitting ~75% of the 4-6S binding to pass through the filter. The remaining 4-6S peak separated by 10–11 fractions from the 11S receptor peak, and we have estimated its contribution to the 11S peak by simply extrapolating the leading edge of the 4–6S peak.

Radioisotope Counting

Samples were counted by liquid scintillation spectrometry in a Packard Tricarb Scintillation Spectrometer (Packard Instrument Co., Downers Groves, Ill.). The scintillation fluor was one part Triton X-100, two parts toluene containing 16 g PPO (2, 5 diphenyloxazole), 0.2 g POPOP (1,4-bis[2-(5 phenyloxazolyl)]benzene) per gallon of toluene. In some experiments, samples were counted in a Packard gamma counter. Data were corrected for crossover between the 131I and 32P channels.

Cell Morphology

Chick sympathetic neurons were cultured on collagen-coated, glass coverslips, washed in Hanks' salt solution, and fixed for 1 h each in two changes of 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.2. The specimens were postfixed in 1% OsO4 for 1 h, dehydrated through alcohols (30%, 50%, 70%, 95%, 100% for 5 min each) and in acetone (50% acetone/50% alcohol, 100% acetone for 5 min each) and dried in a critical-point drying apparatus (Polaron Equipment Ltd., Watford, England). The specimens were then sputter-coated with gold-palladium in a Polaron SEM Coating Unit and examined in a JEOL JSM-35 scanning electron microscope (JEOL Ltd., Tokyo, Japan).

The methods used for light microscope autoradiography have been published previously (5).

RESULTS

Identification of the α-Bungarotoxin Receptor in Solution

Fig. 1 shows the binding of radioactive α-bungarotoxin to membranes from chick sympathetic ganglia. Crude membranes were prepared from 18- to 19-d embryos, and the amount of bound radioactivity was assayed after filtering on cellulose acetate filters as described in Materials and Methods. (O–O) Total binding of 125I-α-bungarotoxin, (O–O) “nonspecific” binding, (O–O) total binding minus nonspecific binding.

The α-bungarotoxin receptor present in membranes from chick sympathetic ganglia can be extracted from these membranes by the non-ionic detergent Triton X-100 (Fig. 2). Detergent extracts of ganglionic membranes incubated with radioactive α-bungarotoxin show a peak of radioactivity that chromatographs in the void volume on BioGel P-60 (filled circles), well-separated from unbound α-bungarotoxin. If D-tubocurarine (10^-4 M) is added to the detergent extract along with radioactive α-bungarotoxin, the amount of radioactivity in the excluded peak is reduced greatly, (open circles), which helps identify this peak as the specific binding component or α-bungarotoxin receptor in neurons. The non-ionic detergent Triton X-100 is more efficient at solubilizing α-bungarotoxin-receptor complexes from the membranes of neurons than either 1 M NaCl or 1 mM EDTA (Table I), suggesting that the receptor is an integral membrane protein.

The α-bungarotoxin-receptor complexes that chromatograph as an excluded peak on BioGel P-60 (Fig. 2) sediment largely as a single peak when assayed after velocity centrifugation (Fig. 3, filled circles). Again, we identify the radioactivity in this peak as α-bungarotoxin-receptor complexes because it is absent in preparations incubated with α-bungarotoxin plus 10^-4 M D-tubocurarine (open circles). The α-bungarotoxin receptor (sedimen-
FIGURE 2 Chromatography of detergent extracts of chick sympathetic ganglia on Biogel P-60. Paravertebral, sympathetic ganglia were dissected from eight 19-d embryos, homogenized in 10 mM Tris HCl pH 7.8, and centrifuged at 27,000 g for 45 min. The resulting membrane pellet was solubilized in 1% Triton X-100, 10 mM Tris HCl, pH 7.8, and divided into two equal aliquots of 500 μl. One aliquot was pre-incubated with 10⁻⁶ M d-tubocurarine for 30 min at 37°C, after which time 0.01 μg/ml of ¹²⁵I-α-bungarotoxin was added to both aliquots for an additional 30 min at 37°C. Blue dextran 0.1% was added to the samples which were layered on a 1 x 25 cm Biogel P-60 (exclusion limit = 60,000 daltons) column equilibrated with 1% Triton X-100, 10 mM Tris, pH 7.8, and eluted with this same solution. The position of chick myotube acetylcholine receptors and radioactive α-bungarotoxin was determined previously. 20-drop fractions were collected and counted in a γ-counter. ●—● ¹²⁵I-α-bungarotoxin; ○—○ ¹²⁵I-α-bungarotoxin + 10⁻⁴ M d-tubocurarine.

tation coefficient ~11S) sediments slightly faster than chick skeletal muscle acetylcholine receptors (5) which have a coefficient of 10S (12). The second (4-6S) peak in these gradients is also present in preparations incubated with α-bungarotoxin plus d-tubocurarine and has been discussed above (Materials and Methods).

Unlike its binding to acetylcholine receptors in skeletal muscle, which is essentially irreversible, α-bungarotoxin dissociates from sympathetic neurons with a half-time of ~2 h at 37°C (5). However, treatment of α-bungarotoxin-receptor complexes with glutaraldehyde (0.1%) “fixes” the α-bungarotoxin to its receptor and greatly increases the stability of the complexes (5). This finding is important for the density-shift studies that employ long periods of centrifugation to separate light from density-labeled receptors.

Kinetics of Synthesis and Insertion of α-Bungarotoxin Receptors into the Plasma Membrane

To label the α-bungarotoxin receptor metabolically, we have used a density-shift methodology adapted in this laboratory for the study of another membrane protein, the acetylcholine receptor in chick skeletal muscle (11, 12, 17, 18). Neurons grown in culture medium containing ²H, ¹³C, ¹⁵N-substituted amino acids incorporate these dense amino acids into their newly synthesized proteins, rendering the proteins more dense so that they can be separated from old, “light” proteins synthesized in normal medium containing ¹H, ¹³C, ¹⁵N-amino acids. Treatment of intact neurons with radioactive α-bungarotoxin identifies the population of receptors that is present in the plasma membrane of chick sympathetic neurons. By extracting α-bungarotoxin receptor complexes and assaying them after velocity centrifugation on sucrose/deuterium oxide gradients, we can determine how many density-labeled and light α-bungarotoxin receptors are on the surface at any time.

Fig. 4 shows the sedimentation profiles of α-bungarotoxin receptor complexes extracted from neurons after incubation for increasing lengths of time in medium containing dense amino acids.

| Solution | Radioactivity on cells (cpm) | Radioactivity in solution (cpm) | Extraction efficiency (% of bound) |
|----------|-----------------------------|--------------------------------|-----------------------------------|
| 150 mM NaCl | 1,782                       | 158                            | 8                                 |
| 150 mM NaCl + 1 mM EDTA | 1,828                       | 157                            | 8                                 |
| 150 mM NaCl + 1% Triton X-100 | 777                          | 1,431                          | 65                                |
| 1 M NaCl | 1,977                       | 156                            | 7                                 |

Four sets of neuronal cultures (4 plates each) were incubated with 0.1 μg/ml of ¹²⁵I-α-bungarotoxin for 1 h at 37°C. They were washed thoroughly to remove unbound toxin and treated with 1 ml of a test solution with constant stirring for 1 min. The solution was removed, centrifuged at 10,000 g for 5 min, and an aliquot of the supernate was counted by scintillation spectrometry. The activity remaining on the plates was solubilized in 1 N NaOH and also counted.
Froction Number

FIGURE 3 Sucrose density centrifugation of the excluded peak from Biogel P-60 chromatography of detergent extracts of chick sympathetic ganglia. Four paravertebral sympathetic chains from 18- to 19-d embryos were incubated with $^{125}$I-$\alpha$-bungarotoxin (0.05 $\mu$g/ml) plus or minus $10^{-4}$ M $\alpha$-tubocurarine for 1 h at 37°C. Then they were washed of unbound $\alpha$-bungarotoxin, and extracted into 300 $\mu$l of 1% Triton 10 mM Tris HCl, pH 7.8. Blue dextran (0.1%) was added to the extracts and each was layered on a Biogel P-60 column. Ten-drop fractions were collected and counted in a $\gamma$-counter. The excluded peak fractions from each column were pooled, carefully layered on a 5-20% continuous sucrose gradient, and centrifuged for 5 h at 5°C and 48,000 rpm in a Beckman SW 50.1 rotor. (O--O) Biogel P-60 excluded peak from ganglia incubated in $^{125}$I-$\alpha$-bungarotoxin; (O--O) excluded peak from ganglia incubated with $^{125}$I-$\alpha$-bungarotoxin plus $10^{-4}$ M $\alpha$-tubocurarine.

The appearance of a faster sedimenting peak by 6 h (filled circles) is due to de novo synthesis of $\alpha$-bungarotoxin receptors from dense amino acids. If cycloheximide (100 $\mu$g/ml) is present from the beginning of the incubation period in the medium containing dense amino acids, then the faster sedimenting peak does not appear (Table II). A computer program has been used to analyze the number of light and density-labeled receptors in each gradient. From these data, a curve has been constructed showing the time course of insertion of newly synthesized $\alpha$-bungarotoxin receptors into the plasma membrane. The results strongly suggests that the 2-h delay is not a result of the time required for the amino acid pools of the cell to equilibrate with dense amino acids. We conclude that transport of receptors through the internal pool takes 2 h and that both transport and insertion into the surface are independent of protein synthesis.

We tested the effects of several drugs on the insertion of $\alpha$-bungarotoxin receptors into neuronal plasma membranes (Table II). As mentioned previously, cycloheximide blocks insertion only indirectly as a result of inhibition of new receptor synthesis. Neither colchicine, at a concentration that blocks axonal elongation (8), nor actinomycin D at a concentration that blocks RNA synthesis (29) has any effect on the synthesis and insertion of receptors.

**Turnover of $\alpha$-Bungarotoxin Receptors**

The kinetics of appearance of density-labeled receptors in the plasma membrane (Fig. 5) reflect the turnover as well as the synthesis and insertion of receptors. The turnover rate was measured directly in a pulse-chase experiment in which a population of surface receptors was density-labeled by a 12-hr incubation in medium containing dense amino acids. The cells were transferred to normal medium, and the number of density-labeled receptors remaining was measured at intervals after the transfer. After 12 h in dense medium, 65% of the surface population of receptors was labeled with dense amino acids (Fig. 5). This population decreased exponentially with a half-time of 11 h or a rate constant equal to 0.06/h (Fig. 7). The same turnover rate has been measured for light receptors in neurons growing in dense medium (unpublished observations, see also...
FIGURE 4 Sucrose gradient velocity centrifugation profiles of α-bungarotoxin receptor complexes from the plasma membrane of chick sympathetic neurons cultured in medium containing 2H, 13C, 15N-substituted (dense) amino acids. Neurons in medium containing dense amino acids were permitted to grow for various periods. At the end of a period, they were incubated with 125I-α-bungarotoxin (0.1 μg/ml) and the α-bungarotoxin-receptor complexes were extracted and prepared as described above (Materials and Methods). Aliquots of light "marker" α-bungarotoxin receptors from neurons grown continuously in normal medium and labeled with 125I-α-bungarotoxin were layered on 25-40% sucrose-deuterium oxide gradients along with 125I-α-bungarotoxin receptor complexes from neurons grown in dense medium. Gradients were centrifuged at 38,000 rpm for 48 h at 13°C in a Beckman SW 41 rotor. 25-drop fractions were collected. The sedimentation profile of the light marker 125I-α-bungarotoxin-receptor complexes was used to determine the position of light receptors in the extracts of neurons grown in medium containing dense amino acids. (••••) 125I-α-bungarotoxin-receptor complexes from neurons grown in dense medium. (○○○○) 125I-α-bungarotoxin receptor complexes from neurons grown in light medium.

reference 18) and also can be calculated from the difference between the rate of insertion of receptors (8% of the surface population per h, Fig. 5) and the rate of increase of receptors (2-3% of the surface population per h, Fig. 10). Thus, labeling of receptors with heavy isotopes does not seem to affect their turnover. The lag in turnover (Fig. 7) is caused by the appearance of density-labeled receptors from the internal pool for 2 h after transferring the neurons into light medium and obscures the turnover of receptors that occurs during this period. (The continued appearance of density-labeled receptors for 2 h after transferring neurons to light medium was shown above to be due to postsynthetic transport of receptors to the surface membrane.) The exponential shape of the curve suggests that receptors are lost at random from the surface population, and all receptors, regardless of age, share an equal probability of turning over within the next unit of time.

The turnover of acetylcholine receptors in chick myotubes in culture has been measured previously by density-labeling (18) and by the decrease in binding of α-bungarotoxin to muscle cells treated with puromycin (9). Acetylcholine receptors in myotubes turn over by being internalized and proteolytically degraded (9, 10). The degradation of acetylcholine receptors has been inferred from observations that radioactive α-bungarotoxin bound to acetylcholine receptors is subject to proteolysis inside the cell, and that the radioactivity diffuses from the cell and into the medium as radioactive iodotyrosine. The rate of iodotyrosine production by muscle cells matches the rate of turnover of acetylcholine receptors (2, 9).

We have used a similar approach to obtain information about the fate of α-bungarotoxin receptors inserted into the plasma membrane of chick sympathetic neurons. Our data can be explained best by a model in which receptors turnover by a process of internalization and subsequent proteolytic degradation.

α-Bungarotoxin dissociates with a half-time of ~2 h from neurons. Thus, gel chromatography of radioactivity appearing in the medium after neuronal α-bungarotoxin receptors on intact neurons are saturated with 125I-α-bungarotoxin shows that 70-75% of the radioactivity co-chromatographs with α-bungarotoxin, but that 25-30% of the radioactivity co-chromatographs with the much smaller molecule [125I]iodotyrosine (Fig. 8). The production of iodotyrosine by neurons does not
FIGURE 5 Kinetics of insertion into the plasma membrane of newly synthesized α-bungarotoxin receptors. The data points for this graph were obtained by analysis of sedimentation profiles of α-bungarotoxin receptor complexes prepared from neurons grown in medium containing dense amino acids as in Fig. 4. The number of density-labeled receptors is plotted as a percent of the total number of α-bungarotoxin receptors labeled in a culture; and, because neurons were plated in equal numbers, this varied little (~10%) between culture plates. The curve drawn through these points is a first-order exponential with $t_\frac{1}{2} = 6$ h. Note that the halftime (6 h) falls at 8 h on the abscissa because there is a 2-h delay before any newly synthesized receptors appear in the surface. Symbols indicate different experimental trials.

occur if the cells are incubated with $^{125}$I-α-bungarotoxin in the presence of $10^{-4}$ M o-tubocurarine (Fig. 9), indicating that the α-bungarotoxin must bind to the receptor to be degraded and is not being simply endocytosed from the medium and degraded. Iodotyrosine production is energy-dependent and is inhibited by treating cells with CCCP (carbonyl cyanide m-chlorophenylhydrazone), an uncoupler of oxidative phosphorylation, or by maintaining cells at room temperature (Fig. 9). Also, we find that a small amount of iodotyrosine is released (11% of the total radioactivity remaining on the cells 10 h after labeling with $^{125}$I-α-bungarotoxin) by solubilizing cells that have been producing iodotyrosine.

**Accumulation of α-Bungarotoxin Receptors in the Plasma Membrane**

Fig. 10 shows the change in the number of α-bungarotoxin receptors in sympathetic neurons as a function of time in culture (see also references 14, 26). The number of receptors increases for the first 2-3 d in culture, peaks at about day 5, then slowly decreases to about day-1 levels after 10 d. We have measured this by scintillation

| Drug          | Treatment with drug | Density-labeled receptors (% of total surface receptors) | Density-labeled receptors (% of control) |
|---------------|---------------------|--------------------------------------------------------|-----------------------------------------|
| Control       |                     | 38                                                     | –                                        |
| Colchicine (0.05 μg/ml) | 3                  | 34                                                     | 90                                      |
| Cycloheximide (100 μg/ml) | 8                  | 0                                                      | 0                                        |
| Actinomycin D (5 μg/ml) | 8                  | 38                                                     | 100                                     |

Cultures of sympathetic neurons were grown in medium containing dense amino acids for 8 h. Cultures were treated with drugs at the concentrations and for the periods shown above. After 7 h, the neurons were incubated with radioactive α-bungarotoxin (0.1 μg/ml) in dense medium for 1 h at 37°C. The cultures were then washed of unbound α-bungarotoxin, the α-bungarotoxin-receptor complexes were extracted, and the number of density-labeled receptors in each culture was determined.
FIGURE 7 Turnover of α-bungarotoxin receptors in the plasma membrane of neurons. A set of neuronal cultures (6 plates) was grown in medium containing dense amino acids (2H, 13C, 15N-amino acids) for 12 h, at which time ~65% of the α-bungarotoxin receptors in the plasma membrane were density-labeled. The cultures then were transferred to medium containing light amino acids (t = 0) (1H, 14C, 14N-amino acids) and, at various times thereafter, cultures were incubated with 125I-α-bungarotoxin and the number of density-labeled and light receptors remaining in the cells was determined. The number of receptors remaining is plotted as a percent of the density-labeled receptors at t = 0. The data are fit by a first-order exponential with τ = 11 h or a rate of ~6% of the surface population per h.

FIGURE 8 Biogel P-2 chromatography of radioactivity in the medium of neurons labeled with 125I-α-bungarotoxin. A set of cultures (3 plates) was incubated with 125I-α-bungarotoxin to label the receptors in the plasma membrane, washed thoroughly to remove unbound α-bungarotoxin, and returned to the incubator. At the end of 10 h, aliquots of the medium were chromatographed on Biogel P-2 column (Materials and Methods). 25–30% of the radioactivity in the medium chromatographs at the same position as iodotyrosine on Biogel P-2.

Site of Insertion of α-Bungarotoxin Receptors into the Plasma Membrane

In light microscope autoradiographs of sympathetic neurons that have been incubated with 125I-α-bungarotoxin, we see exposed emulsion grains over growth cones and a decrease in the number of grains from the cell body to the tip of the axon (Fig. 11). Although we did not mention it at the counting of the amount of radioactive α-bungarotoxin specifically bound to neuronal cultures of increasing ages (Fig. 10, left). We also have estimated the amount of α-bungarotoxin bound to each neuron by light microscope autoradiography (Fig. 10, right) to show that the differences are not due to decreases in cell number that could be caused by cell death. In all our experiments on α-bungarotoxin receptor metabolism, we have used neuronal cultures that were 1–2 d old. During this period, receptors are accumulating in the plasma membrane at a rate of between 2 and 3% net addition to the surface population each hour. This rate of increase fits well with our measurements of the rate of insertion of newly synthesized receptors into the surface (8% of the surface population per h) and the degradation rate of receptors (6% of the surface population per h) or an accumulation rate of 2%/h.
Figure 10: The number of α-bungarotoxin receptors in chick sympathetic neurons during time in culture. (A) Neuronal cultures were plated at equal densities and permitted to settle and grow for 1 d. At each time point thereafter, three plates were incubated with [125I]-α-bungarotoxin (0.1 μg/ml) with or without D-tubocurarine (10⁻⁴ M). The plates were washed of unbound α-bungarotoxin, solubilized in 1% Triton X-100, 10 mM Tris, pH 7.8, and the radioactivity in the solution was counted. Binding not blocked by D-tubocurarine was <10% of control values and was subtracted from controls. (B) Neuronal cultures were made and handled as described above, except that after neurons were incubated with [125I]-α-bungarotoxin they were fixed in 2% glutaraldehyde, 100 mM cacodylate buffer, pH 7.4, dehydrated in alcohols, and coated with Kodak NTB-2 Nuclear Track Emulsion. After 5-7 d, autoradiographs were developed in Kodak D-19 developer and fixed. Grains were counted over neurons, using a compound microscope with phase and brightfield optics.

In the first of these experiments, sympathetic ganglia were cultured whole rather than as dissociated neurons. After several days in culture, an extensive outgrowth or “halo” of axons surrounded each ganglion. This halo was dissected easily from the ganglion to yield a preparation of pure axons. The strategy of the experiment was to culture whole ganglia for several days, transfer them to medium containing dense amino acids for various periods, then separate the ganglia from their axons, label the α-bungarotoxin receptors in the axons with [125I]-α-bungarotoxin, and then analyze the number of density-labeled receptors in the axons vs. ganglia. If newly synthesized receptors were being incorporated at the tips of elongating axons, then the number of density-labeled receptors should be greater in the axons than the ganglia. In three separate experiments, we found that the number of density-labeled receptors in the ganglia was 7-27 times that of the axons. However, the relative rate of insertion of newly synthesized receptors, when normalized to the pre-existing surface population in the axons and ganglia during the labeling period, was almost the same in the ganglia and axons at each time point (Table III). It seems then that receptors are inserted at multiple sites along the cell and not exclusively at the growth cone.

The conclusion that receptors are not inserted predominately into growth cone plasma membrane is strengthened by the results of experiments testing the effects of cytochalasin B. Cytochalasin B blocks axonal elongation by disrupting the structure of the growth cone (Fig. 12) (37). We have observed that when growing axons are exposed to cytochalasin B (2 μg/ml) for as little as 3-5 min, the growth cone filopodia cease their activity and the growth cone takes on a clubbed appearance. This is followed shortly by retraction of the fine branches of elongating neurites. The rate of receptor insertion was measured in cultures of dissociated neurons incubated continuously with cytochalasin B (2 μg/ml) or pulsed for 3 h (5 μg/ml). In both cases the insertion of receptors was unaffected (Fig. 13), suggesting that the structure of the growth cone is of little importance in this process.

DISCUSSION

Neurons in culture extend long processes in a manner that appears identical to neurons growing in vivo. In our early experiments confirming observations of Greene and his colleagues on α-bungarotoxin binding (7, 20, 21), we often saw α-bungarotoxin receptors over growth cones in light microscope autoradiographs of sympathetic neurons. At the start, it seemed likely that α-bungarotoxin was binding to a plasma membrane protein, and its presence over growth cones was consistent with current concepts that new membrane was added at the growth cone of elongating axons. Beyond that, the presence of a unique, identifiable plasma membrane protein in sympathetic neurons (21) gave us the potential to learn...
whether the growth cone was, indeed, the site of insertion of new membrane proteins, and we began our studies of the metabolism of α-bungarotoxin receptors in chick sympathetic neurons.

After transferral of cultures of neurons into medium containing dense amino acids, there is a 2-h delay before density-labeled receptors appear in the plasma membrane. During this delay, re-
TABLE III
Sites of Insertion of Newly Synthesized α-Bungarotoxin Receptors into the Plasma Membrane of Sym pathetic Ganglia and Axons

| Labeling Period | Axons | Ganglia | Axons | Ganglia |
|-----------------|-------|---------|-------|---------|
| h               | cpm   | cpm     | %     | %       |
| 4               | 3,234 | 86,245  | 14    | 18      |
| 6               | 1,968 | 13,808  | 35    | 38      |
| 10              | 2,997 | 49,370  | 45    | 45      |

The paravertebral sympathetic chains of chick embryos (11-13 d) were isolated from the embryo and cut into individual ganglia. The ganglia were cultured for 3-5 d and produced an extensive outgrowth or "halo" of axons. The diameter of a ganglion and its halo was often greater than 2 mm. Individual axons, however, were extensively branched and their lengths are best reflected in their rates of axonal elongation of ~50 μm/hr. 3- to 5-day-old cultures were incubated for various periods in medium containing density-labeled amino acids. The ganglia were then separated from their axons with the aid of a dissection microscope. The ganglia were incubated with 125I-α-bungarotoxin and the axons with 125I-α-bungarotoxin, both at a concentration of 0.1 μg/ml for 1 h at 37°C. Total receptors reflect cpm of 125I-α-bungarotoxin bound (axons) or 125I-α-bungarotoxin bound (ganglia) after correction for the difference in specific activities of the two isotopes. The percent of new receptors was obtained after radioactive α-bungarotoxin-receptor complexes were extracted from axons or ganglia, stabilized with glutaraldehyde, and centrifuged in sucrose-deuterium oxide gradients as described above (Materials and Methods).

Receptors are being inserted into the membrane from a previously synthesized pool of unlabeled receptors. Similar delays have been observed between biosynthesis and the appearance of secretory proteins (28) and other integral membrane proteins at the cell surface (1, 12, 34, 38). These delays generally are attributed to the time required for post-translation modification, packaging and transport to the surface, processes that do not require continued protein synthesis (24). An internal pool of acetylcholine receptors that are precursor to surface receptors has been localized by electron-microscope autoradiography to the Golgi apparatus of muscle cells, and it appears that integral membrane proteins traverse the same cellular pathway as secretory proteins before they appear at the surface (15).

Our attempts to measure the size and turnover of the intracellular pool have met with limited success. There is a large number of α-bungarotoxin binding sites, ~3 times the population in the plasma membrane, that are exposed by solubilizing the cells in Triton X-100 (unpublished observations). We have not determined how much of this population represents precursor to the plasma membrane receptors. Our kinetic measurements indicate that only ~5% of these sites should represent the metabolically active internal pool.

The hypothesis that the growth cone is the major site of addition of membrane is derived from observations of membrane flow and axonal elongation in growing neurons (3, 4, 23, 25). We have tested whether the α-bungarotoxin receptor reflects the hypothesized addition of new membrane at the growth cone. In experiments comparing the insertion of α-bungarotoxin receptors into the plasma membrane of axons and whole ganglia, and also in experiments measuring the insertion of α-bungarotoxin receptors in cultures of dissociated neurons treated with cytochalasin B, the growth cone does not seem to be the exclusive or even the primary site of insertion of this plasma membrane protein. On the other hand, our results are not consistent with insertion exclusively into the plasma membrane of ganglion cell bodies or axon hillocks. We found that the newly synthesized receptors inserted into the plasma membrane during an 8-h period had a distribution in cell bodies and axons identical to that of the entire receptor population. Receptors inserted into cell body plasma membrane could not possibly diffuse far enough in the lipid bilayer to appear in our pure axon fraction. Maximal diffusion rates for membrane proteins are nearly two orders of magnitude too slow. Likewise, rapid flow of bilayer, sweeping along the receptor molecules, is not a likely mechanism for transport of receptors toward the growth cone. Presently available evidence suggests some bulk flow of membrane in the opposite direction. There are, at present, no data suggesting bulk membrane flow rapid enough to distribute receptors all over the several millimeters length of the ganglion cells. Thus, we conclude that it is most likely that new receptors are inserted at multiple sites and that the spatial distribution of receptors reflects the distribution of insertion sites.

An interesting suggestion has been made by Pfenninger and Bunge (31) for the stepwise formation of neuronal plasma membranes. According to their model intra-membrane particles are
The effect of cytochalasin B on the structure of growth cones of growing neurons. Chick sympathetic neurons that were 1 d in culture were treated with cytochalasin B (2 μg/ml) for 5 min at 37°C, fixed, and prepared for scanning electron microscopy. In neurons untreated with cytochalasin B (left), elaborate growth cones (g) with many microspikes are frequently found at the tips of axons. In neurons treated with cytochalasin B the fine processes comprising the growth cone are absent, leaving a truncated structure at the tip of the axon. In the presence of cytochalasin B, processes extending from a non-neuronal cell (F) have begun to bead and also to withdraw. Bars, 10 μm.

After cells are pulse-labeled with dense amino acids, density-labeled α-bungarotoxin receptors disappear exponentially with a half-time of 11 h. This decrease is not due to instability of receptors synthesized with dense amino acids since receptors synthesized in normal medium disappear with the same kinetics when transferred to medium containing dense amino acids (unpublished observations, see also reference 18). The first-order kinetics of turnover suggest that, regardless of age, receptors turn over at the same rate by a mechanism that randomly samples the surface population. The internalization could itself occur randomly, but other steps in the life cycle of the receptor such as movement in the plane of the plasma membrane or randomly distributed sites of insertion could, as well, engender randomization.

The evidence indicates that the receptors that disappear from the surface are degraded. We find that a significant fraction of 125I-α-bungarotoxin used to label receptors on intact cells is metabolized to iodotyrosine, a proteolytic breakdown product of 125I-α-bungarotoxin, and we postulate that the receptor is also being degraded. The kinetics of iodotyrosine production have been shown to be a reliable indicator for the degradation of skeletal muscle acetylcholine receptors.
FIGURE 13 Effect of cytochalasin B on the insertion of newly synthesized α-bungarotoxin receptors into the plasma membrane of neurons. Neurons were incubated for 5 h in cytochalasin B (2 μg/ml) in medium containing dense amino acids. At the end of that period, the α-bungarotoxin receptors in the plasma membrane were labeled with 125I-α-bungarotoxin (○=○) and were layered on 25-40% sucrose-deuterium oxide gradients along with light, "marker" 125I-α-bungarotoxin-receptor complexes (O=O). Analysis of the sedimentation profiles shows that in neurons untreated with cytochalasin B (left), 23% of the surface receptors are density-labeled as compared with 21% of the surface receptors in neurons maintained continuously in cytochalasin B (right). Note that cytochalasin B (2 μg/ml) has a pronounced effect on growth cone morphology after only 5 min.

Several other pieces of evidence suggest that the turnover of receptors is accomplished by internalization and degradation. The production of iodotyrosine and no larger radioactive peptide (Fig. 8) requires that the α-bungarotoxin be degraded very thoroughly to single amino acid residues or that it be cleaved specifically at the peptide bonds on either side of a single tyrosyl residue. It seems unlikely that such a specific protease exists on the cell surface whereas degradation thorough enough to digest a protein to single amino acids would probably take place within the cell in some intracellular compartment. Finally, we find that there is a small amount of iodotyrosine (11% of the total radioactivity associated with the cells 10 h after labeling with 125I-α-bungarotoxin) that is released from neurons after they are solubilized and is presumably within the cells. Further evidence for interiorization comes from experiments demonstrating that degradation is inhibited by CCCP and is highly temperature dependent (Fig. 9).

In chick myotubes, acetylcholine receptors are degraded by a first-order process that is both energy and temperature dependent (9). Ultrastructural observations indicate that α-bungarotoxin-receptor complexes are internalized and subsequently degraded within the cell in secondary lysosomes (10). A similar mechanism has been demonstrated recently for the turnover of lectin receptors in the plasma membrane of cultured neurons (19), and it seems likely that this is the cellular pathway for the degradation of α-bungarotoxin receptors.

The number of α-bungarotoxin receptors in the plasma membrane of neurons increases 100-150% between day 1 and day 3 in culture. During this period, growth cones are abundant and it is our impression that this is the most active period of axonal elongation. The increase in receptors appears to reflect the increase in surface area that occurs during this period. After ~5 d in culture, the number of α-bungarotoxin receptors decreases either by a reduction in the synthesis or by an increase in the turnover of receptors. This sequence of events is superficially similar to the reduction in extrajunctional acetylcholine receptors that accompanies innervation of muscle fibers. However, we do not know whether the α-bungarotoxin receptor in sympathetic neurons is a synaptic protein, although that appears to be the case for some other neurons (27, 35), nor do we know whether the decrease in receptors is related to synapse formation among sympathetic neurons in culture. The decrease in number of receptors also could be a result of increased spontaneous electrical activity in the neurons, just as activity in muscles modulates the number of acetylcholine receptors (16).

We would like to thank Drs. Kenneth Muller, Richard Rotundo, Peter Devreotes, and Mr. John Gardner for their valuable suggestions throughout the course of these experiments. We also thank Mr. Gregory Nelson for his help with the scanning electron-microscopy and Ms. Delores Sommerville and Ms. Barbara Thomas for their excellent technical assistance.

S. Carbonetto is supported by a National Institutes of Health Postdoctoral Fellowship. Research in the authors' laboratories is supported in part by a grant from the Muscular Dystrophy Association.

Received for publication 18 October 1978, and in revised form 5 February 1979.
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