

Role of the Cytoskeleton in the Formation, Stabilization, and Removal of Acetylcholine Receptor Clusters in Cultured Muscle Cells

JOE A. CONNOLLY

Department of Anatomy, University of Toronto, Toronto, Ontario, Canada M5S 1A8

ABSTRACT We have examined the effects of microtubule- and microfilament- disrupting drugs on the stability, formation, and removal of acetylcholine (ACh) receptors and ACh receptor clusters on the surface of aneurally cultured chick embryonic myotubes. (a) In muscle cell cultures, cytochalasin D (0.2 $\mu\text{g/ml}$) or B (2.0 $\mu\text{g/ml}$) causes the dispersal of 50–60% of the existing clusters over a 24-h period (visualized with rhodamine-conjugated α -bungarotoxin); Colcemid (0.5 $\mu\text{g/ml}$) has no effect on these clusters. The total number of cell surface ACh receptors does not decline during this period (measured by [^{125}I] α -bungarotoxin binding) in the presence of either drug. (b) When cells are treated with biotinylated α -bungarotoxin and fluorescent avidin, ACh receptors are cross-linked and rapidly internalized (Axelrod, D., 1980, *Proc. Natl. Acad. Sci. USA.*, 77: 4823–4827). Within 6 h, I have found that 0–15% of the existing large clusters remain. Cytochalasin D or B had no effect on this removal of clusters; however, Colcemid completely prevented the removal of clusters from the cell surface. (c) Addition of chick brain extract to chick myotubes causes an increase in the synthesis and clustering of ACh receptors (Jessell et al., 1979, *Proc. Natl. Acad. Sci. USA.* 76: 5397–5401). Cytochalasin D caused a slight increase in the number of receptors synthesized in the presence of brain extract whereas Colcemid had no effect on the synthesis and insertion of new receptors into the plasma membrane induced by the brain extract. However, both drugs prevented the increase in the number of receptor clusters. These results are consistent with the hypothesis that receptor clusters are stabilized by actin-containing filaments, but that the movement of receptors in the plane of the membrane requires Colcemid-sensitive microtubules.

The conversion of membrane proteins from a random (or homogeneous) to a nonrandom (or heterogeneous) distribution is a basic process in cell development and is perhaps best illustrated by the distribution of acetylcholine (ACh)¹ receptors during the formation of the neuromuscular junction. Before innervation of the muscle cell (both in vivo and in vitro) ACh receptors are widely distributed over the surface of the myotube (8, 20), but once the nerve cell contacts the myotube, there is a reorganization of the ACh receptors and they cluster under the nerve process innervating that muscle cell (8, 20, 21). ACh receptors become largely restricted to the area of membrane around the neuron input and few extra-

junctional receptors are seen. It becomes of fundamental importance then to understand how such restricted domains are established and maintained for membrane proteins.

Primary muscle cell cultures provide an ideal system in which to approach this problem. In myotube cultures, clusters or patches of ACh receptors will form in the absence of nerve input. Fluorescence photobleaching recovery experiments on primary rat myotubes have shown that ACh receptors in such high density patches are immobile, whereas the remaining ACh receptors, uniformly distributed over the plasma membrane, are mobile (4). The mechanisms by which these clusters are formed and maintained is not understood. A passive diffusion-trap mechanism has been suggested to account for the movement and immobilization of ACh receptors, both at the site of nerve-muscle contacts (18, 34, 43) and in clusters in aneural muscle cell cultures (38); these studies suggest that

¹ Abbreviations used in this paper: ACh, acetylcholine; NBD, nitrobenzoxadiazol; R- α BT, rhodamine-conjugated α -bungarotoxin.

simple diffusion to a given site is a plausible mechanism for ACh receptor localization during synaptogenesis and cluster formation in myotubes.

However, the diffusion trap mechanism cannot fully explain the immobilization of ACh receptors in a cluster. It has been postulated that clusters are immobilized by attachment to a submembranous filamentous cytoskeleton (35, 36). Tank et al. (40) have found that when blebs are induced in L6 myotubes, the ACh receptors in these blebbed regions are free to diffuse in the plane of the membrane, whereas receptors in intact cell membranes are much more constrained, with some fractions virtually nondiffusible. They proposed that bleb formation has mechanically broken connections anchoring these receptors to the cytoskeleton. Prives et al. (35, 36) extracted primary rat and chick myotubes with Triton X-100, a treatment which leaves the internal cytoskeleton essentially intact (7). They found that the majority of ACh receptors were extracted by this treatment, but that clustered ACh receptors were retained on the insoluble cytoskeleton. Similarly, in experiments on rat myotubes, Stya and Axelrod (39) found a correlation between the average mobility of ACh receptors in the plane of the membrane and their extractability by Triton X-100. Both of these studies concluded that clustered receptors were attached to the cytoskeleton.

To further investigate this relationship, we have examined the effects of microtubule- and microfilament-disrupting drugs on ACh receptor synthesis and insertion into the membrane and on the formation, stability, and removal of ACh receptor clusters on the surface of aneurally cultured chick embryonic myotubes.

MATERIALS AND METHODS

Cells

Embryonic chick muscle cultures were prepared from dissociated myoblasts as previously described (19) except that no trypsin was used in the dissociation. Cells were plated at a density of 10^5 /ml on collagen- (Vitrogen, Flow Laboratories, Inc., McLean, VA) coated Falcon 96 well/cluster plates (0.1 ml/well, or 24 well/cluster plates (0.5 ml/well), or on collagen-coated glass coverslips in 35-mm Falcon dishes (1.0 ml/dish, Falcon Labware, Becton, Dickinson & Co., Oxnard, CA). Cells were plated (day 0) in Eagle's minimal essential medium with Earle's balanced salt solution, supplemented with 10% horse serum, 5% chick embryo extract, and penicillin (100 U/ml)/streptomycin (100 µg/ml). On day 3, and every 2 days thereafter, cells were fed with the same medium containing 2% chick embryo extract. For cytosine arabinoside treatment (19) of cells, cytosine arabinoside was added at a concentration of 10^{-5} M for 48 h from day 3 to day 5. Unless otherwise indicated, experiments were begun with day 5 or day 6 myotubes.

Fixation

Cells were routinely fixed by the methanol/acetone procedure (29). Cells were rinsed quickly in two changes of PBS, pH 7.1 and fixed for 4 min in methanol, then 2 min in acetone, both at -20°C . After air drying, cells were rehydrated in PBS and then mounted in 50% glycerol in PBS, pH 7.8. Alternative methods of fixation included: (a) Triton/methanol—cells were washed two times in stabilization buffer (0.1 M PIPES, 1 mM EGTA, 4% polyethylene glycol 6000, pH 6.9) (30), incubated in stabilization buffer plus 0.5% Triton X-100 for 5 min, washed three times in stabilization buffer, fixed by immersion in methanol at -20°C for 4 min and then mounted as above; (b) paraformaldehyde—cells were rinsed two times in PBS, fixed for 10 min in 2.5% paraformaldehyde in PBS, pH 6.7, washed three times (3 min each) in PBS and mounted as above; (c) paraformaldehyde/Triton X-100—cells were fixed as in procedure b, but then extracted in 0.5% Triton X-100 in PBS for 5 min, washed again in PBS, and mounted.

ACh Receptor Clusters

RHODAMINE-CONJUGATED α -BUNGAROTOXIN LABELING: Purified α -bungarotoxin (Miami Serpentarium, Miami, Fa) was coupled to

tetramethyl-rhodamine (1) and purified by the method of Ravdin and Axelrod (37). Rhodamine-conjugated α -bungarotoxin (R- α BT) was stored in the presence of 3 mg/ml BSA at -20°C . To label cells, R- α BT was added to cells for 60 min at 37°C in complete medium (plus drugs if any). Cells were then washed five times in balanced salt solution plus 5 mg/ml BSA (3), rinsed two times in PBS, and routinely fixed by the methanol/acetone procedure.

BIOTINYLATED BUNGAROTOXIN LABELING: α -Bungarotoxin was commercially biotinylated (Vector Laboratories, Inc., Burlingame, CA). Biotinylated toxin was added to cells for 15 min (final concentration 9 µg/ml), the cells were washed four times in PBS plus 5 mg/ml BSA, fluorescent avidin (cell sorter grade, Vector Laboratories) was added (final concentration 7.5 µg/ml) for 10 min, and the cells were washed three more times in the same buffer (3) and routinely fixed by the methanol/acetone procedure.

For treatment of biotinylated bungarotoxin/fluorescent avidin-labeled cells with drug, the cells were placed in complete 2% medium after the final BSS rinse, and cytochalasin or Colcemid was added for 6 h. Cells were then fixed by the methanol/acetone procedure. Control labeled cells had no drug added. To determine the effects of the cross-linking avidin on receptor cluster removal, a base-line value was obtained by labeling cells with biotinylated bungarotoxin alone with fluorescent avidin being added just before fixation.

CLUSTER NUMBER: Cluster number was estimated by two methods. First, the total number of discrete ACh receptor clusters was counted for a sample of myotubes (no fewer than 50 per sample) essentially according to the criteria of Bloch (9). Myotubes were defined under phase optics (using Bloch's criteria), and the cells were examined under fluorescence optics for staining with nitrobenzoxadiazol (NBD) phalloidin (see below), and then under rhodamine optics. A "large and discrete" cluster was defined as a discrete area of fluorescence (with Bloch's reservations), i.e., the rhodamine fluorescence was confined to a definable area or mass as opposed to a series of microclusters or dots (compare Fig. 2, a and e). Clusters measured a minimum of 3–4 µm in their shortest dimension. Slight variations in the internal structure of clusters (compare Fig. 2, c and k) were not scored. As a complementary determination, the percentage of myotubes bearing discrete clusters was counted in the same cultures. A myotube was scored as positive if it contained at least one large discrete cluster, and as negative if no discrete cluster was seen. Second, in some samples the number of clusters was estimated per square millimeter of myotube membrane. The total number of receptor clusters was determined for a given sample, and the myotubes were photographed. Cell surface area of these myotubes was estimated from the photographic negatives using the program of Lea (27).

[125 I] α -bungarotoxin labeling

This was done as previously described (15), with the exception that in some experiments 24-well plates were used instead of 96-well plates. To measure the effects of Triton X-100 extraction on toxin binding, 100 µl/well of stabilization buffer plus or minus 0.5% Triton X-100 was added after the final rinse. 5 min later, buffers were removed, and the cells were washed again in basal salt solution and then solubilized and counted as above.

Treatment of Cells

Cytochalasin B, dihydrocytochalasin B, and cytochalasin D (Sigma Chemical Co., St. Louis, MO) were dissolved in DMSO at a concentration of 2 mg/ml and diluted to the indicated concentration in saline before use. DMSO at this final concentration had no effect on the cell cytoskeleton or receptor clusters (not shown). Colcemid (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) was dissolved in water at 5 mg/ml and diluted in saline before use. Lumi-Colcemid was made by the irradiation of a Colcemid solution (at 50 µg/ml) with a 200-W high pressure mercury lamp (HBO-200; Osram, Munich, Federal Republic of Germany) as described by Aronson and Inoue (2).

Chick brain extract was prepared as described (15) except that adult chick brain was used.

Fluorescent Staining

For fluorescent visualization of actin filaments, cells were fixed by the paraformaldehyde/Triton X-100 procedure, and incubated with NBD phalloidin (Molecular Probes, Junction City, OR) (6) for 25–30 min at room temperature. 20 µl of stock NBD phalloidin (in pure methanol) was evaporated and the drug was rehydrated in 100 µl of PBS for each cover slip. Cells were then washed twice in PBS and mounted in 50% glycerol in PBS, pH 7.8. For visualization of microtubules, muscle cells were fixed by the methanol/acetone procedure and then stained with antiserum to tubulin as previously described (16).

RESULTS

[¹²⁵I]α-bungarotoxin-binding studies on primary muscle cell cultures show that the majority of surface ACh receptors are extracted by Triton X-100 treatment; the number of receptors which are resistant to extraction increases with the age of the culture (Fig. 1). When cultures of chick embryonic myotubes are treated with R-αBT and subsequently fixed, discrete large clusters of ACh rhodamine are readily discernible (Fig. 2). The number of clusters visualized (estimated per square millimeter of myotube membrane) did not vary significantly if the myotubes were extracted with Triton X-100 before or after fixation, or if a mild fixation in paraformaldehyde was used (Table I). Thus the majority of ACh receptors in these cells are extracted from the membrane by this treatment but the ACh receptors in clusters are not. These results are in close agreement with those previously obtained by Prives et al. (35, 36) and once again suggest a linkage of these clusters to the Triton X-100-insoluble cytoskeleton. We undertook a more detailed study of such possible links by examining the stability, formation, and removal of receptor clusters as well as the total membrane receptor number in the presence of microtubule- and microfilament-disrupting drugs.

I first examined the effects of cytochalasins and Colcemid on clusters already present in myotube cultures, and these data are presented in Tables II and III. A majority of clusters (as defined in Materials and Methods) in cytochalasin B- and cytochalasin D-treated cells are lost after treatment with the drug for 24 h (Table II). The results seen with cytochalasin D and cytochalasin B treatment were numerically similar, but cytochalasin D was effective at one-tenth of the concentration of cytochalasin B. The less potent cytochalasin dihydrocytochalasin B caused the removal of a significant proportion of receptors, but this was less than that seen with cytochalasin B at the same dose. In these cytochalasin-treated cells, microaggregates of receptors or what may be a dispersing cluster, were sometimes seen (Fig. 2). Cytochalasin B treatment of muscle cell cultures resulted in detachment of >50% of the myotubes from their substratum. However, cytochalasin D treatment for 24 h resulted in much less detachment (24%). Although many of the remaining myotubes had an altered cell shape, a significant proportion remained in good morphological condition and myofibrils were evident (Fig. 2). In that myotubes were screened first under fluorescein optics for the presence of myofibrils, it was only these cells that were scored for the presence of receptor clusters. Given these results, we did a time course of drug action for cytochalasin D. These data (Table III) reveal that cytochalasin D treatment results in the disappearance of clusters in time-dependent fashion.

The effective cytochalasin concentrations employed here would result in the loss of microfilament bundles from fibroblasts in these primary muscle cultures within 1–2 h (assayed by fixation of cultures with paraformaldehyde and staining with NBD phalloidin). However, because of the α-actin-containing myofibrils in the muscle cells, which were not obviously affected by this concentration of cytochalasin, it was difficult to ascertain if the non-α-actin microfilaments were broken down. However, when myotubes were treated with the tumor promoter phorbol myristate acetate (17, 41), myofibrils were lost and there remained a class of actin filaments, which were sensitive to subsequent breakdown by cytochalasin. It is these nonmyofibrillar actin filaments that seem associated with the receptor clusters (Connolly, J. A., manuscript in preparation).

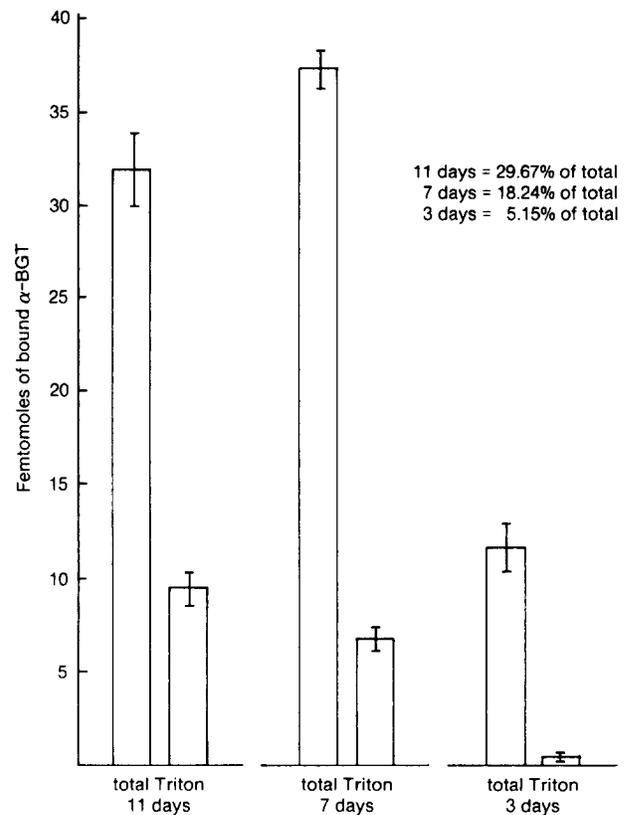


FIGURE 1 Binding of [¹²⁵I]α-bungarotoxin to myotubes at various ages in culture. Total refers to average value (± SD) in femtomoles of bound toxin minus nonspecific binding determined. Triton refers to average values for bound toxin in cultures that were treated with Triton X-100 for 5 min. Values in days refer to number of days in culture.

Long-term treatment of these primary muscle cultures with Colcemid resulted in an altered cell morphology as the myotubes became flattened out and irregular in shape; this treatment detached 10–15% of the myotube within 24 h. However, the ACh receptor clusters seem normal in both number and size (Table II; Fig. 2). The concentration of Colcemid used caused the depolymerization of microtubules in both the muscle cells and fibroblasts within 1–2 h (assayed by immunofluorescence with antiserum to brain tubulin); lumi-Colcemid treatment at this same concentration had no effect (not shown).

We also examined the effect of these two drugs over a 48-h period on the total number of receptors present on the myotube surface. No significant differences were seen ($P < 0.005$) between control cells and those treated with cytochalasin D or Colcemid in the total number of receptors present on myotubes (Fig. 3).

One can induce new receptor clusters in chick myotubes by incubating these cells with brain extracts (15, 26). This increase in cluster number induced by brain extracts is due, at least in part, to the migration of receptors in the plane of the membrane (15). Within 6 h of the addition of chick brain extract, significant increases could be seen in the number of clusters (Table IV). Both cytochalasin D and Colcemid would prevent the increase seen, and cytochalasin D treatment resulted in a decrease of cluster number relative to control cells (Table IV). The result seen with Colcemid was specific as treatment with lumi-Colcemid had no effect (Table IV).

The increase in the number of receptors present on the

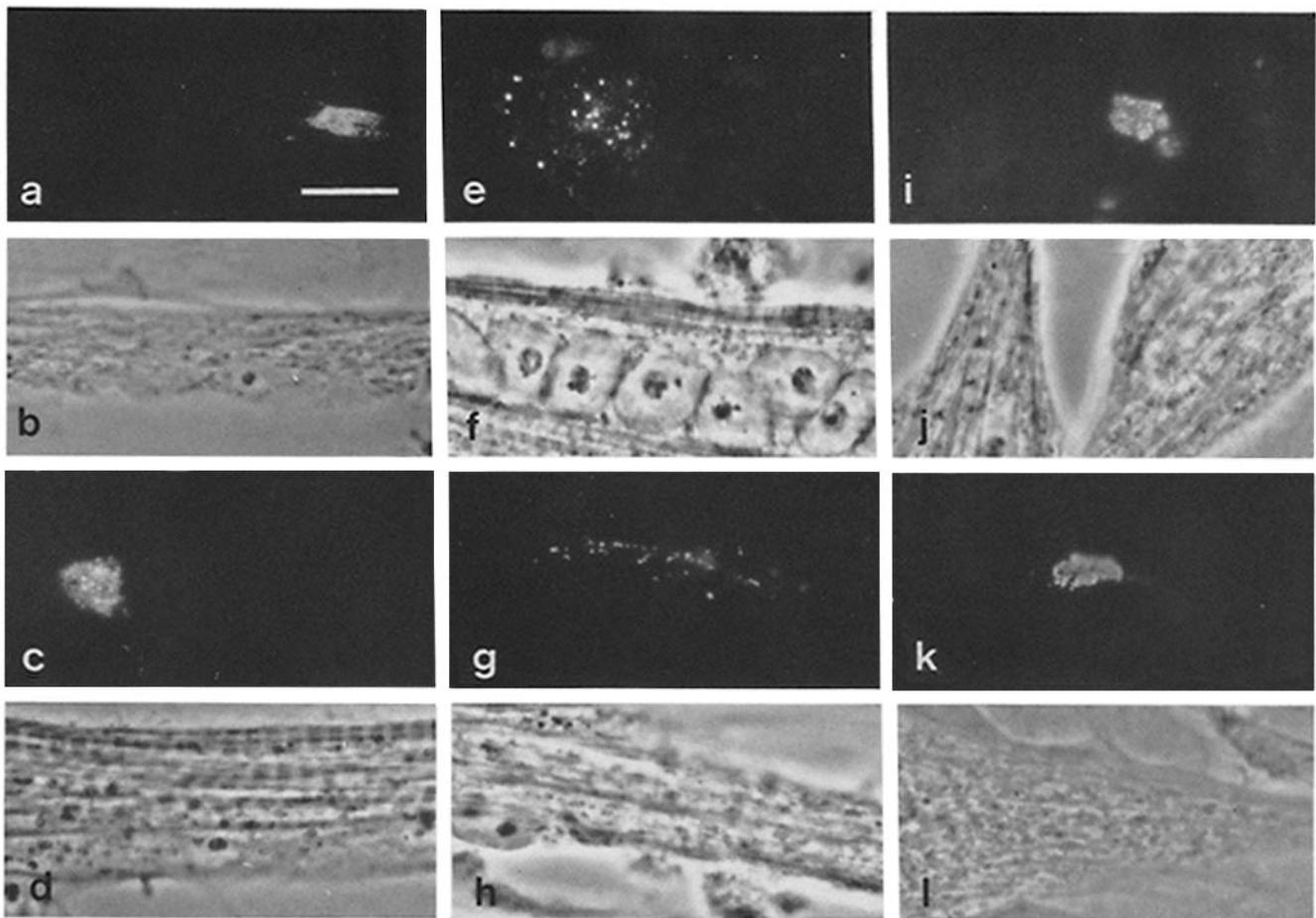


FIGURE 2 R- α BT staining of ACh receptor clusters in chick embryonic myotubes. Cells have been stained with R- α BT, fixed, and photographed under either fluorescence (a, c, e, g, i, and k) or phase-contrast illumination (b, d, f, h, j, and l). Large discrete clusters are seen on nontreated 5-d-old (a and b) and 6-d-old (c and d) myotubes treated with a saline control for 24 h; clusters that appear to be breaking up are seen on 6-d-old myotubes treated with cytochalasin D (0.2 μ g/ml) for 16 h (and f, g, and h); and intact clusters are seen on 6-d-old myotubes treated with Colcemid (0.5 μ g/ml) for 16 (i and j) or 24 (k and l) h. Bar, 10 μ m. (a–l) \times 1340.

TABLE I
Effect of Fixation on Number of ACh Receptor Clusters in Chick Myotubes

Fixation	Cluster number
Methanol/acetone	210
Triton/methanol	221
Paraformaldehyde	190
Paraformaldehyde/Triton	183

Cluster number is expressed as number of clusters/mm² of myotube membrane. Clusters were counted in 15 fields of myotubes (average total = 41 myotubes), the fields were photographed, and myotube area was estimated.

membrane requires a lag period in chick myotubes (26), and thus quantitative increases could not be seen within this 6-h period. We thus followed the effects of these drugs on the incorporation of new receptors into the plane of the membrane at 24 and 48 h after the addition of the brain extract (Fig. 3). At 24 h small, but not significant (results were considered significant at a *P* value of < 0.005 when data were analyzed by the Student's *t* test), increases were seen in all three samples. At 48 h, all three brain extract-treated samples produced significant increases in treated versus control wells. Ratios of specific toxin binding in chick brain extract-treated versus nontreated samples were: 1.59 for chick brain extract

TABLE II
Effect of Cytochalasin and Colcemid on ACh Receptor Clusters in Chick Myotubes

Drug	Control value	Positive myotubes %
Control	100	74.4
Dihydrocytochalasin B (0.2 μ g/ml)	91.6	66.6
Dihydrocytochalasin B (2.0 μ g/ml)	62.5	50.0
Cytochalasin B (0.2 μ g/ml)	54.1	70.7
Cytochalasin B (2.0 μ g/ml)	45.45	28.0
Cytochalasin D (0.02 μ g/ml)	75.0	50.0
Cytochalasin D (0.2 μ g/ml)	41.6	32.5
Colcemid (0.5 μ g/ml)	112.5	67.5

Myotube cultures were treated with drug (or control addition of saline) for 24 h, labeled with R- α BT for 1 h, fixed, and counterstained with NBD phalloidin. Cells were first located under fluorescein optics, and cells showing positive staining for myofibrils were then examined under rhodamine optics to determine the presence or absence of ACh receptor clusters.

alone, 2.21 for chick brain extract plus cytochalasin D, and 1.57 for chick brain extract plus Colcemid; this difference between cytochalasin D treated and control or Colcemid-treated cells was significant. Thus, the cytochalasin D treatment, which appears to destabilize existing clusters and pre-

TABLE III
Time Course of Cytochalasin D Effects on ACh Receptors in Chick Myotubes

Time h	Control value %	Positive myotubes	
		Treated	Control
8	57.1	43.3	72.0
16	61.9	32.4	65.4
24	28.6	24.0	64.0
32	37.5	33.3	74.1

Cells were treated with cytochalasin D (0.2 $\mu\text{g}/\text{ml}$) for the time indicated, then treated with R- αBT and fixed. The first column represents the cluster number expressed as a percentage of the value obtained for a no-drug sister culture control fixed at a similar time point (taken as 100%). The second and third columns represent values for the percentage of positive myotubes in both treated and untreated (control) cultures at each time point.

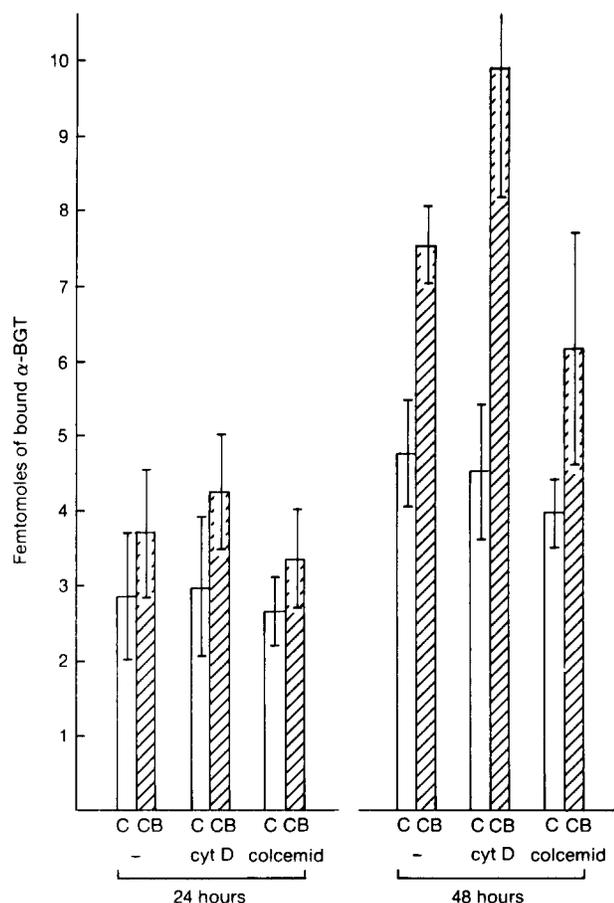


FIGURE 3 Binding of [^{125}I] α -bungarotoxin to chick myotubes treated with cytochalasin D or Colcemid. Binding assays were done after 5-d myotubes were incubated for 24 or 48 h in the presence or absence of drug. Drug treatment was either Colcemid (0.5 $\mu\text{g}/\text{ml}$), cytochalasin D (Cyt D) (0.2 $\mu\text{g}/\text{ml}$), or none (-). In each pair of rectangles on the histogram, the left-hand one (C) represents the drug or control treatment alone, the right-hand one represents this same treatment plus chick brain extract (CB). (10 $\mu\text{l}/\text{well}$). Ordinate values represent specific binding of α -bungarotoxin in femtomoles of bound toxin \pm SD.

vent the formation of new ones, increased beyond control levels the number of receptors that appeared on the cell surface in response to the chick brain extract treatment.

We then examined the effects of these drugs on the removal of clusters from the cell surface by biotinylated bungarotoxin

TABLE IV
Effects of Cytochalasin and Colcemid on Chick Brain Extract-induced Increases in ACh Receptor Clusters in Chick Myotubes

Drug	Control value %
Chick brain control	160.0
Cytochalasin D (0.2 $\mu\text{g}/\text{ml}$)	86.7
Colcemid (0.5 $\mu\text{g}/\text{ml}$)	106.7
Lumi-Colcemid (0.5 $\mu\text{g}/\text{ml}$)	154.2

Cultures were treated with 100 μl of chick brain extract for 6 h (except nontreated control which received saline). Nontreated control was taken as control value of 100%. As brain extract treatment often induced multiple clusters on a single myotube, positive and negative myotubes were not counted.

TABLE V
Effect of Biotinylated α -Bungarotoxin/Fluorescent Avidin Treatment on ACh Receptor Cluster Number in Chick Myotubes

Drug	Control value	
	Expt. 1	Expt. 2
Cross-linked control	0	16.0
Colcemid (0.5 $\mu\text{g}/\text{ml}$)	100.0	104.0
Lumi-Colcemid (0.5 $\mu\text{g}/\text{ml}$)	—	9.1
Cytochalasin B (2.0 $\mu\text{g}/\text{ml}$)	11.1	—
Cytochalasin D (0.2 $\mu\text{g}/\text{ml}$)	—	12.0

Cells were treated with biotinylated α -bungarotoxin plus fluorescent avidin for 6 h. Cross-linked control represents cultures so treated which received no additional drug treatment. Control value was established by incubating sister cultures with biotinylated α -bungarotoxin, but adding the fluorescent avidin just before fixation.

cross-linked with fluorescent avidin (3). When cells were incubated with biotinylated bungarotoxin and then with fluorescent avidin, the large discrete clusters on the surface were essentially all gone within 6 h (Table V). Both cytochalasin B and cytochalasin D had no effect on this removal of clusters from the surface. However, Colcemid completely prevented the removal of clusters from the myotube surface; its inactive analogue lumi-Colcemid had no effect on this process (Table V).

DISCUSSION

Colcemid treatment of embryonic chick myotubes has no effect on the disposition of ACh receptor clusters present in the myotube membrane. In contrast, both cytochalasin B and cytochalasin D caused a gradual loss of these clusters (up to ~60%) in a dose- and time-dependent fashion. [^{125}I] α -bungarotoxin-binding data indicate that neither drug treatment resulted in a significant loss of toxin-binding sites from the cell surface within 24 h. Although cytochalasin can destabilize clusters then, it appears the receptors from these clusters are not lost from the membrane surface. Axelrod et al. (5) previously reported that colchicine and cytochalasin B (at a dose 50 times higher than that used in these studies) had no effect on existing ACh receptor clusters in rat myotubes. However, these cells were examined after only 1 h of drug treatment. One might expect that if clusters are stabilized by links to an intracellular cytoskeleton, then dissolution of cytoskeletal elements (which itself takes on the time course of 1 h) would result in a slow dispersal of receptor clusters as the anchoring fibers are removed. Bloch (9) reported similar experiments on rat myotubes in which he looked at the effects of these same

two drugs for 6 h. He reported small reductions in the number of clusters in cytochalasin treated cells (consistent with our results), but saw a significant reduction (~40%) in the number of clusters in colchicine-treated cells. We report here no effect of Colcemid on existing clusters in chick myotubes with up to 24 h of continuous exposure. It is unclear what might cause this apparent disparity. The possibility that such differences may be accounted for by species differences must be considered. For example, treatment of rat myotubes with sodium azide (9) or Ca^{++} -free medium (10) causes the rapid dispersal of ACh receptor clusters within ~6 h. In chick myotubes, azide treatment resulted in no loss of receptor clusters within 6 h, and ~40% loss after 30 h. Treatment of cells with Ca^{++} -free medium had no discernible effect after 17 h (Connolly, J. A., unpublished observations). In addition, positively charged latex beads, which can induce receptor clusters in cultured *Xenopus* muscle (33), have no apparent effect on chick or rat myotubes (Peng, B., personal communication).

The formation of new ACh receptor clusters, which can be induced by the addition of chick brain extract, is completely blocked by the addition of Colcemid or cytochalasin. We have previously found (15; Connolly, J. A., unpublished data) that brain extract induces clusters to form, at least in part, from receptors already present in the plasma membrane. The [^{125}I] α -bungarotoxin-binding data presented here indicate that the synthesis and insertion of new receptors induced by chick brain extract are not affected by either of these drugs. Thus it is likely we are looking at clusters that are being formed by the movement of receptors in the plane of the membrane. Bloch has previously shown (8) that receptor clusters on rat myotubes will break down in the presence of sodium azide. When he examined the reformation of clusters after azide removal, this process was blocked by both cytochalasin and Colcemid. Recently, Stya and Axelrod (38) have shown that receptor clusters can form in nontreated rat myotubes, at least in part, by the diffusion of clusters in the plane of the membrane, and that such cluster formation was blocked by colchicine. Taken together, these results provide evidence for the involvement of microtubules in the movement of membrane-bound ACh receptors. In our experiments, cytochalasin also blocked the formation of brain extract-induced clusters, but it is unclear from this experiment whether this is because actin-containing filaments are also involved in receptor movement, or that newly forming clusters require the stabilizing forces of actin filaments.

The treatment of myotubes with biotinylated bungarotoxin and fluorescent avidin allows us to look at this question of microfilament involvement in receptor movement more closely. Biotinylated bungarotoxin plus fluorescent avidin treatment results in a near total loss of large, discrete clusters from the cell surface. Cytochalasin treatment had no effect on this internalization, whereas Colcemid, but not lumi-Colcemid, completely prevented it. Inasmuch as no change was seen in the number or distribution of clusters in Colcemid-treated versus untreated cells, this result also implicates microtubules as being involved in the movement of receptors in the plane of the membrane. It appears cytochalasin-sensitive filaments however play no role in this movement or in the internalization mechanism, but are involved in stabilizing receptor clusters.

Although cytochalasin caused the loss of receptor clusters and both cytochalasin and Colcemid would prevent cluster formation induced by chick brain extract, neither drug had

any effect on the synthesis or insertion of new receptors into the membrane in the presence or absence of chick brain extract. In fact, cytochalasin plus chick brain extract treatment resulted in a greater increase in total surface membrane receptor number than treatment with chick brain extract alone. The significance of this particular finding is unclear; however, these data do indicate that ACh receptors, which are believed to move to fuse with the cell surface by means of coated vesicles (13, 14), do so in a manner that is not dependent on microtubules or cytochalasin-sensitive actin filaments in these cells (Connolly, J. A., manuscript in preparation).

The process of receptor clustering in myotubes is of particular significance because this process occurs soon after the innervation of a myotube, both in vitro and in vivo. Our studies have implicated a cytoskeletal role in cluster formation and stabilization in aneural cultures. However, clusters in such aneural cultures could be induced by the close substrate contacts these muscle cells enjoy in vitro, and this is supported by the finding that most clusters are on the ventral surface of the myotube and that these clusters are always located within areas of broad close contact (11). Peng et al. (33) showed that clusters could be induced in *Xenopus* cells by polylysine-coated latex beads at points of bead/myotube attachment, again suggesting a contact phenomenon may be at work. The involvement of the muscle cytoskeleton in the forming neuromuscular junction in vivo remains to be examined. In vitro, Peng has recently examined the appearance of the cytoskeleton at nerve-muscle contacts by whole-mount stereo electron microscopy in co-cultures of *Xenopus* nerve and muscle. In the presynaptic nerve terminal opposite the postsynaptic receptor cluster, synaptic vesicles were suspended in a lattice of 5–12-nm filaments and this lattice was contiguous with the filament bundle forming the core of the axon. Postsynaptically a dense meshwork of 6-nm filaments was specifically associated with the membrane receptor cluster. These results provide strong evidence for a significant cytoskeletal role in the development and/or maintenance of the neuromuscular junction (32).

Immunofluorescence studies with vinculin (11), an intracellular protein believed to participate in the anchoring of microfilament bundles at specific membrane sites (23, 24), indicate it is concentrated in regions of close cell-substrate contact, where the staining pattern of vinculin and that for ACh receptors interdigitate. Bloch and Hall (12) have more recently demonstrated that antibodies prepared against avian smooth muscle vinculin, as well as α -actinin and filamin, gave staining in frozen sections of rat, mouse, chick, and *Xenopus* muscle in the postsynaptic membrane at the neuromuscular junction. At least in the case of the rat, the staining is not identical to that obtained with R- α BT, but rather interdigitates with it. Furthermore, α -actinin and vinculin have been reported not to bind directly to the ACh receptor in the muscle membrane (12). As vinculin and α -actinin are often localized at sites where microfilaments are attached to the cytoplasmic face of the cell membrane (23, 24), these results suggest that receptors may not bind directly to microfilaments, but may rather be stabilized by microfilament insertion into the membrane at sites of formed or forming receptor clusters.

During the growth of primary muscle cell cultures, myoblasts synthesize both the β - and γ -isoforms of actin, and only small amounts of α -actin (22, 42), but soon after fusion the synthesis of β - and γ -actin is drastically curtailed, α -actin

synthesis is increased, and adult skeletal muscle has been reported to contain only α -actin (the constituent actin of myofibrils) by two-dimensional gel electrophoresis (22). However, more recent studies have shown that an antibody to aplysia muscle actin (a γ -isoform of actin) (25, 28), an antibody that does not cross-react with skeletal muscle alpha actin filaments, will stain the myotube membrane with the strongest staining co-localizing with that seen with R- α BT. Pardo et al. (31) have demonstrated by immunofluorescence studies the selective association of γ -actin with skeletal muscle mitochondria. These studies suggest that isoforms of actin, although not biochemically present in large amounts, may play specific roles in the myotube, roles such as the stabilization of acetylcholine receptors at the neuromuscular junction. It is plausible that we are selectively depolymerizing a class of non- α -actin filaments with cytochalasin treatment and this results in a loss of stabilization to the cell surface receptor cluster, a finding supported by the fact that phorbol myristate acetate treatment of myotubes (17, 41), a treatment which results in the loss of α -actin filaments, has no significant effect on the disposition of ACh receptor clusters (Connolly, J. A., manuscript in preparation).

In conclusion, our results support the hypothesis of attachment of ACh receptors to a submembrane cytoskeleton. We further propose that receptor clusters are stabilized by actin-containing filaments within the cytoplasm; because existing clusters cannot be destabilized with Colcemid, cluster stability is not dependent on microtubules, but the movement of receptors in the plane of the membrane is.

I would like to thank Gilles Simard for excellent technical assistance.

This work was supported by grants from the Medical Research Council (MRC) and Muscular Dystrophy Association of Canada. Dr. Connolly is a Scholar of the MRC of Canada.

Received for publication 7 October 1983, and in revised form 20 March 1984.

REFERENCES

- Anderson, M. J., and M. W. Cohen. 1974. Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle. *J. Physiol. (Lond.)* 237:385-400.
- Aronson, J., and S. Inoue. 1970. Reversal by light of the action of *N*-methyl *N*-desacetyl colchicine on mitosis. *J. Cell Biol.* 45:479-477.
- Axelrod, D. 1980. Crosslinkage and visualization of acetylcholine receptors on myotubes with biotinylated α -bungarotoxin and fluorescent avidin. *Proc. Natl. Acad. Sci. USA* 77:4823-4827.
- Axelrod, D., P. Ravdin, D. E. Koppel, J. Schlessinger, W. W. Webb, E. L. Nelson, and T. R. Podleski. 1976. Lateral motion of fluorescently labeled acetylcholine receptors in membranes of developing muscle cells. *Proc. Natl. Acad. Sci. USA* 73:4594-4598.
- Axelrod, D., P. M. Ravdin, T. R. Podleski. 1978. Control of acetylcholine receptor mobility and distribution in cultured muscle membranes. *Biochim. Biophys. Acta* 511:23-38.
- Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole Phalloidin. *Proc. Natl. Acad. Sci. USA* 77:980-984.
- Ben Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. *Cell* 17:859-865.
- Bevan, S., and J. H. Steinbach. 1977. The distribution of α -bungarotoxin binding sites on mammalian skeletal muscle developing in vivo. *J. Physiol. (Lond.)* 267:195-213.
- Bloch, R. J. 1979. Dispersal and reformation of acetylcholine receptor clusters on cultured rat myotubes treated with inhibitors of energy metabolism. *J. Cell Biol.* 82:626-643.
- Bloch, R. J. 1983. Acetylcholine receptor clustering in rat myotubes: requirement for Ca^{2+} and effects of drugs which depolymerize microtubules. *J. Neurosci.* 3:2670-2680.
- Bloch, R. J., and B. Geiger. 1980. The localization of acetylcholine receptor clusters in areas of cell-substrate contact in cultures of rat myotubes. *Cell* 21:25-35.
- Bloch, R. J., and Z. W. Hall. 1983. Cytoskeletal components of the vertebrate neuromuscular junction: vinculin, α -actinin, and filamin. *J. Cell Biol.* 97:217-223.
- Bursztjan, S., and G. D. Fischbach. 1979. Coated vesicles in cultured myotubes contain acetylcholine receptors. *Soc. Neurosci. Abstr.* 5:477.
- Bursztjan, S., and G. D. Fischbach. 1984. Evidence that coated vesicles transport acetylcholine receptors to the surface membrane of chick myotubes. *J. Cell Biol.* 98:498-506.
- Connolly, J. A., P. A. St. John, and G. D. Fischbach. 1982. Extracts of electric lobe and electric organ from *Torpedo californica* increase the total number as well as the number of aggregates of chick myotube acetylcholine receptors. *J. Neurosci.* 2:1207-1213.
- Connolly, J. A., V. I. Kalnins, D. W. Cleveland, and M. W. Kirschner. 1978. Intracellular localization of the high molecular weight microtubule accessory protein by indirect immunofluorescence. *J. Cell Biol.* 76:781-786.
- Croop, J., Y. Toyama, A. A. Dlugosz, and H. Holtzer. 1980. Selective effects of phorbol 12-myristate 13-acetate on myofibrils and 10-nm filaments. *Proc. Natl. Acad. Sci. USA* 77:5273-5277.
- Edwards, C., and H. L. Frisch. 1976. A model for the localization of acetylcholine receptors at the muscle end-plate. *J. Neurobiol.* 7:377-381.
- Fischbach, G. D. 1972. Synapse formation between dissociated nerve and muscle cells in low density cell cultures. *Dev. Biol.* 28:407-429.
- Fischbach, G. D., and S. A. Cohen. 1973. The distribution of acetylcholine sensitivity over innervated and innervated muscle fibers grown in cell culture. *Dev. Biol.* 31:147-162.
- Frank, E., and G. D. Fischbach. 1977. ACh receptors accumulate at newly formed nerve-muscle synapses in vitro. In *Cell and Tissue Interactions*. J. W. Lash and M. M. Burger, editors. Raven Press, New York. 285-291.
- Garrels, J. I., and W. Gibson. 1976. Identification and characterization of multiple forms of actin. *Cell* 9:793-805.
- Geiger, B. 1979. A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell* 18:193-205.
- Geiger, B., K. T. Tokoyasu, A. H. Dutton, and S. J. Singer. 1980. Vinculin an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. *Proc. Natl. Acad. Sci. USA* 77:127-131.
- Hall, Z. W., B. W. Lubit, and J. H. Schwartz. 1981. Cytoplasmic actin in postsynaptic structures at the neuromuscular junction. *J. Cell Biol.* 90:789-792.
- Jessell, T. M., R. E. Siegel, and G. D. Fischbach. 1979. Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. *Proc. Natl. Acad. Sci. USA* 76:5397-5401.
- Lea, P. J. 1983. Image analysis system based on a non-dedicated micro computer. *Micron Microscopica Acta* 14:301-306.
- Lubit, B. W., and J. H. Schwartz. 1980. An anti-actin that distinguishes between cytoplasmic and skeletal muscle actins. *J. Cell Biol.* 86:891-897.
- Osborn, M., W. W. Franke, and K. Weber. 1977. Visualization of a system of filaments 7-10 nm thick in cultured cells of an epithelioid line (ptK2) by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA* 74:2490-2494.
- Osborn, M., and K. Weber. 1977. The display of microtubules in transformed cells. *Cell* 12:561-571.
- Pardo, J. V., M. F. Pittenger, and S. W. Craig. 1983. Subcellular sorting of isoactins: selective association of γ -actin with skeletal muscle mitochondria. *Cell* 32:1093-1103.
- Peng, H. B. 1983. Cytoskeletal organization of the presynaptic nerve terminal and the acetylcholine receptor cluster in cell cultures. *J. Cell Biol.* 97:489-498.
- Peng, H. B., P.-C. Cheng, and P. W. Luther. 1981. Formation of ACh receptor clusters induced by positively charged latex beads. *Nature (Lond.)* 292:831-834.
- Poo, M. M. 1982. Rapid lateral diffusion of acetylcholine receptors in the embryonic muscle cell membrane. *Nature (Lond.)* 295:332-334.
- Prives, J., C. Christian, S. Penman, and K. Olden. 1980. Neuronal regulation of muscle acetylcholine receptors: role of muscle cytoskeleton and receptor carbohydrate. In *Tissue Culture in Neurobiology*. E. Giacobini, A. Vernadakis, and A. Shahar, editors. Raven Press, New York. 35-52.
- Prives, J., A. B. Fulton, S. Penman, M. P. Daniels, and C. N. Christian. 1982. Interaction of the cytoskeletal framework with acetylcholine receptor on the surface of embryonic muscle cells in culture. *J. Cell Biol.* 92:231-236.
- Ravdin, P., and D. Axelrod. 1977. Fluorescent tetramethyl rhodamine derivatives of α -bungarotoxin: preparation, separation and characterization. *Anal. Biochem.* 80: 585-592. Erratum. 83:336.
- Stya, M., and D. Axelrod. 1983. Diffusely distributed acetylcholine receptors can participate in cluster formation on cultured rat myotubes. *Proc. Natl. Acad. Sci. USA* 80:449-453.
- Stya, M., and D. Axelrod. 1983. Mobility and detergent extractability of acetylcholine receptors on cultured rat myotubes: a correlation. *J. Cell Biol.* 97:48-51.
- Tank, D. W., E.-S. Wu, and W. W. Webb. 1982. Enhanced molecular diffusibility in muscle blebs: release of lateral constraints. *J. Cell Biol.* 92:207-212.
- Toyama, Y., S. Forry-Schaudies, B. Hoffman, and H. Holtzer. 1982. Effects of taxol and colcemid on myofibrillogenesis. *Proc. Natl. Acad. Sci. USA* 79:6556-6560.
- Whalen, R. G., G. S. Butler-Browne, and F. Gros. 1976. Protein synthesis and actin heterogeneity in calf muscle cells in culture. *Proc. Natl. Acad. Sci. USA* 73:2180-2022.
- Young, S. H., and M. M. Poo. 1983. Rapid lateral diffusion of extrajunctional acetylcholine receptors in the developing muscle membrane of *Xenopus* tadpole. *J. Neurosci.* 3:225-231.