Metabolism of Acetylcholine Receptors in Skeletal Muscle [1, 2]

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ABSTRACT—The acetylcholine receptor in skeletal muscle is an integral plasma membrane glycoprotein. Its biosynthesis and incorporation into plasma membrane and its degradation are being studied with the use of biochemical, biophysical, and microscopic techniques. In this report, previously published data are combined with new information to yield a consistent and fairly detailed description of the mechanisms involved in receptor metabolism. It is proposed that the biosynthesis, transport, and incorporation of the receptor into plasma membranes involve a mechanism similar, or identical, to that used by the cell for production and secretion of secretory proteins. The receptor is degraded by a random-hit process, which involves internalization, transport to secondary lysosomes, and hydrolysis. Sites of regulation of receptor metabolism are discussed in the context of regulation of the number and distribution of receptors in plasma membranes, particularly with respect to the formation and stability of neuromuscular junctions.—Natl Cancer Inst Monogr 48: 277–294, 1978.

THE ACETYLCHOLINE RECEPTORS

Acetylcholine (ACh) receptors from vertebrate skeletal muscle and electric organs are large glycoproteins tightly associated with cell membranes. The ACh receptors from various species are homologous molecules, antigenically cross-reactive and closely similar in overall physicochemical properties. These receptors are soluble in aqueous solution as detergent–receptor complexes which retain the binding sites for ACh and for various agonists and antagonists (*1–6*). The functions of the ACh receptor in the plasma membrane are to recognize ACh (which is the neurotransmitter substance released by the motor neuron) and to initiate changes in permeability in the muscle or electroplax membrane (*7*) that eventually lead to muscle contraction or to electric discharge.

The ACh receptor is one of a small set of eukaryotic plasma membrane proteins, which has a known function, whose number and distribution on cells can be determined, and whose metabolism can be quantitatively measured and studied. The ACh receptor is even more attractive as an object for study, because the number and distribution of receptors on skeletal muscle fibers vary in different developmental and physiologic states. These states can be experimentally produced in animals and in cell culture, thus providing us with the opportunity to investigate mechanisms controlling the number and distribution of receptors. Although the ACh receptor–skeletal muscle system is a highly specialized one, the molecular mechanisms involved in ACh receptor metabolism and in the determination of the number and distribution of ACh receptor on the cells are likely to be mechanisms common to the metabolism and regulation of many integral membrane proteins.

Great advances in the study of ACh receptors were made possible by the discovery of α-bungarotoxin (*8*), a small protein (mol wt 8,000 daltons) from the neurotoxic venom of the Formosan banded krait, *Bungarus multicinctus*, which is related to the cobras. Chang and Lee (*8*) purified α-bungarotoxin and demonstrated that it blocked neuromuscular transmission in an apparently irreversible manner. Later, Lee and colleagues (*9*) showed that radioiodine-labeled α-bungarotoxin retained the potency of the native toxin and could be used as a probe for determining the number and spatial distribution of ACh receptors. Many different derivatives of α-bungarotoxin, including acetyl-, fluorescein-, and peroxidase-bungarotoxin have subsequently been used. Homologous toxins from other neurotoxic snakes have also been used in receptor purification and characterization (*10, 11*).

ACh RECEPTORS IN MUSCLE DIFFERENTIATION AND MATURATION

The fine details of muscle morphology and differentiation vary from one species to another and even from one muscle to another, but the major features are similar, and the manner in which ACh receptors are produced and distributed in the muscle fiber membrane are probably common to all vertebrates. Figure 1 shows a low-power magnification of a portion of rat diaphragm muscle that has been stained to reveal the location of neuromuscular junctions and the main branches of the phrenic nerve. The muscle fibers run in parallel from one end of the muscle to the other. Each muscle fiber is a multinucleate cell of a diameter about 50 μ, and near the midpoint of each fiber is a single point of functional connection between the muscle fiber and an axon branch from one motoneuron.

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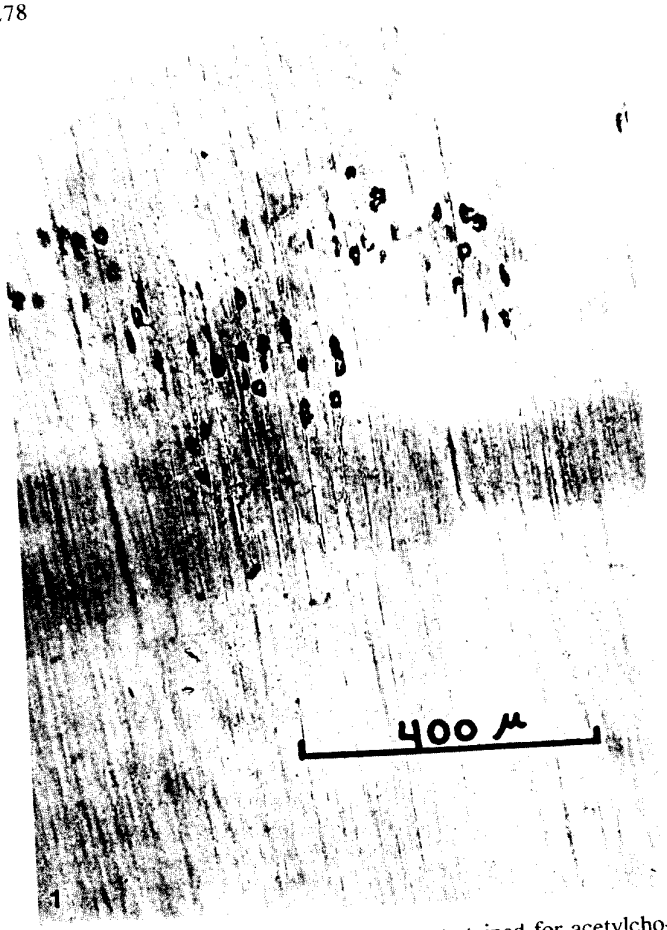


FIGURE 1.—Rat diaphragm whole-mount stained for acetylcholinesterase and to reveal location of major nerve trunk.

This connection, the neuromuscular junction, involves about 1/1,000 to 1/10,000 of the surface area of the muscle fiber. Nearly all the ACh receptors are located in this small area [(*12, 13*); *see* fig. 2]. In fact, receptors are packed into the juxtaneural portions of the muscle plasma membrane at a packing density of about 30,000 receptor sites per μ^2 of membrane area (*14*). This represents close packing, and indeed, it is probable that little other protein is in these areas of membrane. Elsewhere in the plasma membrane of the muscle fiber little or no responsiveness to ACh is found and, judging by the binding of α-bungarotoxin, fewer than 5 ACh receptor sites per μ^2 are present (*15*).

During muscle development, there is a point at which myoblasts cease to divide and then begin to elaborate gene products characteristic of the differentiated state, including ACh receptors. Little definitive information is known about the mechanisms involved in this gene expression and whether the various characteristics of skeletal muscle are coordinately controlled at the gene level. The usual result of these differentiative events is that myoblasts fuse into multinucleate myotubes and ACh receptors appear as functional molecules on the cell surfaces (*16*). These events readily occur in tissue cultures of myogenic cells. The density of ACh receptors in the plasma membranes of the myotubes developing in cell culture rises to about 1,000 sites per μ^2 [(*17*); *see* fig. 3]. During maturation of myotubes into skeletal muscle fibers which resemble those found in adult muscle in vivo, some clustering of ACh receptors into small patches of high receptor packing density occurs (*18–21*). Subsequently, the number of ACh receptors declines, and the receptor patches disappear. When myotubes are innervated by nerve processes from motoneurons, ACh receptor clusters appear at the points of innervation (*22*). The generalized sensitivity declines slowly, so that by several weeks postnatal (in the rat), the ACh receptor distribution is just like in the adult (*23*).

Denervation of adult skeletal muscle results in a dramatic increase in the number of ACh receptors in the

FIGURE 2.—Autoradiograph of end-plate region of a single skeletal muscle fiber after incubation with [^{125}I]α-bungarotoxin and staining for the enzyme acetylcholinesterase.

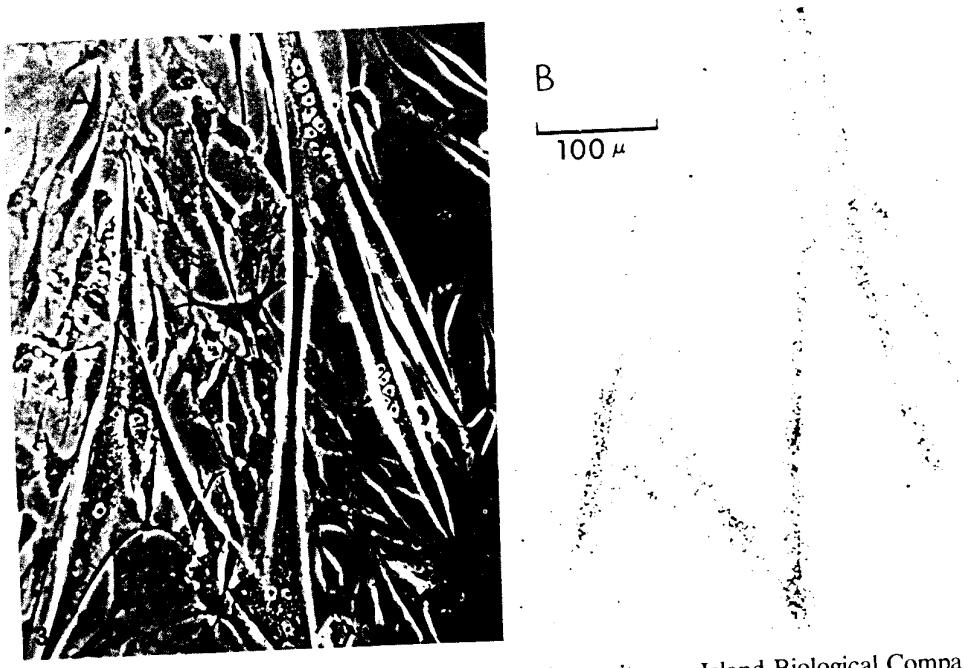


FIGURE 3.—Autoradiograph of tissue-cultured rat muscle labeled with [^{125}I]α-bungarotoxin. A) Phase contrast photomicrograph of a living myotube. Cells were fixed in glutaraldehyde and autoradiography was performed. B) Bright-field photomicrograph of the grains produced by the bound [^{125}I]α-bungarotoxin. [Reproduced with permission from (*17*).]

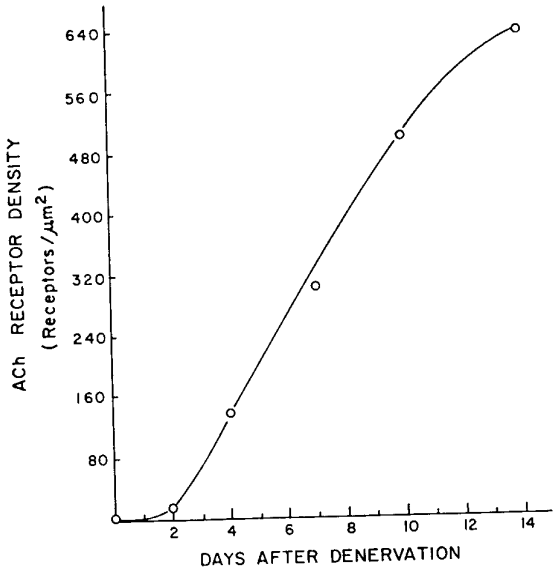
muscle fiber plasma membrane (text-fig. 1). These sites appear as the result of de novo synthesis of ACh receptors (*25, 26*). The extrajunctional ACh receptor site density increases to about 500 sites per μ^2, the exact number and time course depend on the muscle and probably the age of the animal and other factors. The receptors that were located at the neuromuscular junctions before denervation remain in this position, and their number does not change greatly with time (*15, 27*).

It is useful to consider four problems associated with ACh receptor number and distribution. First, there is the problem of gene expression during myogenesis. Whereas the ACh receptor is an excellent marker, it represents only about 1 part in 10^5 of the mass of the cells, and thus few gene copies and few transcriptional and translational events are required. Moreover, the receptor is composed of several subunits. Thus no particular advantages but several disadvantages are present when the ACh receptor–skeletal muscle system is used for studies of gene control during differentiation. Second, there is the problem of ACh receptor metabolism: The production and degradation of ACh receptor molecules results in the accumulation of receptors in the plasma membrane and the maintenance of an ACh receptor population in the membrane. Third, receptor metabolism is modulated by muscle activity and by neurotrophic effects. Fourth, ACh receptors form clusters at neuromuscular junctions and become metabolically more stable. In this paper, the main features of receptor metabolism are discussed, and some data and inferences regarding regulation of receptor number and distribution and the formation of synapses are presented.

MATERIALS AND METHODS

Materials.—Eagle's Minimum Essential Medium (EMEM) and horse serum were obtained from Grand Island Biological Company (Grand Island, N.Y.). ^{2}H-, ^{13}C-^{15}N-amino acid mixtures (98%, ^{2}H; 80%, ^{13}C; 80%, ^{15}N), prepared from algal hydrolysates, as well as deuterium oxide were obtained from Merck Sharp & Dohme (St. Louis, Mo.). Sodium cholate was purchased from Calbiochem (Gaithersburg, Md.). All other chemicals were obtained from commercial sources in the United States.

α-Bungarotoxin was purified, iodinated, and the iodinated form purified and characterized as described in previous publications [(*28*) and references therein].



TEXT-FIGURE 1.—Time course of increase in extrajunctional ACh receptors following denervation of rat hemidiaphragm. Data are from (*24*).

Tissue and organ culture.—Myogenic cells were prepared for tissue culture by dissection of muscle masses from rat and chick embryos and dissociation of cells by trypsin treatment or mechanical disruption, as described in our previous publications (*16, 28*). Rat myogenic cultures were grown in a modified Ham's F12 medium containing 10% horse serum and 2% chick embryo extract. Chick myogenic cells were grown either in this medium or in EMEM with 10% horse serum and 2% chick embryo extract (EMEM 210). These media were buffered with a bicarbonate buffer (18 mM) and maintained in a 5% CO_2–air mixture at 36° C. In some experiments, the medium was replaced with one in which 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) replaced bicarbonate buffer, and the cultures were maintained in a 37° C room without gassing. In some experiments, chick muscle was grown in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids, which was prepared as follows. Each 100 ml medium contained 9 ml of 10X Hanks' balanced salt solution (HBSS), 1 ml minimum essential vitamins, 10 ml horse serum, and 2 ml embryo extract (both exhaustively dialyzed against HBSS), 100 mg dextrose, 1.5 mg cysteine, 0.4 mg tryptophan, 0.7 mg glutamine, and 50 mg of ^{2}H-, ^{13}C-, ^{15}N-amino acid mixture. The commercial amino acid mixture was dissolved in water and put through an Amicon UM-2 filter before use. When cells differentiated in medium containing the ^{2}H-, ^{13}C-, ^{15}N-amino acids, myotubes were thinner than controls, and the extent of fusion did not appear to be as great. ^{2}H-, ^{13}C-, ^{15}N-amino acids had no effect on morphology of myotubes that had fused in normal medium, and the adverse effects of the ^{2}H-, ^{13}C-, ^{15}N-amino acids on differentiating cells could be reversed by switching to normal medium.

Adult skeletal muscles, usually the extensor digitorum longus (EDL) or soleus (SOL) or the left or right hemidiaphragm from mice or rats, were grown in organ culture in Trowell T-8 medium, with or without 0.5% bovine serum albumin (BSA) (*29*). Two types of culture apparatus were employed: culture in petri plates with the muscles stretched by pinning or tying across stainless-steel grids (*30*) and a perfusion chamber in which warm medium dripped over the muscle and was removed at a rate of about 40 ml/hour (*31*). In both, cultures were gassed with 5% CO_2/95% O_2.

Formation of monoiodo-α-bungarotoxin–receptor complexes and preparation of extracts.—Cultures were usually incubated at 37° C for 20–30 minutes in medium containing [^{125}I]- or [^{131}I]-labeled α-bungarotoxin. Unbound toxin was removed by repeated rinses of the cultures with HBSS titrated to pH 7.2 and containing 0.5% BSA. The monoiodo-α-bungarotoxin–receptor complexes were then extracted from the cells with a solution containing 2.5% sodium cholate (wt/vol) or 1% Triton X-100 (wt/vol) and 10 mM Tris–HCl buffer, pH 7.8. At 37° C, the myotubes bind about 130 to 150% as much [^{125}I]monoiodo-α-bungarotoxin as they do at 4°C. All the α-bungarotoxin binding sites have a sedimentation coefficient of about 10S and, therefore, all are associated with ACh receptors. These additional sites react slowly with α-bungarotoxin. The additional binding sites observed at 37° C have been termed "hidden sites" and have been previously reported (*28, 31*). The hidden sites behave metabolically like surface sites; i.e., the rate constant for receptor degradation (which has been previously reported to be 0.03 hr^{-1}) is independent of the point to which the α-bungarotoxin–myotube interaction has progressed. The hidden sites are possibly surface receptors located on complex infoldings of the surface membrane, and the slow rate of binding of α-bungarotoxin to these sites might be due to a diffusional barrier. Complete characterization of the hidden sites will be presented elsewhere. For the present discussion, it is only important to note that at least 6 hours are required to saturate completely all the receptors exposed to the extracellular medium.

Soluble receptors in detergent solution were labeled with α-bungarotoxin under the following condition, and the complexes formed were separated from free α-bungarotoxin by Biogel P-60 chromatography. Nonspecific binding of α-bungarotoxin to detergent extracts of chick myogenic cultures has been an obstacle in reliable preparation of soluble α-bungarotoxin receptor complexes from precursor pools (*28*). Nonspecific binding is characterized as α-bungarotoxin binding that emerges in the void volume of Biogel P-60 columns and sediments in the 2S–6S region of sucrose gradients. Binding of α-bungarotoxin to the nonspecific sites is not *d*-tubocurarine-competible. It presents a range of reaction rates, some of which are higher than for α-bungarotoxin–receptor complexes. Empirically derived methods eliminate or greatly reduce the amount of nonspecific binding. Extracts in 2.5% sodium cholate, 10 mM Tris (pH 7.8), were preincubated at 37° C for 2 to 3 hours. [^{125}I]α-bungarotoxin was then added to the extract at a final concentration of 0.01 μg/ml, and incubation was continued at 37° C for an additional 20 to 30 minutes. The reaction was terminated by loading the reaction mixture onto Biogel P-60 columns equilibrated with 1% sodium cholate, 10 mM Tris (pH 7.8). α-Bungarotoxin–receptor complexes are freed of unbound α-bungarotoxin by chromatography on 13-ml Biogel P-60 columns poured in 10-ml disposable plastic pipettes. Under these conditions, about 70 to 100% of the radioactivity emerging in the void volume in typical pool assays is α-bungarotoxin–receptor complexes. The preincubation at 37° C results in a great reduction in the amount of nonspecific binding with no detectable loss in receptor activity. That is, reactions run at 22° C with no preincubation yield the same number of α-bungarotoxin–receptor complexes but a great deal more nonspecific binding.

Sedimentation value of receptors.—In the course of the experiments reported here, we have repeatedly determined the sedimentation coefficient (S) of α-bungarotoxin–receptor complexes. The S-value of α-bungarotoxin–receptor complexes has been extensively reported to be 9S to 10S, and we find that the S-value of α-bungarotoxin–receptor complexes extracted from embryonic chick myotubes is, similarly, about 10S (*1, 28*). However, we noticed that the manner in which the α-bungarotoxin–receptor complexes are formed and handled can have an effect on the S-value. For instance, when α-bungarotoxin–receptor complexes are formed on intact myotubes and subsequently extracted

in nonionic detergents, the value of the complexes is about 10S. On the other hand, if receptors are first extracted into nonionic detergents and reacted with α-bungarotoxin in solution, the coefficient of the complexes is about 9.5S. Preliminary evidence also suggests that heat treatment of 10S complexes converts them to 9.5S complexes. We do not know the significance of this small shift or whether the shifts produced by the various treatments are equivalent. However, comparisons of α-bungarotoxin–receptor complexes formed or handled by various protocols can lead to artifacts. No difference in S-value has been observed among the α-bungarotoxin–receptor complexes formed from surface, "hidden," pool, and newly incorporated surface receptors as long as the complexes were formed and handled identically. Accordingly, when comparisons were made between [^{125}I] and [^{131}I]monoiodo-α-bungarotoxin–receptor complexes, each type of complex was formed in an identical manner.

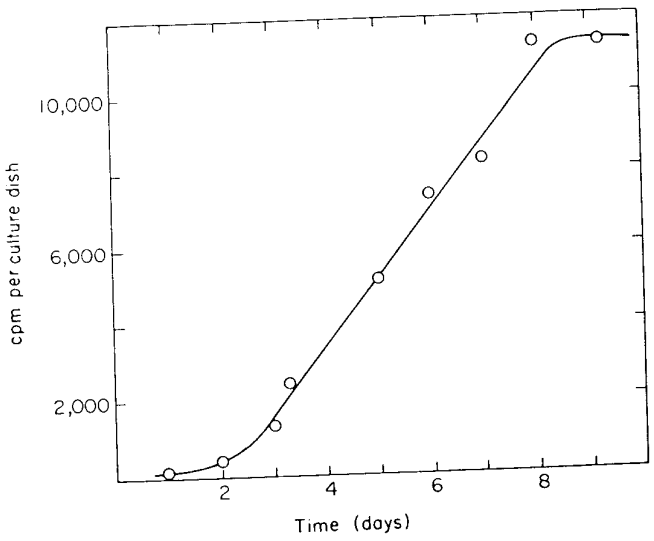
An estimation of the magnitude of the density shift of the receptor was calculated from the rates of sedimentation of ^{1}H-, ^{12}C-, ^{14}N-receptors and ^{2}H-, ^{13}C-, ^{15}N-receptors (α-bungarotoxin–receptor complexes) in shallow sucrose gradients of various average densities. Densities for the detergent–α-bungarotoxin–receptor complexes were derived by extrapolation with the use of the analysis of Martin and Ames (*32*). Corrections were made for detergent binding (*33*) and for bound α-bungarotoxin. The theoretical, maximum density shift was calculated, with the amino acid composition of the eel receptor (*34*) as an approximation of that for the chick receptor. No correction was made for the contribution of carbohydrates to receptor density. The density shift technique as applied to ACh receptor biosynthesis is described in much greater detail elsewhere (*35*).

Other methods.—Also mentioned in this report are experiments requiring various other techniques including electron microscope autoradiography, electrophysiologic procedures, routine biochemistry, and protocols for handling myogenic cell cultures. These techniques were described in previous publications, and references to these publications are made in the text at appropriate places.

RESULTS

Metabolism of ACh Receptors in Tissue Cultured Skeletal Muscle

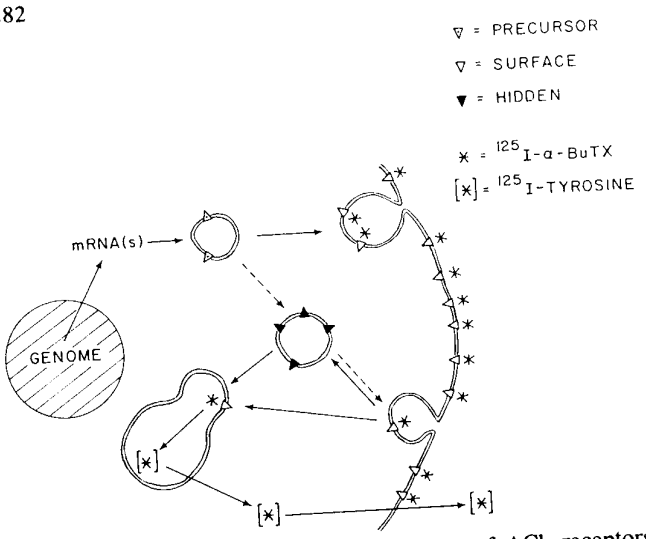
When myogenic cells are plated on collagen-coated petri dishes and cultured in appropriate medium, the cells divide and move about in the culture and then, after about 2 days, begin to fuse to form multinucleate myotubes. At this time, a large increase in the number of ACh receptors begins. ACh receptors accumulate, and the surface area of the myotubes increases by fusion of more myoblasts and by enlargement of cell girth. Text-figure 2 illustrates a time course of ACh receptor accumulation in cultured chick skeletal muscle. Most of our studies of receptor metabolism have been done on cultures that are in the period of rapid accumulation of ACh receptors. The time course of ACh receptor accumulation depends on plating density



TEXT-FIGURE 2.—Accumulation of ACh receptors as a function of culture age. A large set of chick muscle cultures, prepared by mechanical disruption of 11-day chick embryo leg muscle and plating at 5×10^{5} cells/35-mm petri dish, were cultured in F12 at 37° C. At each of the indicated times, [^{125}I]monoiodo-α-bungarotoxin was added to the culture medium of a subset of cultures so that the final concentration was 0.2 μg/ml. After 60 min, unbound [^{125}I]monoiodo-α-bungarotoxin was removed by three 10-min rinses in HBSS containing 0.5% BSA. Amount of bound radioactivity was then determined by scintillation counting. [Reproduced with permission from (*28*).]

and feeding of the cells, the choice of culture medium, selection of horse serum, and other details of culturing. However, there is always a period of relatively rapid ACh receptor accumulation. During this period and later, when the number of ACh receptors is no longer increasing, the rate of accumulation (whether positive, zero, or negative) is the result of the difference in rates of new ACh receptor *incorporation* into the plasma membranes and *degradation* of these receptors. Incorporation and degradation are more fundamental aspects of ACh receptor metabolism. Each can be measured by a simple strategy.

Our studies of ACh receptor incorporation into plasma membranes and degradation have led us to propose a model for the major events in receptor metabolism illustrated in text-figure 3 (*31*). The protein subunits of which the ACh receptor is composed are synthesized with the usual protein anabolic mechanisms of the cells and are rapidly assembled into receptor units resembling those in the plasma membrane. These newly synthesized ACh receptors occur on some membrane system(s) in the cytoplasm of the muscle cells and are collectively referred to as the "precursor pool." The average residence time of a new ACh receptor in the precursor pool is 3 hours. Receptors are transferred from this pool to the surface by an energy-requiring process, and they are then able to function. The average lifetime of an ACh receptor in the plasma membrane is 22 hours. Receptors in the plasma membrane may exchange with a class of receptors termed



TEXT-FIGURE 3.—Hypothetical "life cycle" of ACh receptors in cultured chick skeletal muscle. Cross-section through a myotube with receptors symbolized as triangles in membrane profiles. *Solid arrows* indicate processes consistent with data on receptor metabolism. *Dashed arrows* indicate processes which are neither supported nor strongly counter-indicated by present data. ^{125}I-α-BuTX = [^{125}I]α-bungarotoxin. [Reproduced with permission from (*31*).]

"hidden." The significance of hidden receptors is not clear but their presence complicates the design of experiments that measure biosynthesis and incorporation of new receptors into plasma membrane. The surface and hidden ACh receptors appear to be metabolically equivalent and have the same fate. Receptors are degraded by an energy-requiring process which involves the internalization of the receptors and transport to secondary lysosomes and then proteolytic destruction.

Biosynthesis and Incorporation

A simple strategy was devised (*17*) for the study of just the appearance of recently synthesized ACh receptors on the cell surface. First, all the receptors on the surfaces of the muscle fibers are blocked with α-bungarotoxin.[7] Then the appearance of new receptors is monitored either by iontophoresis and intracellular recording or by the binding of [^{125}I]-labeled α-bungarotoxin (fig. 4). Autoradiographic studies indicate that new binding sites appear uniformly over the myotube surface [(*17*); unpublished observations].

[7] This block is not significantly reversible in the time course of the experiments. Most of the cell surface ACh receptors are saturated by exposure to 0.1 μg α-bungarotoxin/ml for 30 min. A more complete saturation is achieved by overnight exposure to 1 μg α-bungarotoxin/ml. This exposure saturates both surface and hidden receptors. [*See* (*28*) and (*31*) for definition and discussion of hidden receptors.] As far as we know at present, no distinction between surface and hidden receptors is made by the metabolic processes of the muscle fibers. Thus further discussion of hidden receptors in this context would needlessly complicate the issues.

Several lines of evidence indicate that this strategy monitors the appearance of newly synthesized ACh receptors. The most direct evidence comes from pulse labeling cells with deuterated or [^{13}C]-labeled amino acids and demonstrating that the newly appearing receptors are of increased buoyant density (*26*). The density shift method has now been used with triple-labeled amino acids (^{2}H, ^{13}C, ^{15}N) (text-fig. 4), and the kinetics of shift in receptor buoyant density after switching cells to labeling medium have been investigated (*35*). An estimate of the apparent density shift between ^{1}H-, ^{12}C-, ^{14}N-receptors and ^{2}H-, ^{13}C-, ^{15}N-receptors has been obtained from measured sedimentation rates of the respective bungarotoxin–receptor complexes in shallow sucrose gradients of different average densities (*32*). The average density of the gradients was varied by substitution of D_2O for H_2O. The calculated density shift was 0.09 g/cm^3, corresponding to approximately a 6% shift in buoyant density. The maximum attainable shift [calculated from the amino acid composition of purified receptors from *Electrophorus electricus* (*34*)] is about 8.5%. Since the calculated shift is a minimum estimate, a substantial fraction of the amino acyl residues of the receptor must be substituted with ^{2}H, ^{13}C, and ^{15}N.

The model for ACh receptor biosynthesis suggests that receptors undergo a 3-hour transit time before being inserted into the plasma membrane. This feature of the model has been verified by measurement of the kinetics of incorporation of ^{2}H-, ^{13}C-, ^{15}N-amino acids into the precursor pool and into the surface ACh receptors. Receptors in the precursor pool are labeled first, and this labeling saturates at about 4 hours (text-fig. 5). Dense receptors begin to appear in the plasma membrane slowly but after the precursor pool is saturated, essentially all the receptors appearing on the surface are density-shifted ones (text-figs. 6, 7). This confirms the "precursor–product" relationship between the precursor pool and the surface receptors.

Since small amounts of density-shifted receptors appear on the surface at 2 and 3 hours after switching the cells to labeling medium, it is suggested that the intracellular transport of receptors is not a strictly linear, assembly line process. That is, newly synthesized receptors and receptors already in transport can become, to a certain extent, intermixed and inserted at random into the plasma membrane. Alternatively, the appearance of small amounts of receptors at 2 and 3 hours after synthesis might occur in a subpopulation of myotubes in which the transit time is shorter than average.

Metabolic inhibitor studies are consistent with the isotopic labeling experiments (*17, 28, 31, 36*). Thus incorporation of new receptors into plasma membrane is inhibited by low temperature, inhibitors of protein synthesis, and compounds that interfere with ATP synthesis (text-fig. 8). Low temperature and uncouplers of oxidative phosphorylation rapidly inhibit incorporation. Inhibitors of protein synthesis, however, have no immediate reaction on receptor incorporation, but these inhibitors are effective after a delay of 2–3 hours. Similarly, after removal of protein synthesis inhibitors, a lag of 2–3 hours occurs

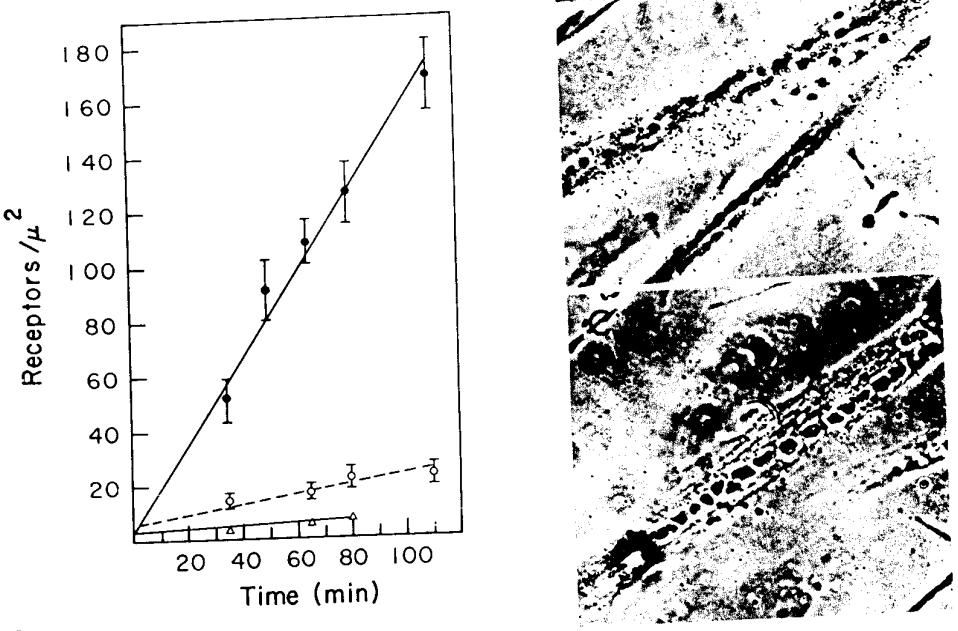


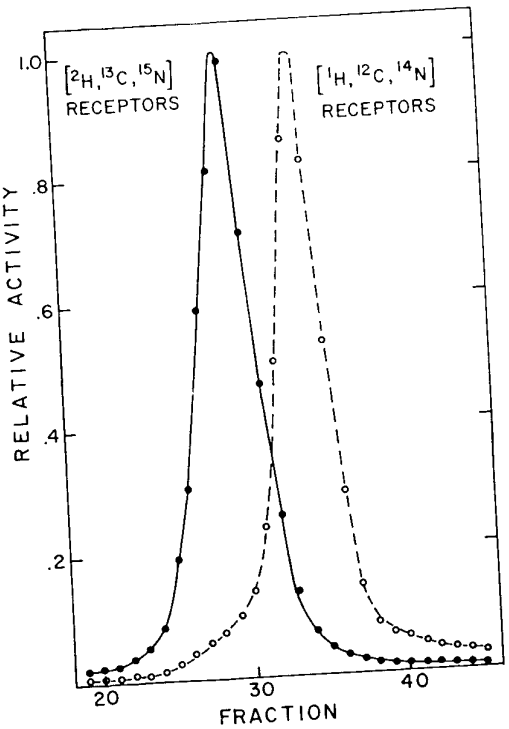
FIGURE 4.—Incorporation of new ACh receptors into myotube surface membranes. Following a saturating exposure to unlabeled α-bungarotoxin, the appearance of new α-bungarotoxin sites was determined by [^{125}I]α-bungarotoxin binding and autoradiography. •——• = control cells. o- - -o = cells pretreated 3 hr in 100 μg cycloheximide/ml. △——△ = cells incubated in 2×10^{-4} M dinitrophenol, 2×10^{-3} M iodoacetate. B), C) Autoradiographs of [^{125}I]α-bungarotoxin-treated myotubes after 90 min recovery from saturating, unlabeled α-bungarotoxin in control medium (B) and in cycloheximide-containing medium (C). [Reproduced with permission from (*17*).]

before incorporation resumes at the control rate (*31*). During inhibition of protein synthesis, the precursor pool is depleted (*28*). There is no time lag before the precursor pool begins to decrease in size. This suggests that no large supply of receptor subunits can, with time, assemble into receptor units capable of binding α-bungarotoxin. Presumably, the receptor precursors represent newly made and rapidly assembled receptor units present on cytoplasmic membranes. These receptors are probably in the process of being transported and glycosylated. As the detergent solubilized α-bungarotoxin–receptor complexes, all the precursor receptors are precipitable by concanavalin A and thus presumably already have some associated carbohydrate residues (unpublished observation). All the precursor receptors are present as membrane-associated molecules, judged by their co-sedimentation with membranes in cell homogenates and solubilization only with nonionic detergents.

Degradation of ACh Receptors

Because α-bungarotoxin is nearly an irreversible ligand for ACh receptor sites, a strategy for studying ACh receptor degradation is available. [^{125}I]α-bungarotoxin interacts with living muscle fibers to form strong complexes with the surface membrane ACh receptors; then the fate of the complexes is followed. Since dissociation of toxin and receptor is negligible on the time scale of hours and α-bungarotoxin is also a protein, the assumption is that the [^{125}I]α-bungarotoxin will remain bound to the receptor site until subjected to cellular degradative processes. Indeed, it was found that radioactivity is released from tissue-cultured muscle and from adult denervated muscle fibers after [^{125}I]α-bungarotoxin binding (text-fig. 9). This release involves mostly the hydrolysis of the radioactive α-bungarotoxin to a low-molecular weight radioactive species that appeared in the culture medium (*28, 38, 39*). Before the destruction of [^{125}I]α-bungarotoxin could be interpreted confidently as revealing the normal cellular process of ACh receptor turnover, it was necessary to show that 1) the breakdown of [^{125}I]α-bungarotoxin is due to cellular processes, 2) its binding does not initiate or stimulate the degradative process, and 3) the destruction of the labeled toxin is paralleled by a loss of cell surface ACh receptors. We have examined these aspects, using tissue cultured embryonic chick skeletal muscle (*28*).

This involvement of cellular degradative processes was first indicated by inhibitor studies. Low temperature, inhibitors of oxidative phosphorylation (cyanide, dinitrophenol, azide, and carbonylcyanide *p*-trifluoromethoxy phenylhydrazone), concanavalin A (unpublished observations), trypan blue (reportedly an inhibitor of lysosomal hydrolases), and cell disruption inhibit the destruction of bound [^{125}I]α-bungarotoxin. More recently, electron micro-

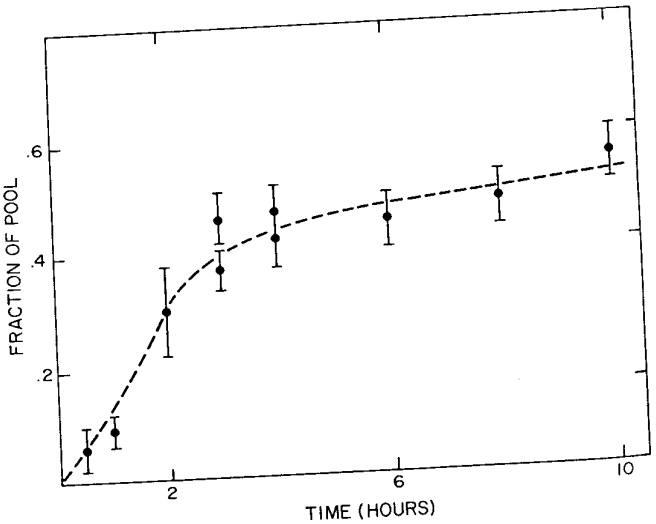


TEXT-FIGURE 4.—Density-shifted receptors from differentiating cells. Twenty large muscle cultures were grown for 18 hr in normal medium. At this time, there were few myotubes in culture and the myoblasts were fed with medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids. After the cells had fused in the density-labeled medium, the surface receptors were complexed with [^{125}I]monoiodo-α-bungarotoxin. Complexes were then extracted in 2.5% cholate and 10 mM Tris and mixed with a marker of [^{131}I]monoiodo-α-bungarotoxin–receptor complexes prepared from myotubes grown in normal medium. A 0.4-ml aliquot of the mixed extract was carefully layered over a 25–40% sucrose–deuterium oxide gradient containing 1% Triton X–100, 1 mM EDTA–phenylmethylsulfonyl fluoride, 10 mM Tris pH 7.8. Centrifugation was for 2 days in a Beckman SW41 rotor at 36,000 rpm at 4° C. Gradients were pumped into scintillation vials (0.2-ml fractions were collected), and the activity of each isotope was determined.

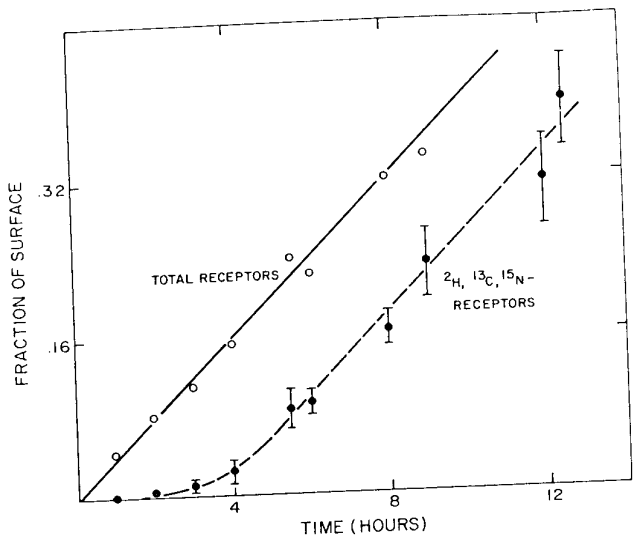
scope autoradiographic studies (*31*) implicate secondary lysosomes as the sites of degradation.

Determining whether [^{125}I]α-bungarotoxin degradation reflects the normal rate and mechanism of receptor degradation is more difficult, and, so far, the evidence is indirect. When protein synthesis is blocked by puromycin, incorporation of receptors into plasma membrane continues for 2–3 hours and then ceases. However, the degradation of [^{125}I]α-bungarotoxin bound to ACh receptors is only slightly affected by puromycin, and the rate of degradation does not change at the time incorporation ceases. Under these conditions, the loss of radioactivity from cultures to which [^{125}I]α-bungarotoxin had bound at 3 hours paralleled the loss of ACh receptors from muscle cells *which had not been exposed to α-bungarotoxin* (*28*). Thus the destruction of a molecule of α-bungarotoxin reflects the disappearance of an ACh receptor site, and the binding of the labeled toxin has no detectable effect on the rate of ACh receptor degradation. We are using the density-shift technique to investigate this matter further.

Many observations suggest that extrajunctional ACh receptors in cultured chick muscle have a biologic half-life of about 22 hours and are degraded by a "random-hit" process. This process involves the internalization of ACh receptors by the cell, transport to secondary lysosomes, and proteolytic destruction there. First, there are the inhibitor studies, which show an energy requirement for degradation and possibly implicate lysosomal hydrolases in the process. A second line of evidence is that when [^{125}I]monoiodo-α-bungarotoxin (bound to ACh receptors) is degraded by the cell, the major radioactive species liberated into the culture medium is [^{125}I]monoiodotyrosine (*28, 39*). The production of iodotyrosine from iodinated α-bungarotoxin requires at least two proteolytic cleavages, one on either side of the iodotyrosyl residue in the polypeptide chain. If degradation were extracellular, one would expect to find large fragments of degraded toxin in the medium. The degradation of iodinated proteins has been studied in other cell systems. For example, macrophages ingest [^{125}I]-labeled albumin, concentrate it in lysosomes, degrade it to yield [^{125}I]tyrosine, which rapidly appears in the culture medium (*40*). Similarly, when cell surface proteins of mouse L-cells are iodinated, they are likewise degraded to yield [^{125}I]tyrosine. When these cells ingest latex beads (and thus internalize much of their surface), degradation of iodinated cell-surface proteins is



TEXT-FIGURE 5.—Kinetics of appearance of ^{2}H-, ^{13}C-, ^{15}N-receptors in the intracellular pool. [^{125}I]monoiodo-α-bungarotoxin–receptor complexes involving the pool receptors from cultures incubated for the designated times in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids were prepared. These receptors were analyzed by sucrose gradient velocity sedimentation to determine the fraction of pool receptors which were ^{2}H-, ^{13}C-, ^{15}N-receptors.



TEXT-FIGURE 6.—Kinetics of appearance of total (^{1}H-, ^{12}C-, ^{14}N- plus ^{2}H-, ^{13}C-, ^{15}N-) and density-shifted (^{2}H-, ^{13}C-, ^{15}N-) receptors on myotube surfaces during incubation in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids. After long-term incubation in medium containing unlabeled α-bungarotoxin, cultures were rinsed to remove unbound α-bungarotoxin and cultured in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids. Receptors appearing on the myotube surfaces were labeled by addition of 0.15 μg [^{125}I]monoiodo-α-bungarotoxin/ml to the culture medium during the final 30 min. Unbound [^{125}I]monoiodo-α-bungarotoxin was removed by rinsing cultures in the cold, and the [^{125}I]monoiodo-α-bungarotoxin–receptor complexes were extracted and analyzed by sucrose gradient velocity sedimentation. The receptors from three to five 100-mm (diameter) cultures were used for each data point. Data from 2 experiments were combined by first subtracting the small background present at zero time and then normalizing the slope for total receptor appearance to 4% addition of new receptors to the total surface receptor population per hour, a typical rate of incorporation. The fraction of ^{2}H-, ^{13}C-, ^{15}N-receptors at each time point was calculated from areas under curves on sucrose gradients (*see* text-fig. 7).

accelerated, a reaction in which the lysosomal system has been implicated (*41, 42*).

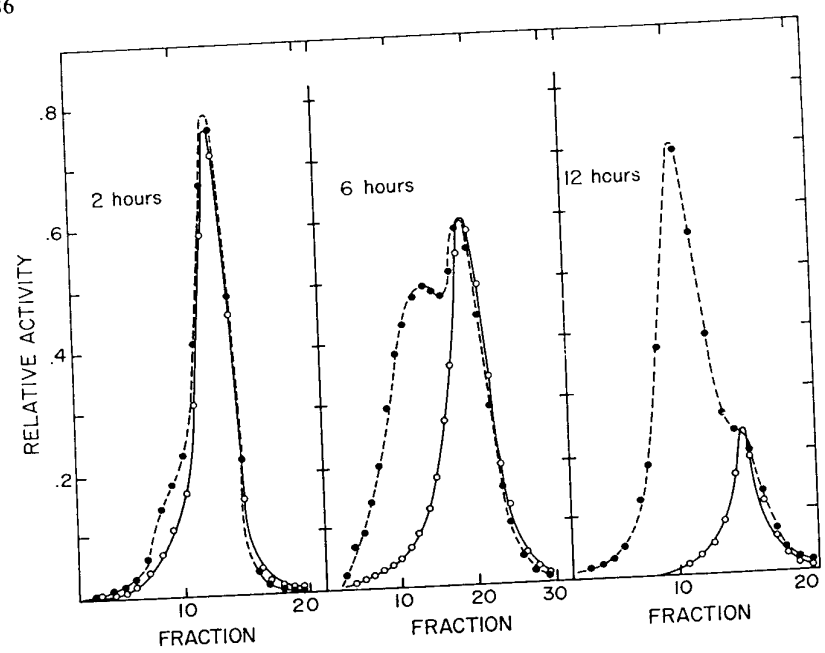
A third line of evidence for an intracellular degradation mechanism comes from high time-resolution analysis of the initial phase of degradation. Muscle cells were exposed to [^{125}I]α-bungarotoxin for 5 minutes, briefly rinsed, and then the medium was analyzed periodically for the amount of [^{125}I]tyrosine by chromatography on Biogel P-2. The rate of [^{125}I]tyrosine appearance in the medium accelerated with time, reaching a steady rate after about 90 minutes [(*28*); text-fig. 10]. Such a lag is predicted by a model of degradation in which internalization, transport, hydrolysis, and the outward diffusion of iodotyrosine occur before [^{125}I]tyrosine can be detected in the medium.

A fourth line of evidence for an intracellular degradation mechanism is provided by electron microscope autoradiographic studies [(*31*); previously unpublished observations]. In these studies, the location of radioactivity on and within cultured muscle cells was determined as a function of time after brief exposure of cells to [^{125}I]α-bungarotoxin. Most pertinent to the present discussion is that the large secondary lysosomes of the muscle cells accumulated radioactivity to a plateau level of slightly over 2% of total cellular radioactivity (fig. 5, text-fig. 11). This accumulation occurred with kinetics consistent with a model of degradation in which the transport of [^{125}I]α-bungarotoxin–receptor complexes to lysosomes and their hydrolysis in lysosomes results in the production of [^{125}I]tyrosine.[8] In the continued presence of trypan blue, cells that have been treated overnight with medium containing this stain transport [^{125}I]α-bungarotoxin–receptor complexes from their surfaces to secondary lysosomes but are unable to degrade these complexes. Thus radioactivity builds up in secondary lysosomes, amounting to about 20% of the radioactivity associated with the cells 6 hours after a brief treatment with [^{125}I]α-bungarotoxin. In detergent extracts from such cells, we found no evidence of partial breakdown products or free [^{125}I]α-bungarotoxin. Virtually all the radioactive material behaves like [^{125}I]α-bungarotoxin–receptor complexes when subjected to velocity sedimentation in sucrose gradients.

Turnover of Other Integral Membrane Proteins

Two points researchers need to know are: 1) whether the mechanism used by skeletal muscle fibers for the turnover of ACh receptors is also used in the turnover of other cell protein, and 2) whether the turnover rates of various plasma membrane proteins are the same or if some mechanism independently regulates the turnover rates of different membrane proteins. So far, no observations or findings suggest that the ACh receptor is unique in its mechanism or rate of turnover. Unfortunately, no other plasma membrane proteins in cultured skeletal muscle whose turnover rates can be measured with ease are known. Lacking a specific protein candidate, we have been using the lactoperoxidase iodination method to label some unidentified cell surface proteins, a technique developed (*41, 42*) for measuring the turnover of some membrane proteins in cultured mouse L-cells and HeLa cells. The application of this method to cultured chick muscle has been fraught with difficulties, but it has been possible to label muscle cultures (depleted of fibroblasts) by the method of Fischbach (*43*) and then to measure the production of iodotyrosine. As indicated above, this accompanies the degradation of some iodinated membrane proteins.

[8] In control experiments with *d*-tubocurarine added to the medium to prevent the specific interaction of [^{125}I]α-bungarotoxin with receptors, this association of radioactivity with lysosomes did not occur. However, when cells were maintained in the presence of high concentrations of free [^{125}I]α-bungarotoxin, uptake of the free labeled toxin into lysosomes did occur.



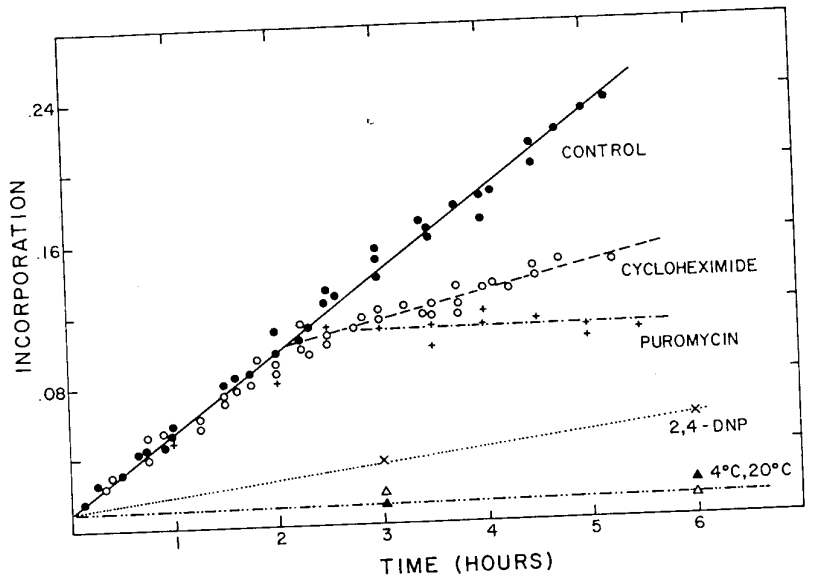
TEXT-FIGURE 7.—Sucrose-gradient, velocity sedimentation profiles of surface receptors appearing during culture of myogenic cells in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids. Gradient profiles illustrated here are those from which the data for the 2-, 6-, and 12-hr time points in text-figure 6 were obtained. A marker of [^{131}I]monoiodo-α-bungarotoxin-^{1}H-, ^{12}C-, ^{14}N–receptor complexes was mixed with sample before centrifugation, and its position in the gradient is identical to that of the [^{125}I]monoiodo-α-bungarotoxin–^{1}H-, ^{12}C-, ^{14}N-receptor complexes. *Note* the small number of ^{2}H-, ^{13}C-, ^{15}N-receptors present at 2 hr.

After labeling cells with I-125 by the lactoperoxidase method and then using [^{131}I]α-bungarotoxin to label ACh receptors, we measured the production of [^{125}I]tyrosine and the loss of I-131 from the cells (text-fig. 12). The [^{125}I]tyrosine represented a small fraction of the radioactivity released into the medium at any time, the remainder consisting of high-molecular weight material and a large amount of iodide. Apparently, some iodinated components are lost from the cells or substratum directly into the culture medium, whereas others are degraded to produce iodotyrosine. (We have not detected any labeling of the ACh receptor by lactoperoxidase technique.) The kinetics of production of iodotyrosine are similar to those of ACh receptor degradation. These data are consistent with the suggestion that many membrane proteins are degraded by the same mechanism and at the same rate as the ACh receptor.

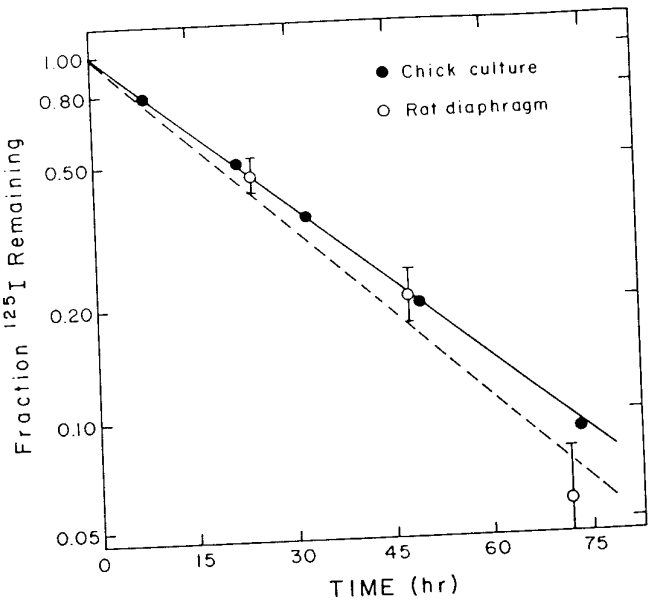
Regulation of ACh Receptor Metabolism

A Simple Mathematical Model

As indicated above, new extrajunctional ACh receptors enter the plasma membrane at a rate that is approximately constant. This is to be expected if the number of mRNA molecules for receptor subunits is not changing appreciably with time and if the rate of initiation and elongation of polypeptide chains is fairly constant. Let us define this rate of entry of receptors into the plasma membrane by the constant r, with units as receptors per hour. The



TEXT-FIGURE 8.—Incorporation of new ACh receptors into plasma membranes of cultured chick skeletal muscle after blockage of old receptors with unlabaled α-bungarotoxin. Unlabeled α-bungarotoxin was removed and inhibitors added at zero time (t = 0). Number of newly incorporated receptors has been normalized to total number of original surface receptors at t = 0. Data are from 5 experiments for cycloheximide, 3 for puromycin, 2 for 2,4-dinitrophenol (DNP), 1 for each temperature. Data for each individual experiment have been normalized so that control rate is 4.5%/hr. Actual control rates ranged from 3.8 to 5.1%/hr.

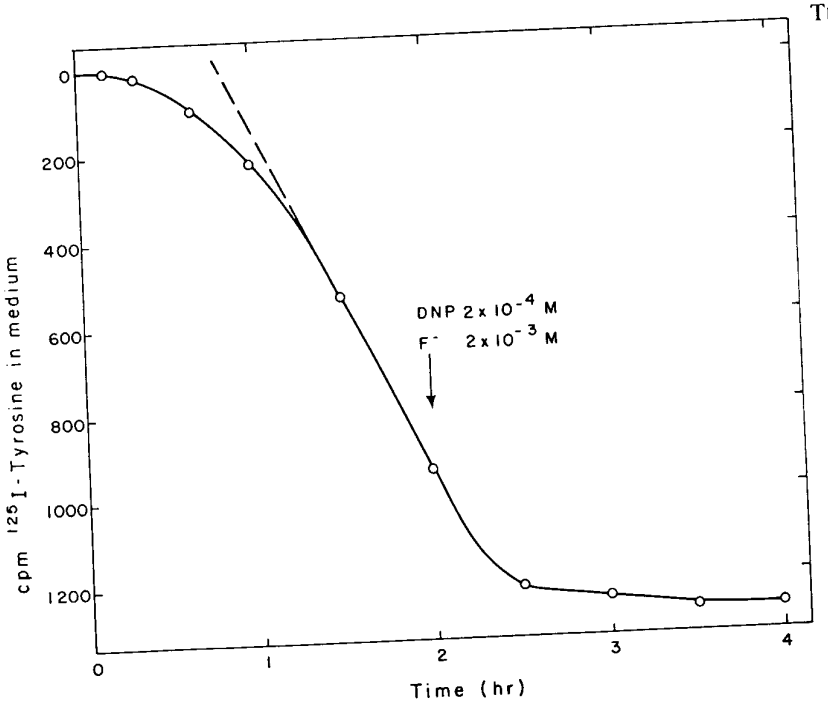


TEXT-FIGURE 9.—Release of radioactivity from cultured chick muscle (-•-•-) and from denervated rat diaphragm in vivo (- -o- -o- -) after blockage of ACh receptors with [^{125}I]α-bungarotoxin. Check data are from (*28*); rat data from (*37*).

kinetics of degradation of ACh receptors are not linear but are first-order exponential. Let us define the rate constant for degradation by the constant k, with units being reciprocal hours. (The half-life of ACh receptors is then 0.7/k.) In any steady state, i.e., when r and k have been fixed for a long time compared with the half-life of the ACh receptors, the number of receptors in the plasma membrane is constant and is equal to r/k.

Now let us consider circumstances in which the number of ACh receptors changes. Many occur, including the appearance of receptors during differentiation of skeletal muscle, the disappearance of extrajunctional receptors after innervation of embryonic muscle fibers, the appearance of extrajunctional receptors after denervation of adult skeletal muscle, and the disappearance of extrajunctional receptors caused by electrical stimulation of either denervated adult muscle or cultured embryonic muscle. In all, the skeletal muscles are displaying their ability to control ACh receptor metabolism. Controls that operate on the synthetic phase of receptor metabolism, regulating the production of mRNA, its translation, or the incorporation of newly synthesized receptors into plasma membrane, will cause a change in rate constant r in our formalization. Control mechanisms, which operate by changing the rate of receptor degradation or which cause receptors to disappear by any other random-hit process, will cause a change in rate constant k in our formalization. Any single perturbation of the system (i.e., a rapid change in r and/or k) should lead to a predictable new steady-state level of ACh receptors, and this would occur with predictable kinetics. Thus if we start with R_1 receptors being produced and degraded with rate constants r_1 and k_1, and then the rate constants suddenly change to r_2 and k_2, the number of receptors when a new equilibrium is established will be:

$$R_2 = r_2/k_2$$



TEXT-FIGURE 10.—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 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 [^{125}I]α-bungarotoxin/ml was added to the cultures. After 5 min, it was removed by six (30-sec) rinses with warm medium, which was then replaced at the indicated times and the used medium (3 ml/dish) was centrifuged briefly to remove cell debris. Then carrier I-tyrosine was added, an aliquot was fractionated on Biogel P-2, and the fractions were assayed for radioactivity by scintillation spectrometry. Radioactivity in the I-tyrosine peak was calculated and the cumulative total [^{125}I]tyrosine released was plotted as a function of time. After the maximal rate of [^{125}I]tyrosine release was obtained (as judged from other experiments of this type), medium containing 2,4-dinitrophenol and fluoride (F) replaced the normal medium. Zero time is the time of initial contact of [^{125}I]α-bungarotoxin and myotubes. [Reproduced with permission from (*28*).]

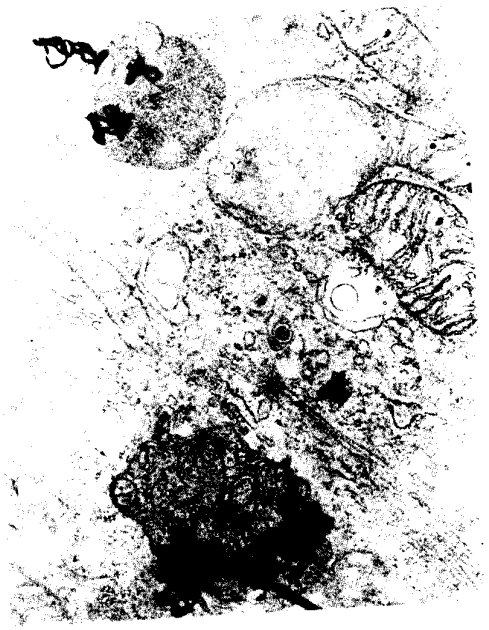
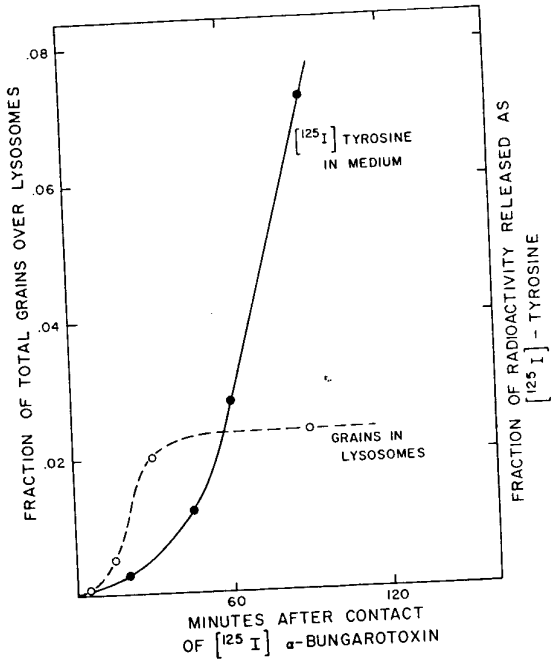


FIGURE 5.—Electron microscope autoradiograph of chick myotube after saturation of ACh receptors with [^{125}I]α-bungarotoxin, showing grains over electron-dense organelles resembling secondary lysosomes. [Reproduced with permission from (*31*).]



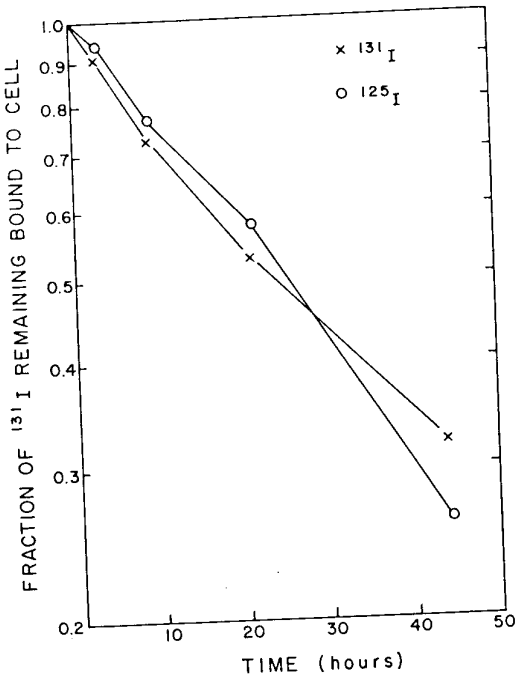
TEXT-FIGURE 11.—Kinetics of transport of radioactivity to secondary lysosomes (- -o- -o- -) and kinetics of liberation of iodotyrosine from chick muscle cultures (-•-•-) after brief exposure of muscle to [^{125}I]α-bungarotoxin. Data obtained from electron microscope autoradiograms and from chromatographic analysis of culture medium.

and at any intermediate time the number of receptors (R_t) will be:

$$R_t = r_2/k_2(1-e^{-k_2 t}) + R_1 e^{-k_2 t}.$$

Control Mechanisms Governing Extrajunctional Receptors

The above formalizations are useful in helping us to think about which steps in receptor metabolism are subject

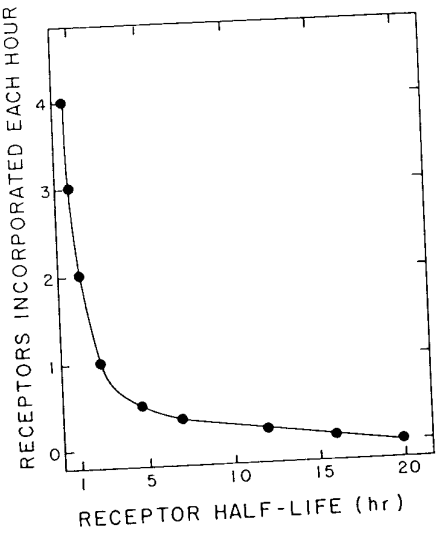


TEXT-FIGURE 12.—Loss of radioactivity from cultured chick myotubes after iodination and saturation of ACh receptors with iodinated α-bungarotoxin. Five-day muscle cultures, treated days 3–5 with 1 μM cytosine arabinoside (*43*), were rinsed three times with HBSS buffered with 18 mM HEPES, pH 7.2. Cells were then treated for 15 min at 22° C with 0.5 ml of a mixture containing 400 μCi/ml ^{125}I-Na, 20 μg/ml lactoperoxidase, 10 U glucose oxidase, and 10 mM glucose in HBSS. The reaction was stopped by addition of $Na_2S_2O_5$ to 40 μg/ml. After three rinses in HBSS, cells were rinsed overnight at 15° C in medium containing 0.1 μg [^{131}I]α-bungarotoxin/ml. After four 5-min rinses in HBSS, cells were incubated in culture medium at 37° C. At indicated times, medium was replaced and cells returned to 37° C. At 48 hr, cells were scraped from plates in phosphate-buffered potassium iodide solution and centrifuged at 2,000 rpm for 10 min, and pellets were dissolved in 1% Triton X-100. Medium samples and cell extract were fractionated on Biogel P-2 columns to separate iodotyrosine from iodide and high-molecular weight materials. The ratio of [^{125}I]iodotyrosine to [^{131}I]iodotyrosine was determined by scintillation spectrometry. This ratio remained constant, suggesting that [^{125}I]iodotyrosine was being produced by a cellular process in first-order exponentially decreasing amounts, as is true for the [^{131}I]iodotyrosine. Therefore, the [^{125}I]iodotyrosine data were fit to a first-order exponential curve and plotted (—o—o—); [^{131}I]iodotyrosine (—×—×—).

to regulation in real situations. This is illustrated in the following analysis of a particular circumstance: the disappearance of extrajunctional ACh receptors during electrical stimulation of denervated adult skeletal muscle [*see* (*44*) and references therein]. Following denervation, the number of ACh receptors in extrajunctional regions increases as a result of de novo receptor synthesis (*25, 26*) and ACh sensitivity of rat soleus muscle increases to a plateau level of about 250 millivolts/nanocoulomb (mV/nC). Electrical stimulation at 100 Hz, given once every 100 seconds, causes a fall in ACh sensitivity to about 1 mV/nC in 4 days and 0.2 mV/nC in 7 days (*44*). One can convert the sensitivity values into approximate ACh receptor numbers by using (as a relationship) that ACh sensitivity is proportional to the square of the number of ACh receptors per unit area of muscle plasma membrane (*15, 24, 45*).

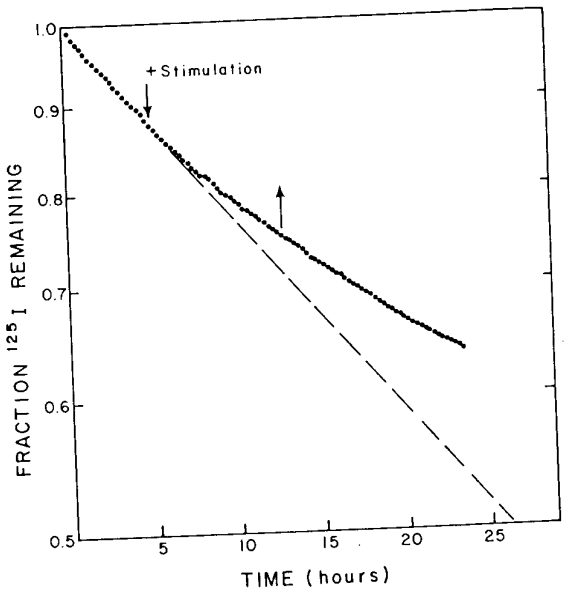
Now we can use pairs of these receptor numbers as R_1 and R_2 in the above equation and think about what changes in r and/or k are required to shift the number of receptors in this period of time. The changes in ACh sensitivity found by Lomo and Westgaard (*44*) are comparable to a change in receptor number from 100 to 3 receptors per unit area in 100 hours. This could be accomplished by 1) a complete inhibition of the production of new ACh receptors ($r = 0$) with no change in the degradation rate, assuming a receptor half-life of 20 hours, 2) at the other extreme, no change in new receptor production rate ($r = 3.5$ receptors/hr) but an acceleration of the degradation rate by a factor of more than 20, i.e., decreasing the half-life of the average receptor to less than 1 hour. If both incorporation and degradation rates were affected by electrical stimulation, the decrease in receptor number could be achieved, e.g., by an 80% inhibition of incorporation rate with a decrease in receptor half-life to 4 hours. Text-figure 13 is a plot of paired incorporation rates and receptor half-lives that will lead to a decline in extrajunctional receptors from 100 to 3 in 100 hours.

Although the incorporation rate is technically difficult to measure, the degradation rate can certainly be estimated within a factor of two, and much smaller changes in degradation rate could hardly go undetected in individual experiments. Measurement of ACh receptor degradation rates during electrical stimulation [(*46, 47*); text-fig. 14] show that the degradation rate is *not* stimulated; in fact, it is decreased. Thus the decline in ACh receptors during electrical stimulation must involve a large decrease in the rate of incorporation of receptors in the plasma membrane. In addition, the rate of change in receptor number during electrical stimulation should approximately match the kinetics of receptor degradation (see above). As shown in text-figure 15, this expectation is met.



TEXT-FIGURE 13.—Theoretical curve of rate of receptor incorporation into plasma membrane (new receptor sites per unit area per unit time) versus half-life of receptors in plasma membrane. Pairs of incorporation rates and half-lives falling on this curve represent those combinations which will lead to a decline in receptors per unit area from 100 to 3 in 100 hr. [Reproduced with permission from (*48*).]

The reversal potentials for cholinergic responses at junctions and extrajunctional sites are similar or identical (*48*), and the general pharmacologic characteristics are also similar. No differences in sedimentation coefficient, electrophoretic mobility, chromatographic behavior, or interaction with concanavalin A have been reported for the

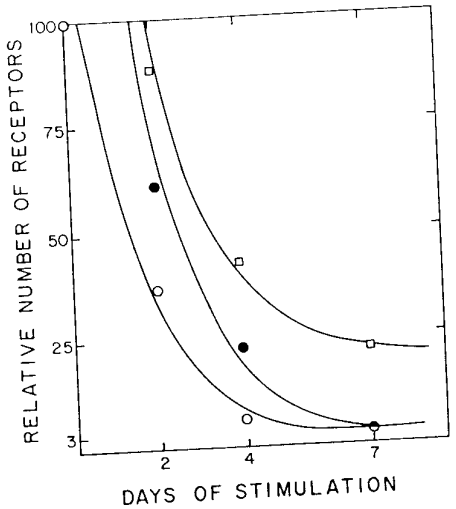


TEXT-FIGURE 14.—Release of radioactivity from denervated (6 days) mouse EDL muscle in perfusion culture after brief exposure to [^{125}I]α-bungarotoxin. Effect of electrical stimulation at 20 Hz given once every 10 sec is illustrated. [Reproduced with permission from (*47*).]

Acetylcholine Receptors at Neuromuscular Junctions

Relation Between Extrajunctional and Junctional ACh Receptors

In many respects the ACh receptors in neuromuscular junctions and in extrajunctional areas are indistinguishable.



TEXT-FIGURE 15.—Decline in number of ACh receptors in denervated rat SOL muscle due to electrical stimulation. Data on ACh sensitivities (*44*) were converted to relative number of ACh receptors per unit area of muscle surface, with the use of the direct proportionality between ACh sensitivity and the square of the packing density of ACh receptors (*15, 24*). Symbols refer to three patterns of electrical stimulation: 1 Hz continuous (-□-); 10 Hz given once every 10 sec (-●-); and 100 Hz given once every 100 sec (-○-). The curves, fitted by eye to experimental points, are all first-order exponentials with a half-time of 24 hr. These are curves predicted for the decline in receptors if such a decline were due to complete or partial inhibition of biosynthesis of receptors and a degradation rate slightly slower than published values. That these curves do not extrapolate back to the beginning of electrical stimulation may be significant. It is also clear that a decline in receptor number faster than reported degradation rates for [^{125}I]α-bungarotoxin bound to ACh receptors does not occur under any of these stimulation regimes.

detergent-solubilized forms of these receptors (*48*). However, a difference in curare sensitivity was discovered by Beranek and Vyskocil (*49*), and a difference in affinity for curare was reported by Brockes and Hall (*48*). Some difference was found in unit conductance, as determined by ACh noise analysis (*50*). It is not known whether these differences reside in the receptor molecules or in the different membranous environments of junctional and extrajunctional receptors. A major contribution to the resolution of this problem was the discovery by Brockes and Hall (*48*) that detergent-solubilized junctional and extrajunctional receptors from rat diaphragm differ in isoelectric point. The difference in isoelectric point may represent a net difference of about 20 charges per receptor unit. Whether these charge differences are intrinsic to the protein or carbohydrate moieties of the receptor or are due to differences in associated lipids or other bound entities has not been established.

These findings raise the question: How are extrajunctional and junctional receptors related? One extreme possibility at present is that the two types of receptors are products of separate genes and, therefore, may be regulated by entirely different control mechanisms. A more conservative possibility is that these receptors have a common origin but the receptors are secondarily modified in different ways, e.g., by addition of different carbohydrate groups or by differential proteolytic cleavage. Such modifications obviously could be important in the organization of post-synaptic ACh receptors into densely packed clusters opposite the nerve terminal. Recent work by M. J. Anderson, M. W. Cohen, and E. Zorychta (personal communication) involving prelabeling of receptor with fluorescent α-bungarotoxin suggests that extrajunctional ACh receptors in embryonic *Xenopus laevis* somite muscle become organized into post-synaptic areas during the formation of neuromuscular junctions in vitro.

Turnover of Junctional ACh Receptors

We know from the extensive anatomical literature, including many histochemical studies in which stains were used for acetylcholinesterase (*51*) and many electron microscopic studies (*52–56*), that the architecture and esterase activity of post-synaptic areas are still recognizable months after denervation of skeletal muscles. Experiments with chickens suggest that the acetylcholinesterase is not replenished at such regions after denervation (*57*), whereas experiments with rats (*58*) indicate a low rate of addition of acetylcholinesterase to these areas. Although these experiments do not directly address the question of receptor metabolism, one must conclude that 1) even in the absence of cues from the nerve terminal, the muscle fiber attempts to maintain the architecture of the former post-synaptic surface, or 2) the post-synaptic surface is metabolically inert, i.e., resistant to catabolism. Since the receptor is a major element in this post-synaptic structure, what is true for the overall structure is likely to be true for the ACh receptors in it.

Hartzell and Fambrough (*15*) reported data on changes in the number of receptors per end-plate following denervation. In 10-day, denervated rat diaphragm fibers, the high packing density of receptors at former end-plates remained comparable to receptor density at normal neuromuscular junction. Chang, Chuang, and Huang (*59*) suggest that there is an increase of about 50% in the number of receptors per end-plate region compared with more distant parts of the muscle, which occurs within the week following denervation. Frank et al. (*27*) found that the number of receptors at former end-plates declines slowly following denervation of rat SOL muscle and at 5 weeks after denervation, it is approximately half the normal value.

Several groups have measured the loss of radioactivity from muscle following blockade of junctional ACh receptors with radioactively labeled α-bungarotoxin (*37–39, 60*). The reported time courses are less than 80% release in 8 days from mouse sternamastoid muscle (*60*) and 50% release in 6–7.5 days from rat diaphragm (*37–39*). Chang and Huang (*37*) cite data on the partial inhibition of loss of radioactivity by administration of dactinomycin to the animals, in support of the hypothesis that this loss of radioactivity reflects ACh receptor turnover. [Fertuck et al. (*60*) reported that dactinomycin impairs the recovery of neuromuscular transmission in a mouse after α-bungarotoxin treatment.] There is little indication that [^{125}I]α-bungarotoxin is not released in active form. Indeed, the

reported time courses of release are not dissimilar from the time course for dissociation of [^{125}I]α-bungarotoxin–receptor complexes at 37° C, and it seems probable that simple dissociation must make a substantial contribution to the observed phenomena.

The radioactive species released from muscle after blockade of junctional ACh receptors with [^{125}I]α-bungarotoxin have recently been characterized in our laboratory. Unlike the [^{125}I]α-bungarotoxin-labeled extrajunctional ACh receptors in denervated muscle, the radioactive species released from the tissue consist mostly of components of molecular weight greater than 2,000 daltons as judged from chromatography of Biogel P-2. This material is likely to be free [^{125}I]α-bungarotoxin dissociated from binding sites at the neuromuscular junction. A small proportion of low-molecular weight material, including just a trace of iodotyrosine is also present. This suggests that the loss of radioactivity from the muscle after [^{125}I]α-bungarotoxin binding is not by the same process as that involved in the turnover of extrajunctional ACh receptors. The loss of radioactivity from normal muscle after [^{125}I]α-bungarotoxin binding follows approximately the same kinetics as loss of radioactivity from denervated muscle after [^{125}I]α-bungarotoxin binding when the latter occurs under anoxic, hypertonic conditions, which result in the rapid death of the muscle fibers. All the above observations are consistent with dissociation of [^{125}I]α-bungarotoxin from junctional ACh receptors being the main mechanism for loss of radioactivity from the muscle. Thus the turnover rate for junctional ACh receptors may be much slower than previously suggested.

In summary, it is likely that we do not yet know how to measure either the biosynthesis or the degradation of junctional ACh receptors. In view of the importance that knowing these rates would be to our thinking about the formation, maintenance, and plasticity of these synapses, greater efforts to surmount the technical difficulties are certainly warranted.

CONCLUSION

The location and numbers of ACh receptor sites in skeletal muscles have been described for muscles in different physiologic and developmental states. It is clear that some of the changes in ACh receptor distribution and number, which accompany physiologic and developmental changes, result from regulatory mechanisms operating on receptor metabolism. Through the use of α-bungarotoxin and more recently by our direct labeling of receptors with labeled amino acids, the major events in the metabolism of extrajunctional ACh receptors have been identified. Preliminary data and theoretical considerations suggest that muscle activity can serve as one regulatory stimulus by inhibition of the biosynthesis of ACh receptors. On the other hand, the formation of closely spaced arrays of receptors in post-synaptic membrane is associated with at least an order-of-magnitude reduction in receptor turnover rate.

These observations suggest a possible mechanism for the transition in receptor distribution as neuromuscular junctions form on embryonic muscle. Receptors in the restricted area of the end-plate are stabilized metabolically, leading to increases in packing density. Biosynthesis of new receptors is repressed, and extrajunctional areas of the membrane are cleared of receptors by the normal degradative mechanisms. In addition, existing extrajunctional receptors might be gathered in the end-plate region, contributing to the increase there, while concomitantly contributing to removal of receptors from extrajunctional regions.

Prospects remain bright for additional advances in our understanding of receptor metabolism. The next steps will include our 1) determining the biochemical relationships between junctional and extrajunctional receptors, 2) refining the description of extrajunctional receptor metabolism through the use of the density-shift technique, electron microscope autoradiography, inhibitor studies, and further chemical characterization of receptors, 3) examining the effects on receptor metabolism produced by procedures which result in altered receptor distribution and number, and 4) developing strategies appropriate for studying the metabolism of junctional ACh receptors.

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DISCUSSION

U. Z. Littauer: I wonder whether you tested inhibitors of initiation of protein synthesis rather than elongation, and what are their effects on receptor synthesis?

D. M. Fambrough: I have not tried these.

Littauer: My other question is: Do you only find iodotyrosine in the medium?

Fambrough: You mean are there other components? In our system, about 80–90% of radioactivity is in the form of iodotyrosine, and there is some free iodide, but only a small amount. Possibly, the muscle has an enzyme that can remove the iodine from iodotyrosine; it should have that enzyme to some extent. Then there is a small amount of high-molecular weight material. The only characterization of that is that it can be sedimented under conditions in which the microsomal fraction is pelleted; presumably that is not free receptors, but bits of cells. In fact, I am doing experiments to find out if viruses budding off cells can carry some receptors in their envelopes.

N. Galanti: I understand from your presentation that receptors are glycoproteins. Do they contain sialic acid?

Fambrough: The information that I have on glycoproteins is this: All the receptors that people have looked at can be precipitated by concanavalin A (Con A) in what looks like a specific manner. Also, we have heard from Dr. Raftery and his colleagues at California Institute of Technology. The sugars have been characterized for the *Torpedo* receptor, and a manuscript on this subject is in press, but the word is that about 3% of every kind of subunit is composed of carbohydrate residues; sialic acid is not present.

Galanti: My second question is: Have you studied the fate of the carbohydrate moiety after denervation?

Fambrough: I have told you a couple of things. We were hoping the precursor might have no carbohydrate in the early stages. We also hope that in the lysosomes of cells treated with trypan blue, the carbohydrates were stripped off. The answer is no; these are still Con A receptors, as well as α-bungarotoxin receptors. There is a small component of Con A nonprecipitable receptors in the trypan blue-treated cultures, and I am trying to figure out what that means.

S. Roseman: What he was really driving at, I think, is after innervation, i.e., after you make the synapse. Could that explain what is really going on? It is focused on the right group of compounds I would say.

Fambrough: Well, according to Dr. Changeux, the difference is in the phosphorylation of two receptor components of different isoelectric points. According to Dr. Stan Appel, junctional and extrajunctional receptors from rat muscle can be separated on Con A columns, where one or the other can be forced to go through a Con A column. I have never been able to get a receptor off a Con A, but they have used rather drastic techniques to do that.

M. R. Escobar: It may be interesting to determine the effect of hybridization or cybridization on receptor number and distribution. Has any work been done in this regard? Would you expect differences between transformed and normal cells as there appeared to be differences between embryonic and adult cells?

Fambrough: I do not think you can transform cells that are making ACh receptors.

Escobar: Have ACh receptors, similar to the ones which you described, been reported for cells other than skeletal muscle cells?

Fambrough: I do not know of any instance when a dividing cell population that you could use for this kind of experiment would have receptors on them.

J. W. Littlefield: Are there clues you can get from the inherited diseases of this neuromuscular junction? Has anybody studied the chick-inherited muscular disease or of human or rat?

Fambrough: There are no known cases of inherited defects involving ACh receptors directly.

H. R. Herschman: Is there any evidence that localization will occur without nerve contact with muscle? The experiments you cite suggest that localization occurs as a consequence of innervation.

Fambrough: Let me tell you more about Anderson and Cohen's experiment. When they put the fluorescent toxin on their muscle, before the nerve goes in, they see bright spots of specific toxin binding that tend to be along the edge of the cells or on little processes going out into contact in the culture dish before nerves are in the system. Then when the nerves make contact, those spots dissolve or fade out. What they think happens is that the spots break up into smaller and smaller bits, and they see a stage when there may be little dots of fluorescence on the cell. Then these fluorescent receptors coordinate to make a new cluster on the line where the nerve runs through the system. Also, Drs. Eric Frank and Gerry Fischbach find that functions of nerve-muscle contacts in tissue culture are not formed at positions on the muscle where receptor clusters preexist, but receptors rapidly cluster at the new contacts.

R. T. Christian: You are suggesting from those experiments that as a consequence of innervation at one point some sort of information is transferred to a distant point that tells the receptor complex to break up and reassemble somewhere else. Is that right?

Fambrough: That is what it looks like.

Roseman: Do you know whether the localization process is an active one or is it simple diffusion?

Fambrough: Nothing is known about that yet.

Roseman: Can you prevent it, or is it in any way inhibited by metabolic inhibitors?

Fambrough: I believe that Anderson and Cohen are doing such experiments now.

Roseman: I am intrigued by the idea of surface membranes constantly circulating within the cytoplasm in little packages. It is not my idea, by the way.

Fambrough: We have a lot of information about receptor cycling that was just totally obliterated from this talk. However, it seems to me that a constant internalization and externalization of receptors is going on, perhaps independent of the synthesis and degradation processes. Always in our culture system, about 30 or 40% of the receptors are not immediately available to interaction with extracellular α-bungarotoxin. We call these "hidden sites," and we know that morphologically they are located on membrane profiles in the cytoplasm of myotubules when viewed in thin-section electron microscopy. I believe these profiles are probably vacuoles made from plasma membrane by internalization.

Roseman: That is great, because it fits a lot of other information.

M. Nirenberg: Do they ever come out?

Fambrough: Oh, yes, they come out and you can measure the kinetics of their coming out by the binding of [^{125}I]-labeled α-bungarotoxin because it is really limited by their reappearance on the surface.

Roseman: I happen to think that is a general model. I think what you are saying applies to a lot of situations, not just this particular one.

C. Shopsis: Have you done experiments in which you pulse-labeled with heavy amino acids and then dissolved cells and used the α-bungarotoxin to determine the normal rate of loss of receptors?

Fambrough: Yes, we have done about six sets of those, but we are not ready to turn in a manuscript yet about it. Dr. John Merlie, who has worked with Dr. Changeux and Dr. Francois Gros in labeling receptors with [^{35}S]methionine, measured the rate at which those receptors disappear versus toxin being degraded, and those rates matched exactly. The data are most interesting. I cannot say we have done as well. We can get first-order kinetics, but it looks as though the kinetics are slightly different, whether or not the receptor has toxin on it.

J. P. Changeux: Considering the degradation of the toxin, where receptor clusters have formed, could it be that their degradation rate is much slower than for more dispersed receptors? For instance, is there a residual, slower component in your kinetics of toxin degradation?

Fambrough: It would be most difficult to determine that kinetically. I think receptor clusters represent maybe 3% of the sites in our culture, but you can pick them up autoradiographically. You can see whether hot spots remain after the surrounding receptors are gone, and they do not, as far as I can tell. Maybe they are in equilibrium with those sites that are more free.

J. Uhr: A propos of your comment about hidden sites, I think it is interesting that in iodinated intact murine splenocytes, about one-third of the H2 alloantigen is not available to interact with antibody in the intact cells. Even if you add antibody to H2 and antibody to the antibody to cap it and then come in with a second wave of H2 antibody, you still cannot increase that percentage; it is as though the H2 alloantigens are hidden by the kind of mechanism you propose.

The second point is: Would it not be a simple procedure in your case to purify the receptor, at least in terms of radioactivity in a one-step procedure with iodinated cells, unlabeled α-bungarotoxin, and antibody to the bungarotoxin?

Fambrough: This kind of procedure looks good on paper, but it will not give you complete purification in practice. However, it will give a substantial enrichment. We could not tell at the level of 1:500 that the receptor is not one of the major iodinated species. If you put α-bungarotoxin on the cells, it is not one of the major iodinated species as far as we can tell.

Roseman: Do you need an intact growing nerve to get localization? For instance, you can isolate these nerve ending-rich fractions, so-called synaptosome fractions. Would they give you localization?

Fambrough: We do not need a nerve to get localization. We get them in muscle cultures without nerves present. I do not know if these preparations would cause specific localization.