

NEWLY SYNTHESIZED ACETYLCHOLINE RECEPTORS ARE LOCATED IN THE GOLGI APPARATUS

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ABSTRACT

Chick skeletal muscle cells in tissue culture were fixed and treated with saponin to allow [125 I] α -bungarotoxin access into the cells while preserving ultrastructure. The kinetics of binding of iodinated α -bungarotoxin to intracellular acetylcholine (ACh) receptors and to surface ACh receptors were comparable. About half of the intracellular ACh receptors are newly synthesized and in the pathway leading to incorporation into the plasma membrane. Correlated electron microscope autoradiographic and kinetic studies of this receptor population suggest that a substantial fraction of the newly synthesized ACh receptors are located in the Golgi apparatus, where they reside for approx. 2 h.

KEY WORDS acetylcholine receptor · Golgi apparatus · muscle · membrane biogenesis · membrane proteins · glycoproteins

Acetylcholine (ACh) receptors in developing embryonic myotubes are synthesized about 3 h before their appearance in the plasma membrane (5-8, 12). During this 3 h period, receptors reside in an intracellular membrane system. The intracellular transport of receptors from their sites of biosynthesis to the plasma membrane has several properties in common with intracellular transport of secretory proteins (9, 10). Both processes are independent of protein synthesis but dependent upon oxidative phosphorylation, and the time required for intracellular transport is several hours in each case. As shown below, newly synthesized ACh receptors appear in the Golgi apparatus very soon after their synthesis. This finding supports the hypothesis that integral membrane glycoproteins reach the cell surface by a pathway similar to that taken by secretory proteins.

MATERIALS AND METHODS

Chick skeletal muscle cultures were initiated by plating mechanically dissociated 11-day chick embryo leg muscle cells at about 2×10^5 cells per dish in 35-mm collagen-coated tissue culture dishes, using Eagle's minimum essential medium supplemented with 2% chick embryo extract and 10% horse serum. Usually, 5-day cultures were used for experiments.

α -Bungarotoxin was purified and iodinated to give specific activities up to about 10^6 Ci/mol (see reference 5 and references therein) and was used within a few days of iodination.

To preserve ultrastructure, maintain receptor site integrity, minimize the production of nonspecific α -bungarotoxin binding sites, and allow α -bungarotoxin access to intracellular ACh receptors, we devised the following procedure for fixation (see reference 4) and saponin treatment. Fresh fixative was prepared by hydrolysis of paraformaldehyde in 20 mM sodium phosphate buffer, pH 7.2 (80°-85°C) followed by addition of the other components to the cooled solution to give a final composition: 2% formaldehyde, 100 mM lysine, 60 mM sucrose, and 20 mM sodium phosphate buffer, pH 7.2. This solution was again heated and 10 mM sodium

periodate added. Chick muscle cultures were fixed for 1 h at room temperature. The cultures were then rinsed with basic salt solution (BSS). At this point, the cultures could be returned to culture medium buffered at pH 7.2 with 18 mM tricine or HEPES (sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate) buffer and kept at 4°C without change in α -bungarotoxin binding properties for at least 1 wk. To reveal intracellular binding sites, the fixed cultures were treated for 3 min with 0.5% saponin in 20 mM sodium phosphate buffer, pH 7.2, with or without 150 mM KCl, NaCl, or 300 mM sucrose. (This procedure does not extract receptors from the cells.) The cultures were then rinsed twice with BSS and returned to HEPES-buffered complete medium at room temperature (25°–27°C) with or without 1.5×10^{-4} M *d*-tubocurarine for at least 15 min.

For binding studies, the [¹²⁵I] α -bungarotoxin ([¹²⁵I]BuTX) was diluted to a final concentration of 0.03–0.05 μ g/ml (3.6–6.0 nM) in HEPES-buffered complete medium with or without 1.5×10^{-4} M *d*-tubocurarine. The medium was removed from the fixed cultures, and this [¹²⁵I]BuTX-containing medium was added. The specific binding was complete in about 1.5–2 h at room temperature. The medium was removed, and unbound [¹²⁵I]BuTX was rinsed away by immersion of the cultures in large-volume baths (>1 liter) of BSS. Three 10-min rinses removed virtually all unbound or readily dissociated [¹²⁵I]BuTX. The bound radioactivity either was measured in a gamma counter designed to accept culture dishes or the total radioactivity was dissolved in 1 N NaOH overnight, and the solution was counted in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). When we wished to study the saponin-revealed sites alone, cultures were grown in the presence of 0.5 μ g/ml unlabeled α -bungarotoxin and rinsed to remove unbound α -bungarotoxin just before fixation.

Specific binding was initially defined as the difference between the amounts of [¹²⁵I]BuTX bound in the absence and in the presence of 1.5×10^{-4} M *d*-tubocurarine. This interpretation was strengthened by many observations discussed below. The number of specific [¹²⁵I]BuTX binding sites is proportional to (perhaps equal to) the number of ACh receptor sites, and these terms are used interchangeably in this report.

When cultures treated as described above were to be used for electron microscope autoradiographic studies, the cultures were subsequently fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in buffered 1% osmium tetroxide, stained with 0.5% aqueous uranyl acetate and embedded in Epon. Carbon-coated thin sections on Formvar-coated 200-mesh grids were covered with Ilford L4 emulsion, by use of a loop method (3). Photographic emulsion was developed by the EAS gold intensification process (14). Specimens were examined in a JEOL 100S electron microscope.

Area measurements and grain counts were generally

made on photographic prints (at about total magnification of 10,000) assembled into montages representing single grid squares. Areas were measured with a Numonics graphic calculator (Numonics Corp., Lansdale, Pa.). In analysis of autoradiographs, we scored silver grains over the following cell compartments: (a) rough endoplasmic reticulum (RER), (b) nucleus, (c) Golgi apparatus, (d) remaining perinuclear area, (e) myofibrils, (f) surface membrane, and (g) other cytoplasmic area, including mitochondria and areas rich in ribosomes, loosely organized filaments and sarcotubular systems. Any silver grain covering any area of nucleus or nuclear membrane was assigned to the nucleus, even if the grain also was overlying a Golgi apparatus. (The Golgi apparatus are consistently located adjacent to nuclei in these cells.) This was done to bias the data (if at all) away from assignment of grains to the Golgi apparatus. The perinuclear category included silver grains outside the nucleus within a grain diameter of the nuclear membrane and not associated with a definite Golgi apparatus, grains within a grain diameter of a Golgi apparatus but not directly over it, and grains over perinuclear membranous structures which were possibly Golgi-related but identification of which was uncertain.

RESULTS

(1) Preservation of Surface ACh Receptors during Fixation

The specific binding of [¹²⁵I] α -bungarotoxin ([¹²⁵I]BuTX) (3.6 nM) to formaldehyde-fixed cultures at 25°C approximated first-order exponential kinetics with half-maximal binding in 20 min (data not shown). This binding was blocked by pretreatment and coincubation of the cells with either 10^{-3} M ACh (together with 3×10^{-5} M eserine sulfate) or 10^{-4} M *d*-tubocurarine. When cultures were treated with unlabeled α -bungarotoxin before fixation, few specific binding sites for [¹²⁵I]BuTX were observed on the fixed cultures (Fig. 1). A study of the kinetics of loss of specific [¹²⁵I]BuTX binding sites and of generation of nonspecific binding sites during fixation in formaldehyde at 25°C demonstrated that about 50% of the specific binding sites present on unfixed cultures were lost in 10 h and that nonspecific binding (which typically was 10–20% of total binding) rose only slightly between 15 min and 10 h of fixation. These changes were approximately linear with time. Thus, fixation for 1 h, as employed in the studies reported below, provided nearly complete preservation of ACh receptor sites.

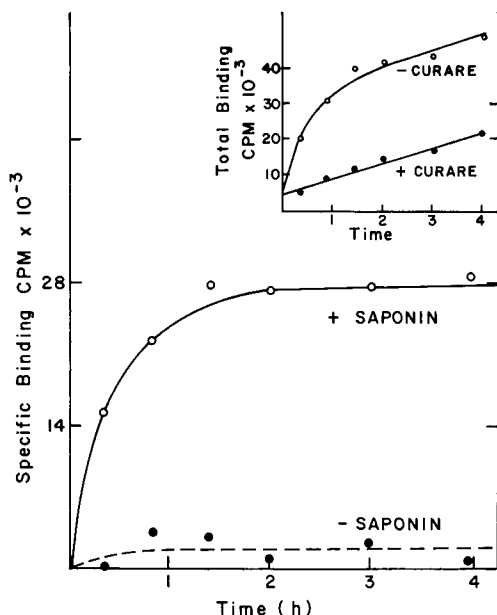


FIGURE 1 Kinetics of specific binding of [^{125}I]BuTX to formaldehyde-fixed chick skeletal muscle cultures (in which surface ACh receptors were blocked with unlabeled α -bungarotoxin before fixation) before (\bullet - \bullet) and after (\circ - \circ) saponin treatment. *Inset*: Kinetics of total [^{125}I]BuTX binding to saponin-treated cultures in the absence (\circ - \circ) or presence (\bullet - \bullet) of 1.5×10^{-4} M *d*-tubocurarine. Each point is the average value from four culture dishes. These data were used to construct the specific [^{125}I]BuTX binding curve (+saponin) of the main figure. [^{125}I]BuTX concentration was 3.6 nM. Binding at 26°C.

(2) Exposure of Internal ACh Receptors by Saponin Treatment of Fixed Cells

Saponin treatment of fixed muscle cultures revealed a new set of specific α -bungarotoxin binding sites (Fig. 1). These sites were revealed whether or not the cultures had been grown in the presence of unlabeled α -bungarotoxin before fixation. The inhibition of [^{125}I]BuTX binding to the specific sites was studied as a function of *d*-tubocurarine concentration and ACh concentration. Inhibition curves were similar for the surface sites and the saponin-revealed sites, with half-maximal inhibition at about $3\text{--}5 \times 10^{-7}$ M *d*-tubocurarine or $1\text{--}4 \times 10^{-6}$ M ACh. Electron microscope autoradiography (see section 4 below) was used to demonstrate that these saponin-re-

vealed receptor sites are intracellular.

The number of saponin-revealed ACh receptors was usually 20–25% of the number of surface ACh receptors (Table I). The saponin-revealed ACh receptor population was progressively diminished to about half normal size when protein synthesis was inhibited for a period of time before fixation and saponin treatment (Fig. 2). These observations are consistent with our earlier findings: (a) in living muscle, about 20% of the ACh receptors are unable to interact with extracellular α -bungarotoxin (7); (b) these consist of about equal populations of newly synthesized receptors in transit to the cell surface and “hidden” receptors which are only slowly labeled with isotopically labeled amino acids (7); and (c) the newly synthesized receptors continue to be incorporated into the plasma membrane even when protein synthesis is inhibited by puromycin (references 5, 6, and our unpublished observations). We conclude that about 50% of saponin-revealed sites are ACh receptors in the pathway of biosynthesis and transport to the cell surface.

TABLE I
Revelation of Specific [^{125}I] α -Bungarotoxin Binding Sites after Blockade of Surface Receptors

Treatment of cultures after formaldehyde fixation*	Specific [^{125}I]BuTX sites (expressed as fraction of surface sites)†
None	0.02, 0.02
Saponin 0.5%, 3 min	0.21, 0.29, 0.23
Freeze-thaw (-70°C , 25°C)	0.05, 0.14
Triton X-100§ 1%, 1 min	0.11, 0.15
Sodium cholate§ 1%, 1 min	0.14

* Cultures were grown in medium containing unlabeled α -bungarotoxin, rinsed in cold BSS to remove unbound α -bungarotoxin, and fixed in formaldehyde fixative. Saponin, Triton X-100, and cholate treatments were done in 10–20 mM Tris or phosphate buffers; freeze-thaw in BSS.

† Specific binding to surface ACh receptors was measured on fixed cells not prelabeled with α -bungarotoxin. In all cases, specific binding was defined as binding which was blocked by 1.5×10^{-4} M *d*-tubocurarine. Separate numbers refer to different experiments, each consisting of a large set of cultures. Values for saponin-revealed sites come from the three largest experiments contributing data for Fig. 2.

§ Smaller number of ACh receptor sites revealed by these procedures as compared with saponin treatment may have resulted from extraction of some receptors by the detergents.

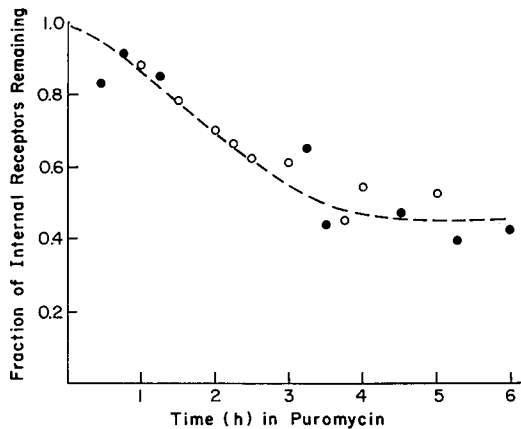


FIGURE 2 Decline in saponin-revealed ACh receptors in cultured chick skeletal muscle after inhibition of protein synthesis by puromycin. Cultures were treated with unlabeled α -bungarotoxin to block all surface ACh receptors, and puromycin (10 or 20 $\mu\text{g}/\text{ml}$) was added to the medium of subsets of cultures at different times. Then, all cultures were rinsed briefly to remove medium and were fixed for 1 h at room temperature in fresh formaldehyde fixative. After fixation, the cultures were treated with 0.5% saponin solution for 3 min and rinsed again. Then, the cultures were incubated at room temperature in medium containing 0.03–0.05 $\mu\text{g}/\text{ml}$ [^{125}I] α -bungarotoxin for 90 min, rinsed for 30 min with large volumes of Hanks' balanced salt solution, and the bound radioactivity was counted. Specific binding was defined as binding not blocked by preincubation of cultures for 15 min with 1.5×10^{-4} M *d*-tubocurarine and inclusion of this concentration of *d*-tubocurarine in the [^{125}I] α -bungarotoxin-containing medium. Open circles represent averaged data from two to four experiments involving four to eight culture dishes for each point. Closed circles represent data from single experiments with four to eight culture dishes for each point.

(3) Exposure of Internal ACh Receptors by Cycles of Freeze-Thaw and by Extraction with Triton X-100 or Sodium Cholate

We explored methods other than saponin treatment for allowing α -bungarotoxin access to the intracellular ACh receptor sites. These methods revealed a more variable and generally smaller

number of ACh receptor sites than did saponin treatment (Table I), and they resulted in inferior preservation of cell ultrastructure.

(4) Location of Saponin-Revealed ACh Receptors

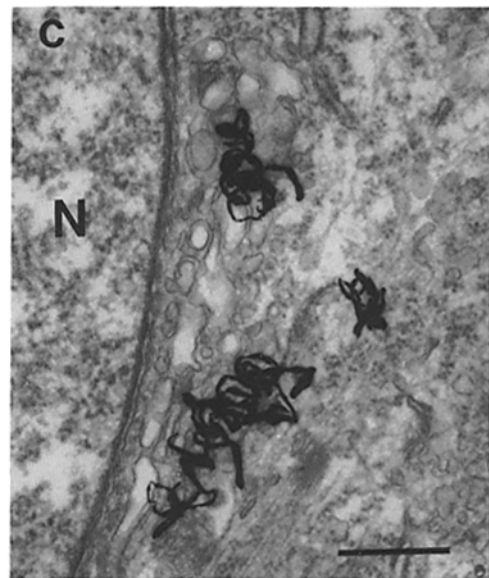
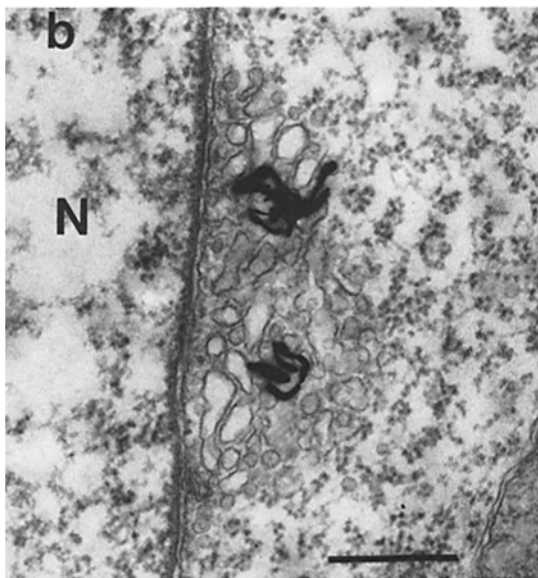
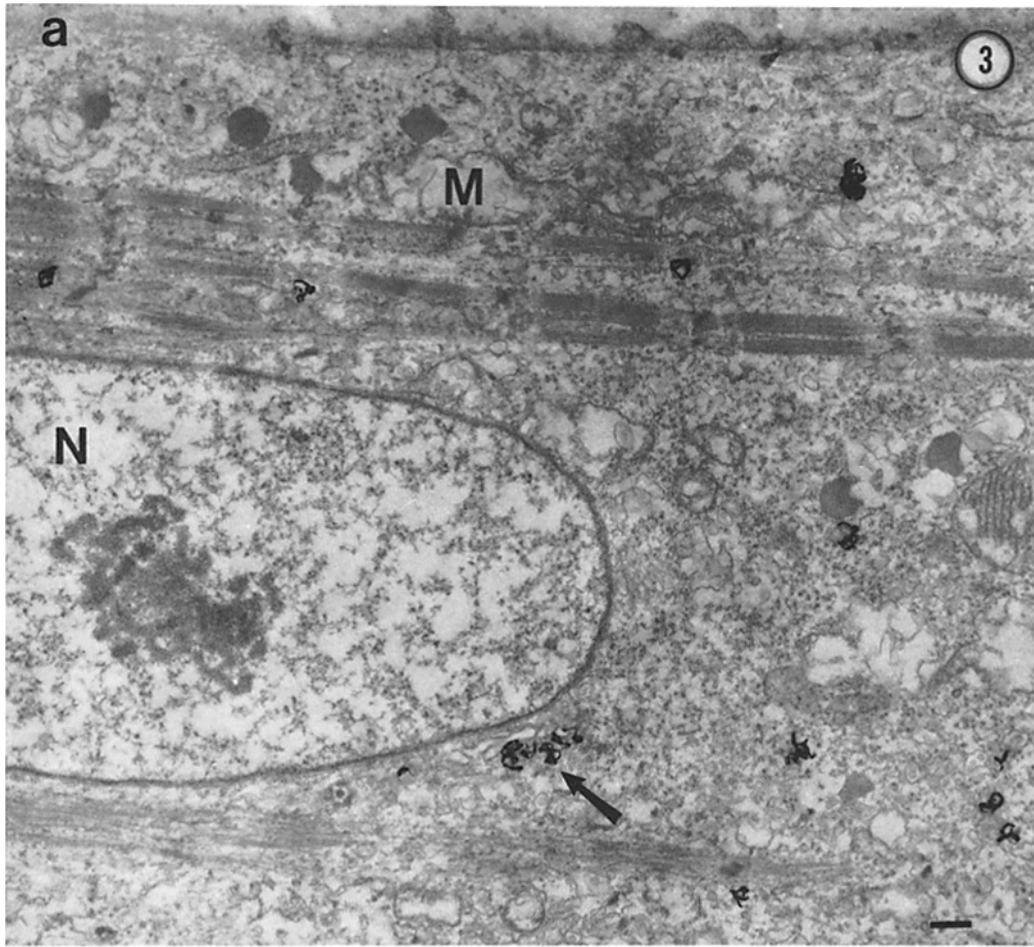
Muscle cultures were grown in the presence of unlabeled α -bungarotoxin to block all of the surface ACh receptors and then fixed, saponin treated and exposed to [^{125}I]BuTX. In electron microscope autoradiographs of thin sections of such cells, only 1.3% of all silver grains were directly over plasma membrane or outside the cells but within a grain diameter of the cell surface. Thus, the great majority of [^{125}I]BuTX binding sites (specific and nonspecific) in these cultures occurred within the myotubes. It should be pointed out that the ultrastructure of the saponin-treated cells was nearly as well preserved as after conventional glutaraldehyde fixation (see Fig. 3). All major elements such as plasma membranes, nuclei, mitochondria, Golgi apparatus, RER, myofibrils, and polysomes retained their familiar general appearance and characteristic positions in the myotubes, although there appeared to be some fragmentation of cell membranes, particularly the outer mitochondrial membrane.

The relative areas of the different cell compartments are tabulated in Table II, and the specific association of silver grains with these areas is presented in Table III. Although the Golgi apparatus occupied only 0.3% of the area of autoradiographs, 14% of silver grains associated with specific [^{125}I]BuTX binding occurred directly over the Golgi apparatus (see Fig. 3). The binding of [^{125}I]BuTX to the Golgi apparatus was drastically reduced by inhibiting protein synthesis in the cultures for 4–5 h before fixation (Table III).

Involvement of the Golgi Apparatus in the Pathway of ACh Receptor Biosynthesis and Transport to the Cell Surface

Newly synthesized ACh receptors continue to be incorporated into the plasma membrane for several hours after protein synthesis is blocked

FIGURE 3 Electron microscope autoradiographs of saponin-revealed [^{125}I]BuTX binding sites. (a) General view of cytoplasm. Arrow points to Golgi apparatus with overlying silver grains. (b and c) Silver grains over Golgi apparatus. Note typical small elements of RER in Fig. 3c. N, nucleus. M, mitochondrion. Bars, 0.5 μm . (a) $\times 10,400$; (b) $\times 37,000$; and (c) $\times 30,000$.



by puromycin (5, 8, 12). An equal number of receptors disappear from the population of saponin-revealed receptors when the cells are treated with puromycin for several hours before fixation (Fig. 2). We found that the Golgi apparatus and perinuclear compartments lost receptors during puromycin treatment, while other compartments failed to show this trend (Table III). To determine the kinetics of these changes, we grew muscle cultures in the presence of unlabeled α -bungarotoxin to block all surface receptors, and then at various times before fixation we added 20 $\mu\text{g/ml}$ puromycin to the culture medium to block protein synthesis (5). All the cultures were fixed

at the same time, saponin treated, incubated with [^{125}I]BuTX (with and without 1.5×10^{-4} M *d*-tubocurarine), rinsed to remove unbound [^{125}I]BuTX, and further prepared for electron microscope autoradiography and analyzed as outlined in Materials and Methods. As illustrated in Fig. 4 and partially tabulated in Table III, the specific association of ACh receptors with the Golgi apparatus was drastically reduced by inhibition of protein synthesis. "Perinuclear" ACh receptor sites were also progressively fewer as the inhibition of protein synthesis was prolonged.

DISCUSSION

Proteins destined for secretion are synthesized on polysomes bound to the endoplasmic reticulum, are processed in the endoplasmic reticulum, are transferred to the condensing vacuoles of the Golgi apparatus where further glycosylation may take place, and are later released from the cell by exocytosis (10). A similar mechanism is generally considered to be the most plausible one for the biogenesis of integral membrane proteins, the major difference being that these membrane proteins are tightly associated with the lipid bilayer. Thus, if secretory-like vesicles containing integral membrane proteins fuse with the plasma membrane, the membrane proteins would be revealed to the extracellular milieu (9, 13). However, there is little data to substantiate this hypothesis. The

TABLE II
SIZES OF SUBCELLULAR COMPARTMENTS (% OF TOTAL AREA*)

RER‡	Golgi apparatus	Perinuclear area	Nucleus	Myofibrils	Other§
0.12%	0.30%	2.4%	9.1%	22.0%	66.1%

* Most computations are based upon a total area of 37,150 μm^2 . RER was computed from a total area of 23,940 μm^2 , since puromycin-treated cells could not be used for measurements of RER.

‡ The paucity of RER in chick myotubes has been noted in numerous electron microscope studies.

§ Area includes mitochondria, ribosome-rich cytoplasm, sarcotubular systems, and cytoplasmic areas rich in loosely organized contractile filaments.

TABLE III
DISTRIBUTION OF SPECIFIC INTRACELLULAR α -BUNGAROTOXIN BINDING SITES

Treatment of muscle cells	Density of silver grains* over						Total area μm^2	Total silver grains
	RER‡	Golgi apparatus	Perinuclear area	Nucleus	Myofibrils	Other cytoplasm		
			<i>grains/100 μm^2</i>					
A. Cells grown in medium containing unlabeled α -bungarotoxin, fixed, saponin treated, incubated with [^{125}I]BuTX.	0	167	32	2	5	3	19,150	1,728
B. As in A, except protein synthesis blocked 4-5 h before fixation.	§	26	8	10	10	7	14,900	1,755

* Grain densities were corrected for nonspecific binding, which was (in grains/100 μm^2) RER, 79; Golgi apparatus, 32; perinuclear area, 8; nucleus, 5; myofibrils, 4; and other cytoplasm, 4. For determination of nonspecific binding densities (silver grains due to [^{125}I]BuTX bound in the presence of 1.5×10^{-4} M *d*-tubocurarine) 489 grains were scored over 11,950 μm^2 .

‡ Due to the small area of RER and small number of silver grains overlying RER, the computation of specific binding to RER is subject to large error.

§ Ribosomes are released from RER by puromycin treatment.

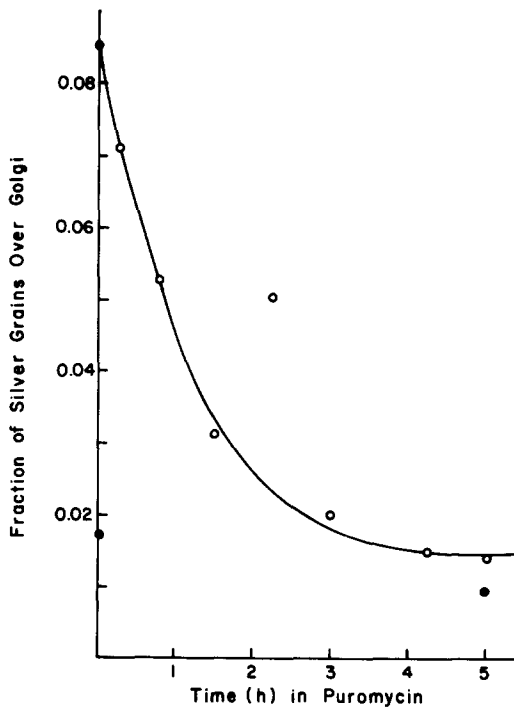


FIGURE 4 Association of ACh receptors with Golgi apparatus after inhibition of new receptor synthesis with puromycin (20 $\mu\text{g}/\text{ml}$). Cells were treated as described in the legend of Fig. 2 and further processed for electron microscope autoradiography, and the distribution of silver grains was determined. From left to right, the total grains scored for each point (O) were 1,728, 693, 1,846, 1,004, 701, 247, 780, and 1,157. Closed circles are controls in which specific binding was blocked by 1.5×10^{-4} M *d*-tubocurarine. Data are expressed as fraction of all silver grains (due to nonspecific as well as specific binding of [^{125}I]BuTX) directly over Golgi membranes and not covering any portion of nuclear membrane.

major reasons for the lack of firm evidence include difficulties in completely purifying plasma membranes and cell organelles and in identifying *bona fide* plasma membrane proteins.

Besides the ACh receptor, a few other membrane proteins (including antigen receptors [15], the membrane glycoprotein of vesicular stomatitis virus [2] and HeLa cell integral membrane proteins [1]) are known to be synthesized many minutes to several hours before their appearance in plasma membranes. However, the intracellular membrane systems involved in the transport of these proteins to the cell surface are unknown. There is strong evidence that the membrane glycoprotein, rhodopsin, is synthesized in the rough endoplasmic reticulum and passes through the

Golgi apparatus before insertion into rod outer segment plasma membranes (11, 16).

In the present study, we sought the subcellular location of the newly synthesized ACh receptors in chick skeletal muscle. These receptors are characterized by the criteria that they (a) are unavailable for interaction with extracellular receptor ligands, (b) are made available for ligand binding by techniques which render lipid bilayers permeable to large molecules, (c) constitute about 10–15% of all the receptor sites and (d) decrease steadily in number when protein synthesis is inhibited until there are few such sites after 3–4 h (5, 6, 7, 12). Furthermore, we know from previous studies that the pool of newly synthesized intracellular receptors represents about 50% of the intracellular receptor sites and that these new receptors are quantitatively transferred to the plasma membrane in the absence of protein synthesis (5, 6, 7, 12).

In this report, we demonstrate that an intracellular population of ACh receptors equal to 20–28% of surface receptors is revealed by saponin treatment of fixed muscle cells. About 50% of these receptors (thus, about 10% of all receptor sites in the system) disappear from the intracellular compartment after inhibition of protein synthesis, with kinetics similar to the kinetics previously found (5, 6, 7, 12) for the appearance of a comparable number of receptors on the cell surface. The receptors associated with the Golgi apparatus and perinuclear regions of the cells account for most of the receptors lost from the intracellular compartment when protein synthesis is inhibited. Judging both from the fraction of silver grains directly over the Golgi apparatus and from the time-course of decline in the association of ACh receptors with the Golgi apparatus when protein synthesis is blocked, the proportion of newly synthesized ACh receptors normally located in the Golgi apparatus must be large, perhaps 50–70% of these receptors. The Golgi apparatus must be the predominant location of new ACh receptors during the first half of their intracellular residence time.

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