Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3488–3492, July 1978
Neurobiology

Regulation of acetylcholine receptor levels by a cholinergic agonist in mouse muscle cell cultures

(neurotrophic relations/carbamylcholine/α-bungarotoxin binding/turnover kinetics)

MARK D. NOBLE*, THOMAS H. BROWN, AND JOHN H. PEACOCK†

Department of Neurology, Stanford University School of Medicine, Stanford, California 94305

Communicated by Donald Kennedy, February 21, 1978

ABSTRACT **The effects of continuous exposure to carbamylcholine (CbCho) on regulation and stabilization of acetylcholine receptors (AcChoR) were studied in cell cultures of G8, a continuous mouse muscle cell line. Exposure of cultures to 10–100 μM CbCho for 24–48 hr produced a 30–50% reduction in ^{125}I-labeled α-bungarotoxin binding. CbCho was not found to alter cell morphology, protein metabolism, or amino acid incorporation. Electrophysiological experiments demonstrated a 75% reduction in the maximum sensitivity of the myotubes to iontophoretic application of acetylcholine (AcCho). The reduction in AcCho sensitivity appeared to represent a true loss of functional receptors because there were no changes in the passive electrical properties of the cells or in the AcCho reversal potential and because receptor desensitization appeared not to be involved. Tetrodotoxin had no effect on receptor levels, either alone or in combination with CbCho. Receptor degradation in control cells could be described kinetically as a first-order process with a half-time of 19.2 hr; turnover rate in receptors remaining after prolonged exposure to CbCho was indistinguishable from that in control cells. We conclude that a receptor-active ligand can exert negative control over AcChoR levels and that prolonged exposure to an AcCho analog is not sufficient to induce a stable population of receptors in these cells.**

An important recent generalization to emerge from studies of various membrane receptors is that their number and functional properties are normally controlled by the concentration of receptor-active ligands reaching the cell surface (1–4). The number of functional receptors typically bears an inverse relationship to the ligand concentration. Currently there is considerable interest in the control and metabolism of acetylcholine receptors (AcChoR) in muscle cells (5–16); however, thus far there have been no investigations demonstrating changes in the number or turnover of AcChoRs directly induced by a cholinomimetic ligand.

In the present work we have examined the effect of a stable acetylcholine (AcCho) analog, carbamylcholine (CbCho), on AcChoR levels in cell cultures of G8, a continuous mouse muscle cell line (17). Our first question was whether CbCho can exert negative control over AcChoR levels. Second, we wished to know whether prolonged exposure to CbCho would induce a more stable population of receptors, because it has been suggested (18–20) that agonist binding to the AcChoR may be responsible for converting extrajunctional-type receptors, which have fast turnover rates, into junctional-type receptors, which are degraded much more slowly. On the basis of binding studies and electrophysiological analysis we find that continuous exposure to CbCho for 24–48 hr significantly decreases AcChoR levels and that the turnover rate in the remaining population appears unaltered.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

METHODS

Cell Cultures. G8 cell stocks were maintained in collagen-coated T-75 flasks (Falcon 3024) and dissociated in trypsin (0.25% in Puck's saline D1) every few days to prevent fusion. Growth medium contained 90% Dulbecco's modified Eagle's medium (DMEM), 4.5 g of glucose per liter, 10% horse serum, and 10% fetal calf serum (designated medium 10/10). Cells were plated out on collagen-coated 35-mm culture dishes (Falcon 3005) at a density of 1.5–2.0 × 10^6 cells per dish in 2 ml of medium 10/10. When cells were confluent, medium was switched to DMEM containing 2% each of horse and fetal calf serum (designated medium 2/2). After 24–72 hr, cells were irradiated with 4 krads (40 Gy) from cesium-137 γ rays to kill dividing cells, and the medium was changed to medium 10/10. Many myotubes were present 48 hr after irradiation. Cells were used in experiments 7–10 days after irradiation, at which time test drugs, diluted to the desired concentration in fresh medium 10/10, were added directly to the cultures. Control cultures always received fresh medium 10/10 when test cultures received drugs. Test and control cultures received the appropriate fresh solutions every 48 hr.

In some experiments, cells were grown on collagen-coated multiwell trays (Falcon 3008). Cells, plated at 5–7 × 10^5 cells per well in 0.5 ml of medium 10/10, were not irradiated and were maintained in medium 2/2 in order to slow cell division and promote myoblast fusion. Drugs were added as for dishes but in medium 2/2.

Purification and Iodination of Toxin. α-Bungarotoxin (α-BuTx) was purified from crude venom (21) and iodinated (22). Specific activity of the iodinated toxin ranged from 6.4 × 10^4–8.5 × 10^5 Ci/mol. Potency of ^{125}I-labeled α-BuTx (^{125}I-α-BuTx) was tested by its blockade of the indirect twitch response in the phrenic nerve-diaphragm preparation (23) or by assay of native toxin's ability to inhibit binding of labeled toxin to hemidiaphragm preparations (24).

Toxin Binding Studies. All binding and washing procedures were carried out at room temperature on a rotary platform. Prior to incubation with toxin, cells were washed three times at 10-min intervals with DMEM containing 5% horse serum (designated medium 5/0) to remove any drugs that would interfere with toxin binding. By using a radiolabeled tracer, it was determined that agents present at 0.1 mM prior to the washes would be diluted to less than 0.1 nM after three washes. After

Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; α-BuTx, α-bungarotoxin; ^{125}I-α-BuTx, ^{125}I-labeled α-BuTx; CbCho, carbamylcholine; DMEM, Dulbecco's modified Eagle's medium; dTC, d-tubocurarine; R_{in}, input resistance; TTX, tetrodotoxin; V_m, membrane potential; V_{rev}, AcCho reversal potential.

* Present address: Medical Research Center Neuroimmunology Unit, Department of Zoology, University College, London, England.
† To whom to address reprint requests.

the third wash, 1 ml of medium 5/0 and 0.2 μg of α-BuTx in 10 μl of medium 5/0 were added to each dish. The toxin incubation period was 90 min which was found to exceed saturation requirements. After incubation with toxin, cells were washed 10 times with 1–1.5 ml of medium 5/0 per dish and twice with 3 ml of phosphate-buffered saline (no serum) per dish and then dissolved in 1 ml of 1 M NaOH for 45 min. An aliquot was transferred to flint glass tubes, and radioactivity was measured in a Nuclear-Chicago gamma emission counter (efficiency = 50%). Binding assays were also performed on cells grown in trays, following the same methods but with suitable reductions in amounts and volumes.

Other studies have shown (9, 12) that receptor degradation kinetics can be evaluated by measuring the extracellular release of ^{125}I after receptor binding with ^{125}I-α-BuTx (9, 12). By using this method, AcChoR degradation at 37° in control medium was compared in control cells and in cells previously incubated in CbCho for 48 hr. The cells were labeled by toxin and washed in the usual manner at 20°. The rate of receptor degradation in chicken myotubes is steeply dependent on temperature ($Q_{10} \simeq 8$) and approaches zero at 20° (10). Similarly, we found toxin loss at 20° to be <0.5%/hr, indicating that little toxin is degraded during labeling and washing. After toxin labeling, medium was removed and replaced with fresh medium at selected time points, and the ^{125}I released during each interval was determined. At the end of the experiment, the total ^{125}I retained by the cells was measured, the total ^{125}I released was calculated, and the fractional amount of receptor degradation F' as a function of time t was determined as

$$F'(t) = \frac{\text{total cpm released by time } t}{\text{total cpm released + total cpm remaining}}.$$

The results are expressed in terms of the fraction F of remaining receptors ($F = 1 - F'$) as a function of time after completion of the labeling procedure.

To determine the spatial distribution of toxin binding sites, after the standard binding procedure the cultures were fixed in 10% formalin, dipped in Kodak nuclear track emulsion NTB-2, exposed for 7–14 days at 4°, and developed in Dektol (1:2 in water) for 1.5 min.

Protein Levels and Amino Acid Incorporation. Total protein levels were determined for every culture (test and control) by the method of Lowry *et al.* (25). In some cultures, amino acid incorporation was evaluated by adding 1 μCi of [^3H]leucine (1 mCi/ml, New England Nuclear) in 10 μl of DMEM directly to wells, incubating the wells at 37° for 2–4 hr, and then washing as in the toxin binding assays. Cells were digested in NaOH; 100-μl aliquots were pipetted onto glass fiber discs, dried overnight, and assayed for radioactivity in a Beckman scintillation counter with 5 ml of Aquafluor (Packard Instruments). Results obtained with this method were similar to results obtained by analysis of hot trichloroacetic acid-precipitates, indicating that the [^3H]leucine remaining in the cells after the 15 washes of the binding assay was almost entirely incorporated into protein.

Electrophysiology. Conventional techniques for intracellular recording (26) were used. Control and test culture pairs were coded and presented as unknowns to the investigator for comparison of AcCho sensitivity on the same day, beginning 15 min after a change to fresh control medium. Iontophoretic pipettes contained 2 M AcCho (Sigma), had resistances of 150–200 MΩ, and required ≤1 nA of negatively biased current to prevent leakage of AcCho. AcCho release pulses of 1-msec duration and 1- to 40-nA current were routinely used for sensitivity mapping. After preamplification (WP-Instruments, M701R) intracellular recording signals were displayed on an oscilloscope, photographed, and simultaneously recorded on a chartwriter (Brush 260). For determination of AcCho reversal potentials and input resistances two intracellular electrodes were placed in the cell within 50 μm of each other; current was injected through one member of the pair and voltage responses to current injection and AcCho pulses were recorded through the other electrode.

Statistical Analysis. Data are reported throughout the paper in terms of the mean ± standard error of the mean. Linear or exponential functions were fitted to the data in all cases by the method of least squares.

RESULTS

Characteristics of AcChoRs in G8 Myotubes. Under the present growth conditions, G8 myotubes showed a nonuniform distribution of α-BuTx binding sites (Fig. 1 *A* and *B*). Much of the binding occurred in focal patches or hot spots, as in primary muscle cell cultures (see ref. 27 for review), although diffuse binding also was evident. Iontophoretic mapping of the cells similarly indicated that AcCho sensitivity was nonuniformly distributed. Maximum sensitivity was usually 2–5 mV/pC in one or more regions of the myotube (Fig. 1*C*) and up to an order of magnitude lower in other regions. This appearance of hot spots in the absence of innervation contrasts with findings with

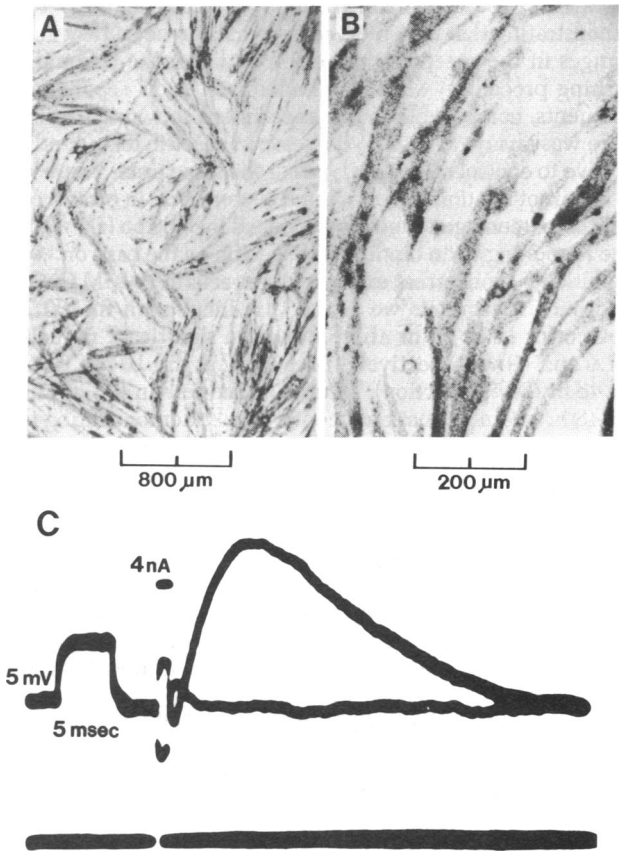

FIG. 1. Nonuniform spatial distribution of AcChoRs in G8 myotubes as revealed autoradiographically at low (*A*) and high (*B*) magnification power by the pattern of ^{125}I-α-BuTx binding. (*C*) Typical recording from a discrete area of high sensitivity, >3 mV/pC. Bottom trace: current; top trace: AcCho response after a calibration pulse. Two successive traces are superimposed; in the second sweep (flat trace) the polarity of the iontophoretic current pulse was reversed.

Table 1. α-BuTx binding after incubation in 100 μM carbachol

Experimental condition	Toxin binding, fmol/mg protein	% of control	Protein levels, mg/dish	n
Control	165.9 ± 8.0	100	1.37 ± 0.22	8
Carbachol (0.5 hr)	167.1 ± 11.6	101	1.36 ± 0.22	8
Carbachol (48 hr)	97.7 ± 4.3	59	1.31 ± 0.17	8

another muscle cell line, L6 (28). Toxin binding was rather variable among cultures grown at different times, commonly ranging from 20 to 170 fmol/mg of protein; but, within a series of cultures, variability was less than ±15%. The toxin binding was specific in that it could be inhibited by more than 95% by concurrent exposure of the cells to 100 μM *d*-tubocurarine (dTC) or CbCho. The apparent dissociation constant, K_D, for dTC was determined from inhibition of α-BuTx binding by the methods of Brockes and Hall (29). In three separate experiments the lowest calculated K_D was 3.6×10^{-7} M, which is an order of magnitude greater than Brockes and Hall reported for junctional receptors (4.5×10^{-8} M) but comparable to their estimate for extrajunctional receptors (5.5×10^{-7} M).

α-BuTx Binding in CbCho-Treated Cells. Cells exposed to 100 μM CbCho for 48 hr showed significantly less toxin binding than did control cells assayed at the same time (Table 1). Short-term exposure (30 min) did not decrease binding levels, demonstrating that the observed decreases do not reflect acute changes in the receptor population and that the prebinding washing procedure was indeed adequate. In 17 separate experiments, comparable to the experiment shown in Table 1, there was always a 30–50% decrease in specific toxin binding relative to control dishes analyzed at the same time. There was no apparent relationship between receptor levels in control cells and the percentage reduction produced by CbCho ($r^2 = 0.07$). The remaining toxin binding was specific on the basis of >95% inhibition by concurrent exposure of the cells to 100 μM CbCho. In other experiments we found that incubation in 100 μM CbCho for 18 or 24 hr also produced a significant reduction (17% and 49%, respectively) in binding as did 10 μM CbCho for 48 hr (37% reduction). Incubating the cells in 100 μM dTC for 48 hr had no effect on receptor levels, demonstrating that prolonged receptor occupancy *per se* is not sufficient to decrease subsequent toxin binding. Similar incubations in 100 μM choline were also without effect on receptor levels.

Total protein levels were never significantly lower in CbCho-treated cells than in controls (for example, see Tables 1 and 2). Furthermore, radiolabeled leucine incorporation by the cells was unchanged by 24-hr exposure to 100 μM CbCho (Table 2). These findings suggest that decreased toxin binding is not associated with a general reduction in metabolic processes.

AcCho Sensitivity in CbCho-Treated Cells. To determine whether the decrease in toxin binding was associated with a loss of functional AcChoRs, AcCho sensitivity was mapped iontophoretically in control and CbCho-treated cells. Maximum sensitivity for each cell was sought by adjusting the microelectrode position and the backing current to give a maximum response and minimum time to peak (5–6 msec in the best cases). The data presented in Table 3 represent the sum of six separate experiments and a total of 91 cells. As indicated, the maximum sensitivity of cells exposed to 100 μM CbCho for 2–5 days was decreased to about 26% of control values. Because there was no tendency for longer exposures to CbCho to produce greater decreases in sensitivity ($r^2 = 0.05$), the data were combined. There was no strong relationship between the mean sensitivity of the control cells and the percentage decrease produced by CbCho ($r^2 = 0.14$), similar to the conclusion reached on the basis of the binding studies. Resting potentials were unchanged by prolonged exposure to CbCho (Table 3). The CbCho treatment produced no detectable changes in cell morphology and no significant differences in the mean input resistance (R_{in}) or the AcCho reversal potentials (V_{rev}) (Fig. 2), both of which were comparable to values reported elsewhere (17) for G8 myotubes.

Table 2. Effects of 100 μM carbachol on protein levels and leucine incorporation

Experimental condition	Toxin binding, fmol/mg protein	Protein, mg/well	[^{3}H]Leu incorp., cpm	n
Control	85.9 ± 4.9	0.42 ± 0.01	5775 ± 358	12
Carbachol (24 hr)	49.8 ± 1.9	0.44 ± 0.01	5674 ± 241	12

Table 3. AcCho sensitivity and resting potentials after incubation in 100 μM carbachol

Experimental condition	Resting membrane potential, mV	Maximum AcCho sensitivity, mV/nC	% of control	n
Control	−49.3 ± 1.2	3667 ± 281	100	39
Carbachol (48–120 hr)	−48.6 ± 0.9	965 ± 178	26	52

In principle, prolonged desensitization of CbCho cells after returning to control medium might mimic receptor loss, giving an apparent decrease in the number of toxin binding sites (30, 31) and in the AcCho sensitivity. Careful consideration was given to this possibility, which was controlled for and evaluated by testing the cells 1–9 hr after a change to control medium. If desensitization were an important factor, there should be some tendency for the sensitivities to increase over this 8-hr period. A least squares linear regression analysis was run on the maximum sensitivites of the 52 CbCho treated cells (Table 3) recorded from during this 8-hr period. Contrary to what would be expected on the basis of a desensitization hypothesis, there was absolutely no tendency for the sensitivities to increase up to 9 hr after a change to control medium; the slope of the regression equation was actually slightly, but not significantly, negative (−66 mV/nC·hr).

Role of Muscle Activity and Depolarization in Receptor Down-Regulation. Muscle activity produced by electrical stimulation is known to decrease receptor levels both in denervated muscle (11, 16) and in cultured aneural myotubes (15); conversely, inhibition of spontaneous activity by tetrodotoxin (TTX) in cultured chicken myotubes has been shown to increase AcChoR levels (15). However, it is unlikely that the CbCho effect on AcChoR levels is mediated by changes in activity. The G8 cultures we have been using show relatively little spontaneous activity, and depolarizing active cells by intracellular current injection or with cholinergic agonists tends to decrease or abolish activity, probably by inactivating inward action currents. Thus, TTX (1 μg/ml), which does abolish spontaneous activity in G8 (unpublished data), has no effect on AcChoR levels, either alone or when combined with CbCho (Table 4).

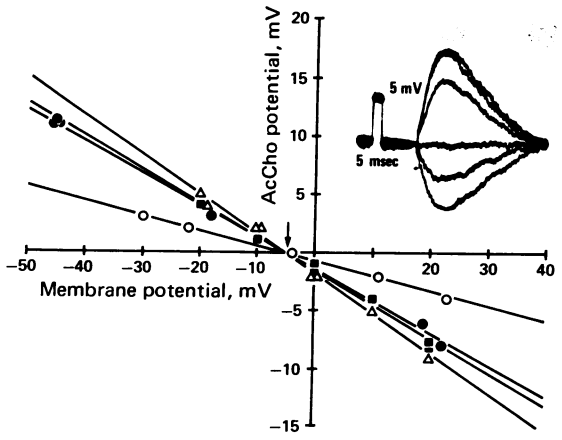

FIG. 2. AcCho reversal curves for several cells, indicated by different symbols, incubated in CbCho and tested in control medium. Arrow, mean reversal potential for control cells. Mean values for the V_{rev} and R_{in} for control cells were -4.7 ± 0.9 mV and 5.6 ± 1.4 MΩ, respectively, and for CbCho cells were 4.5 ± 1.3 mV and 7.7 ± 1.3 MΩ, respectively. (*Inset*) Alternating current coupled recording showing reversal of AcCho potentials.

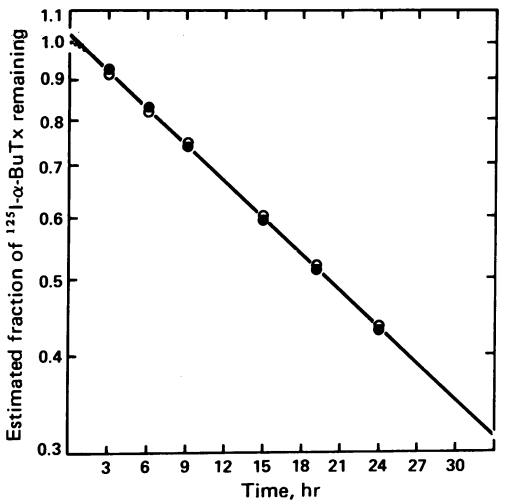

FIG. 3. Fraction of bound α-BuTx remaining as a function of time in control cells (●) and cells incubated for 48 hr in 100 μM CbCho (○). Each data point is the mean of five dishes. The solid line shows the least squares exponential fit to the control data; the degradation curve for CbCho is not shown because it was graphically indistinguishable from the control curve.

Passive depolarization of the cells for 18–24 hr by 30–60 mM KCl also did not decrease AcChoR levels. However, the level of depolarization produced by 60 mM KCl ($V_m = 18.2 \pm 1.4$ mV) was less than that produced by 100 μM CbCho ($V_m = 8.8 \pm 2.4$ mV). Comparable KCl depolarization was difficult to achieve because high KCl concentrations (100 mM or more) caused cells to separate from the surface of the dish.

AcChoR Turnover Kinetics before and after CbCho Treatment. Receptor degradation was followed by measuring the release of ^{125}I into the medium after saturation binding with ^{125}I-α-BuTx (9). The fraction F of toxin remaining bound to the cells was calculated for different time points after labeling and the data were fitted by the exponential function $F = \beta e^{-\gamma t}$. As shown in Fig. 3 (solid circles), the decay could be fitted closely by a single exponential (solid line) with parameters $\beta = 1.03$ and $\gamma = 0.03614$ hr^{-1}, which is consistent with the presence of a single population of receptors whose degradation follows first-order kinetics. The steady-state half-time, taken from the regression equation ($t_{1/2} = 0.6931\ \gamma^{-1}$), was 19.2 hr, which falls in the range reported for extrajunctional receptors (6, 7, 12). Note that the regression equation extrapolated to $t = 0$ slightly overestimates the zero-time bound fraction. This is not surprising because the exponential rate of appearance of ^{125}I in the medium does not begin immediately but accelerates to the steady-state value over the course of about 90 min (9), as illustrated by the dotted line in Fig. 3. This has the effect of introducing about a 1-hr time lag in the steady-state degradation curve (9).

Table 4. Effects of TTX on receptor levels and total protein

Experimental condition*	Toxin binding fmol/mg protein	% of control	Protein mg/dish	n
Control	36.4 ± 5.9	100	1.40 ± 0.09	12
Carbachol (30 hr)	24.3 ± 3.5	67	1.44 ± 0.10	12
Carbachol* + TTX (30 hr)	25.3 ± 3.5	70	1.34 ± 0.04	14
TTX (30 hr	34.5 ± 4.9	95	1.38 ± 0.07	13

* Carbachol was at 100 μM and TTX was at 1 μg/ml.

It has been suggested that extrajunctional recepters may be precursors of junctional receptors and that the interconversion involves some simple covalent modification (29). Changeux and coworkers (18–20) have specifically proposed that extrajunctional receptors are catalytically converted to junctional receptors via a protein kinase reaction and that transmitter released from the nerve may promote or permit the phosphorylation of extrajunctional receptors, thus stabilizing junctional receptors at the endplate region. We were therefore interested in knowing whether prolonged exposure to CbCho would produce a dramatic increase in the turnover half-times. As shown in Fig. 3 (open circles), there was no evidence, based on this method, that the receptors remaining after 48-hr exposure to 100 μM CbCho were stabilized. An exponential fit to the decay yielded parameters $\beta = 1.03$ and $\gamma = 0.03547$ hr^{-1}, giving a turnover half-time of 19.5 hr, which is within 2% of the value determined for control cells.

DISCUSSION

The present results demonstrate by two separate methods that cell surface concentrations of AcChoR can be down-regulated in mouse myotubes by prolonged exposure to a cholinomimetic ligand.‡ Comparable concentrations of dTC did not alter AcChoR levels, demonstrating that active site occupation *per se* is not sufficient to produce receptor down-regulation. The possibility that the observed decreases in toxin binding and AcCho sensitivity are due to receptor desensitization is made unlikely by the finding that there was no tendency for AcCho sensitivities to increase over the course of 9 hr after removal of CbCho.

The effects of CbCho appear to be restricted to the AcChoR in that there were no detectable alterations in protein levels, amino acid incorporation, or cell morphology. Furthermore, after a change to control medium, cells that were incubated in CbCho had normal resting potentials, continued to give

‡ The greater percentage decrease in AcCho sensitivity than in toxin binding may be due to several possible nonlinearities in the relationship between these two measures and is consistent with the empirically demonstrated (32, 33) logarithmic relationship between them.

anode-break action potentials, and showed no changes in input resistance. It is important to note that the apparent selectivity of the consequences of CbCho incubation does not necessarily imply that the receptor down-regulation is mediated by a direct action on the AcChoR. The failure of CbCho incubation to increase the resting potentials and decrease the input resistance in our experiments is consistent with existing evidence (34, 35) that AcCho is not the "neurotrophic factor" responsible for the changes in passive electrical properties of the membrane which accompany innervation.

The effective concentrations of CbCho in the present experiments (10–100 μM) are considerably lower than recent estimates (36) of the peak intracleft AcCho concentration (250 μM) reaching the endplate receptors during synaptic transmission. Furthermore, the efficacy of CbCho as a nicotinic agonist in cultured muscle may be an order of magnitude lower than that of AcCho (30, 37). In the only previous related study, Miledi (38) found that continuous exposure to 1 μM AcCho for several days failed to abolish denervation supersensitivity in denervated frog muscle. The many differences between Miledi's work and the present experiments preclude direct comparison of the results.

It now appears that AcChoRs can be down-regulated in two different ways. Antireceptor antibody appears to accelerate degradation (14, 39, 40) whereas electrical stimulation inhibits synthesis (11, 15). It appears from the present results that CbCho exposure does not produce a prolonged increase in the rate of degradation, because the initial degradation rate constant during the first few hours after removal of CbCho was unaltered (Fig. 3). However, we cannot rule out the possibility that the rate of degradation follows the CbCho concentration with a very short time constant. In spite of the superficial differences between the effects of CbCho and electrical stimulation, both may be acting via the same intracellular "symbols" (41)—for example, calcium ion influx or cyclic nucleotides, as suggested by Lömo (16).

It has been proposed (18–20) that stabilization of AcChoRs may be mediated by the action of transmitter on labile extrajunctional-type receptors. Although the failure of prolonged exposure to CbCho to induce a stable population of receptors in G8 does not disprove this hypothesis, it does indicate that prolonged receptor activation is not *sufficient* to induce stabilization in G8 myotubes. It is conceivable that these cells are not competent to produce stable junctional-type receptors. Alternatively, it is possible that "anterograde factors" other than neurotransmitter are required for induction of junctional-type receptors (18).

In summary, we conclude that the AcChoR, like other receptors, can be negatively controlled by receptor-active ligand. The receptors normally present in G8 myotubes have certain properties analogous to those of extrajunctional receptors; prolonged exposure to a cholinomimetic ligand does not convert them to stable junctional-type receptors.

We thank Daphne Rush for preparation of cultures, Phillip Nelson for his critical review of the manuscript, and Luca Cavalli-Sforza for the use of his laboratory for the binding studies. This study was supported by National Institutes of Health Grant NS12151 and National Science Foundation Grant GB43526 to J.H.P. This work was done during the tenure of a Research Fellowship of the Muscular Dystrophy Association to T.H.B.

1. Kahn, C. R. (1976) *J. Cell Biol.* **70,** 261–286.
2. Kolata, G. B. (1977) *Science* **196,** 747–800.
3. Raff, M. (1976) *Nature* **259,** 265–266.
4. Tata, J. R. (1975) *Nature* **257,** 740–741.
5. Berg, D. K. & Hall, Z. W. (1975) *J. Physiol. (London)* **244,** 659–676.
6. Berg, D. K. & Hall, Z. W. (1975) *J. Physiol. (London)* **252,** 771–789.
7. Chang, C. C. & Huang, M. C. (1975) *Nature* **253,** 643–644.
8. Chang, C. C., Su, M. J. & Tung, L. H. (1977) *J. Physiol. (London)* **268,** 449–465.
9. Devreotes, P. N. & Fambrough, D. M. (1975) *J. Cell Biol.* **65,** 335–358.
10. Devreotes, P. N. & Fambrough, D. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 161–164.
11. Hogan, P. G., Marshall, J. M. & Hall, Z. W. (1976) *Nature* **261,** 328–330.
12. Merlie, J. P., Changeux, J. P. & Gros, F. (1976) *Nature* **264,** 74–76.
13. Merlie, J. P., Sobel, A., Changeux, J. P. & Gros, F. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4028–4032.
14. Appel, S. H., Anwyl, R., McAdams, M. W. & Elias, S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2130–2134.
15. Shainberg, A. & Burstein, M. (1976) *Nature* **264,** 368–369.
16. Lömo, T. (1976) in *Motor Innervation of Muscle*, ed. Thesleff, S. (Academic, New York), pp. 289–321.
17. Christian, C. N., Nelson, P. G., Peacock, J. & Nirenberg, M. (1977) *Science* **196,** 995–998.
18. Changeux, J. P. & Danchin, A. (1976) *Nature* **264,** 705–712.
19. Teichberg, V. I. & Changeux, J. (1976) *FEBS Lett.* **67,** 264–268.
20. Teichberg, V. I., Sobel, A. & Changeux, J. (1977) *Nature* **267,** 540–542.
21. Lee, C. Y., Chang, S. L., Kau, S. T. & Luh, S. H. (1972) *J. Chromatogr. Sci.* **72,** 71–82.
22. Marchalonis, J. J. (1969) *Biochem. J.* **113,** 299–305.
23. Chang, C. C., Chen, T. F. & Chuang, S. T. (1973) *Br. J. Pharmacol.* **47,** 147–160.
24. Berg, D. K., Kelly, R. B., Sargent, P. B., Williamson, P. & Hall, Z. W. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 147–151.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
26. Nelson, P. G., Peacock, J. H., Amano, T. & Minna, J. (1971) *J. Cell Physiol.* **77,** 337–352.
27. Bekoff, A. & Betz, W. J. (1976) *Science* **193,** 915–917.
28. Steinbach, J. H., Harris, A. J., Patrick, J., Shubert, D. & Heinemann, S. (1973) *J. Gen. Physiol.* **62,** 255–270.
29. Brockes, J. P. & Hall, Z. W. (1975) *Biochemistry* **14,** 2100–2106.
30. Dryden, W. F. & Harvey, A. L. (1974) *Br. J. Pharmacol.* **51,** 456–458.
31. Colquhoun, D. (1975) *Annu. Rev. Pharmacol.* **15,** 307–325.
32. Hartzell, H. C. & Fambrough, D. M. (1972) *J. Gen. Physiol.* **60,** 248–262.
33. Fambrough, D. M. (1974) *J. Gen. Physiol.* **64,** 468–472.
34. Engelhardt, J. K., Ishikawa, K., Lisbin, S. J. & Mori, J. (1976) *Brain Res.* **110,** 170–174.
35. Engelhardt, J. K., Ishikawa, K., Mori, J. & Shimabukuro, Y. (1977) *Brain Res.* **128,** 243–248.
36. Kuffler, S. W. & Yoshikamo, D. (1975) *J. Physiol. (London)* **251,** 265–282.
37. Harvey, A. L. & Dryden, W. E. (1974) *Eur. J. Pharmacol.* **27,** 5–13.
38. Miledi, R. (1960) *J. Physiol. (London)* **151,** 24–30.
39. Kao, I. & Drachman, D. B. (1977) *Science* **196,** 527–529.
40. Heinemann, S., Bevan, S., Kullberg R., Lindstrom, J. & Rice, J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3090–3094.
41. Tomkins, G. M. (1975) *Science* **189,** 760–763.