Evidence That Coated Vesicles Transport Acetylcholine Receptors to the Surface Membrane of Chick Myotubes

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ABSTRACT Coated vesicles are present in the myoplasm of embryonic chick myotubes grown in vitro. They are most numerous beneath regions of the surface membrane that contain a high density of acetylcholine receptors (AChR). Prolonged exposure of myotubes to saline extract of chick brain increases the number of intracellular AChR and the number of coated vesicles. This suggests that coated vesicles contain AChR, and this hypothesis was tested with horseradish peroxidase-α-bungarotoxin (HRP-αBTX) conjugates. The conjugates enter saponin-permeabilized cells and, as judged by the inhibition of [^{125}I]αBTX binding, they label the entire intracellular AChR pool. ~50% of the coated vesicles contained HRP-αBTX reaction product. In addition, reaction product was detected in Golgi cisternae and along membranes that bound a subsurface tubulovesicular network. The majority of labeled vesicles are probably involved in exocytosis rather than endocytosis of AChR because very few coated vesicles were labeled when HRP-αBTX was added to the medium bathing intact cells. Moreover, inhibition of protein synthesis with puromycin resulted in a large decrease in the number of labeled vesicles. These results suggest that a subpopulation of coated vesicles ferry newly synthesized AChR to the cell surface.

One of the hallmarks of the adult, vertebrate neuromuscular junction is the presence of a high density of acetylcholine receptors (AChR)[1] packed in the postsynaptic membrane immediately opposite sites of ACh release (17, 18, 25, 26, 29). The assembly of the postsynaptic membrane can be studied in culture because embryonic muscle cells can be innervated by cholinergic neurons in vitro and AChR rapidly accumulate at nerve-muscle synapses (3, 20, 21).

In principle, AChR might accumulate at synapses by migration within the plane of the membrane or by local insertion of newly synthesized molecules. A large fraction of the diffusely distributed AChR in membranes of mammalian and amphibian muscle cells are mobile (4, 31), and at least some of the receptors present at *Xenopus* nerve-muscle contacts in vitro were initially located in extrasynaptic regions (2). No information is available concerning the relative rates of insertion of AChR at synapses compared to extrasynaptic sites. The existence of an intracellular precursor pool of AChR in cultured myotubes has been clearly documented (12–14, 28). Fambrough and Devreotes (16) located some of the intracellular receptors [^{125}I]α-bungarotoxin-binding sites) in Golgi membranes by electron microscopic autoradiography of saponin-permeabilized myotubes, but no data were presented concerning the route by which these receptors reach the surface membrane. While studying the ultrastructure of nerve muscle synapses in chick cultures, we noted a relatively large number of coated vesicles in the myoplasm within 2 μm of the postsynaptic membrane (9). This observation, together with earlier reports of submembrane-coated vesicles in embryonic target neurons (1, 35), raises the possibility that coated vesicles ferry newly synthesized receptors to the postsynaptic membrane.

It is relatively difficult to relocate and thin section physiologically identified synapses in vitro, so as a first step in investigating the role of coated vesicles in assembly of the postsynaptic membrane we have examined uninnervated myotubes treated with embryonic brain extract. Factors in brain extract increase the synthesis and promote the aggregation of AChR in chick myotubes (7, 24). Our working hypothesis is that the same or similar material present in brain

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptors; BSA, bovine serum albumin; BSS, Earle's balanced salt solution; HRP, horse radish peroxidase; HRP-αBTX, HRP–α-bungarotoxin.

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extract is also present in cholinergic neurons and is responsible for induction of receptor aggregates at newly formed synapses. Preliminary accounts of some of these experiments have been published (8, 9).

MATERIALS AND METHODS

Cell Cultures: Chick myotubes were grown in culture as previously described (19). Pectoral muscle from 11-d-old chick embryos were cut into small pieces and incubated in a Ca^{++}- and Mg^{++}-free salt solution (Pucks, D_1G) without added proteolytic enzymes. After 20–30 min, the tissue fragments were pelleted, resuspended in complete medium (see below), and triturated by repeated passage through a pasteur pipette. To reduce the number of fibroblasts in the initial cell suspension, the cells were plated in Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) for 30 min at 37°C. Unattached cells were collected and plated in 35-mm tissue culture dishes that contained one or more collagen-coated Gold Seal #0 coverslips (B-D Labware, Oxnard, CA). Fibroblasts were reduced in number but not eliminated by this preplating technique, so after 4–5 d in culture, the multinucleated myotubes were situated on top of a nearly confluent layer of fibroblasts. Spinal cord explants were added to some of the cultures. The methods for preparing thin slices of 4- or 7-d-embryonic chick spinal cord and for adding them to established myotube cultures have been described (21). Techniques for stimulation of the explants and for intracellular recording from the myotubes have also been described (21).

Cells were plated in Eagle's minimum essential medium made up in Earle's balanced salt solution (BSS) and supplemented with glutamine (2.4 mM), horse serum (10% vol/vol), chick embryo extract (2% vol/vol); penicillin (500 μg/ml), and streptomycin (50 μg/ml). They were fed every other day in the same medium.

Preparation of Brain Extract: Some cultures were supplemented with a saline extract of embryonic chick brain and spinal cord (24). Brains dissected from 14- to 17-d embryos were homogenized in BSS (3 ml per g of tissue) and the suspension was centrifuged at 20,000 *g* for 60 min. The supernatant, which contained ~7 mg protein/ml, was stored at −70°C before use. 100 μl was added to the 1.5 ml of medium in each 35-mm culture dish for 4 d, beginning on the fourth day of culture.

Electron Microscopy and Morphometry: For routine ultrastructural examination, cells were fixed for 1 h in 1% formaldehyde plus 1% glutaraldehyde in 100 mM sodium cacodylate (pH 7.2) plus 6% sucrose, postfixed in 2% osmium tetroxide, dehydrated in alcohol and then embedded in Epon 812. Epon was separated from glass coverslips by immersion in liquid nitrogen. The glass-plastic interface usually fractured cleanly, even though the collagen-coated coverslips were not coated with carbon. Success depended on preventing Epon from flowing over the edge of the coverslip, and this was accomplished by inverting coverslip cultures over a smaller-diameter, Epon-filled well in a rubber mold. Thin sections cut at 60 nm were stained with uranyl acetate and lead citrate and examined in a JEOL 100s microscope.

Areas of myoplasm were determined by superimposing a calibrated sheet containing a square array of intersecting lines over each micrograph (43). At a magnification of 15,000, each grid point was separated by 1 μm. The number of coated vesicles was counted in each micrograph and normalized to an area of 100 μm^2. The length of membrane (l) surrounding cisternae was estimated from the number of membrane-grid intersections, (a), according to the formula

$$l = d \times \frac{a}{2} \times \frac{\pi}{2},$$

where d = distance between grid points (39).

Preparation of Horseradish Peroxidase–α-Bungarotoxin (HRP-αBTX) Conjugates: Pilot experiments were performed with HRP-αBTX conjugates prepared and generously supplied by Dr. Thomas Lentz (Yale University) (27). In subsequent experiments we used conjugates prepared according to the method of Avrameas and Ternynck (5) as described by Vogel (42). We gratefully acknowledge the advice and help of Dr. Cheryl Weill (Louisiana State University) in this procedure. In brief, 10 mg of HRP previously activated with glutaraldehyde was mixed with αBTX (Miami Serpentarium, Miami, FL) in a molar ratio of 1:2.5 (HRP:αBTX) at pH 9.0 for 5 h at 4°C. To estimate the final concentration of αBTX and to monitor recovery, we added 3.26 × 10^6CPM of (^{125}I) αBTX (S.A. = 466 Ci/mM) to the reaction mixture.

After neutralization and incubation in 10 mM $NaCNBH_3$ (Alpha Chemicals, Danvers, MA), free αBTX was removed by filtration through Biogel P-150, equilibrated with 0.15 M NaCl and 10 mM $NaPO_4$ at pH 7.4. Finally, HRP-αBTX was separated from uncoupled HRP by cation exchange chromatography using CM Sephadex C-50. This column was developed with a gradient of Na acetate from 25 mM to 250 mM at pH 5.0. We recovered 7% of the [^{125}I]-αBTX, and the concentration of HRP-αBTX was calculated assuming a 1:1 conjugation ratio (42). The conjugates were stored at 10^{-5} M in BSS–bovine serum albumin (BSA) at −70°C. Tests for the efficacy and specificity of the conjugates are described in the text.

Labeling with HRP-αBTX Conjugates: To expose intracellular ACh receptors, myotubes were permeabilized with saponin as described by Fambrough and Devreotes (16). Cells were fixed for 1 h at room temperature in formaldehyde (a 2% solution in 20 mM sodium periodate [11]); washed several times over the course of 1 h with BSS containing 0.1 mg/ml BSA, exposed for 3–4 min to 0.5% saponin in 150 mM KCl plus 20 mM phosphate buffer, pH 7.2, and finally washed again in BSS plus BSA. HRP-αBTX conjugates were added to permeabilized cells and to intact cells for 2 h at room temperature, and the cultures were then washed several times over the course of 2 h with BSS-BSA solution.

The HRP reaction was performed as described by Graham and Karnovsky (22). After unbound HRP-αBTX was washed away, the cells were fixed for 1 h in 2% glutaraldehyde (in 10 mM sodium cacodylate pH 7.2), washed in BSS-BSA, and then incubated for 1 h at room temperature in 0.5 mg/ml 3,3′ diaminobenzidine and 0.005% H_2O_2 made up in tricine-HCl pH 7.6. After a final wash, the myotubes were postfixed in 2% OsO_4 (in 100 mM sodium cacodylate, pH 7.2), dehydrated in alcohol, and embedded as described above. To maximize the contrast between the HRP reaction product and the tissue, neither the whole cells nor the thin sections were stained with uranyl acetate or lead citrate.

(^{125}I) αBTX Binding and Autoradiography: Permeabilized and unpermeabilized cells were incubated in 5 × 10^{-9} M [^{125}I]αBTX (S.A. = 13 Ci/mmol; New England Nuclear, Boston, MA) in BSS-BSA for 1 h at room temperature and then washed five times with BSS-BSA. The cells were dissolved in 1 M sodium hydroxide plus 0.5 g/ml sodium deoxycholate, and the radioactivity was assayed in Beckman gamma counter (Beckman Instruments, Palo Alto, CA). Specific binding was taken as the difference between the total bound and the amount bound in the presence of 10^{-8} M–10^{-7} M αBTX or 40 mg/ml of tubocurare.

Some cultures were incubated in [^{125}I]αBTX and washed as described above, and then fixed in 1% formaldehyde and prepared for light microscopic auto-

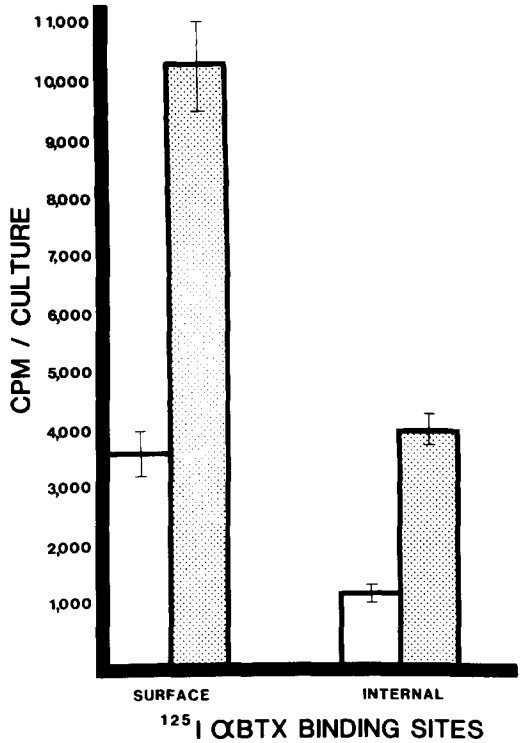


FIGURE 1 Effect of brain extract on the number of surface membrane and internal [^{125}I]αBTX-binding sites. The surface ACh receptors were blocked by incubating cells for 1 h with unlabeled αBTX at 37°C. Internal αBTX-binding sites were revealed by exposing formaldehyde-fixed myotubes to 0.5% saponin for 4 min and incubating with 5 × 10^{-9} M of ^{125}I αBTX for 1 h at 37°C. Bars indicate average ±SEM of eight cultures. Control, open bar. Brain extract-treated, stippled bar.

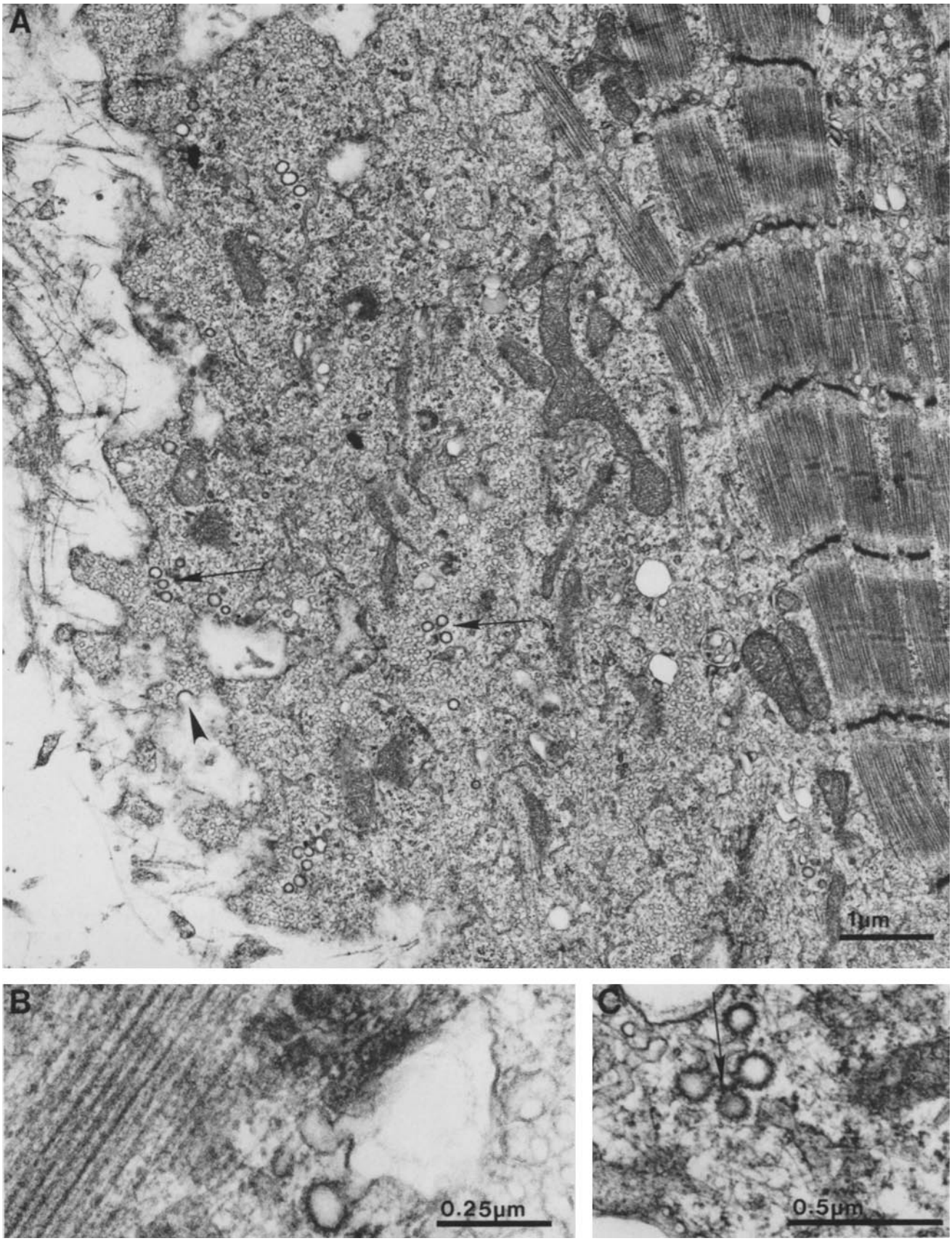


FIGURE 2 Coated vesicles in myotubes treated with brain extract (arrows in *A*). A coated invagination (pit) is marked by the arrowhead. Coated vesicles were also found in contact with internal membranes (*B*) or in contact with one another in a rosette-like configuration (*C*). (*A*) × 16,800; (*B*) × 82,500; (*C*) × 55,000.

radiography. Coverslip cultures were attached to glass slides, allowed to dry, and dipped in Kodak NTB3 emulsion diluted 1:1 with distilled water. The emulsion was exposed in dessicated boxes at 4°C for 3 d and then developed in D-19.

RESULTS

As previously reported (24), when culture medium was supplemented with brain extract for 4 d, the total number of myotube surface membrane AChR increased three- to four-fold compared with the control (Fig. 1). Brain extract also produced a large increase in the number of intracellular receptors (Fig. 1). In this experiment, surface receptors were blocked with unlabeled αBTX and then the cells were permeabilized with saponin, as described in Materials and Methods, and labeled with [^{125}I]αBTX. In both control and brain extract-treated cultures, the receptor pool revealed by brief exposure to saponin amounted to ~30% of the total number present in the surface membrane. This is the same percentage observed by Devreotes and Fambrough (12) following vigorous homogenization of chick myotubes in detergent. Experiments in which [^{125}I]αBTX binding was assayed on each day following addition of brain extract showed that the number of intracellular and surface sites increased over the same time course.

The ultrastructure of brain extract-treated myotubes was similar to that of myotubes grown in control medium. Most fibers exhibited thick and thin filaments arranged in clearly defined sarcomeres. A complex system of interconnected vesicles and tubules was prominent near the cell surface. Diad and triad junctions between tubules of this system and cisternae of the sarcoplasmic reticulum were evident near Z lines (15).

Many coated vesicles ~100 nm across were evident near the surface membrane (Fig. 2*A*), and also near Golgi cisternae in extract-treated cells. The diameter of the coated vesicles near Golgi lamellae was slightly smaller (40–60 nm) than that

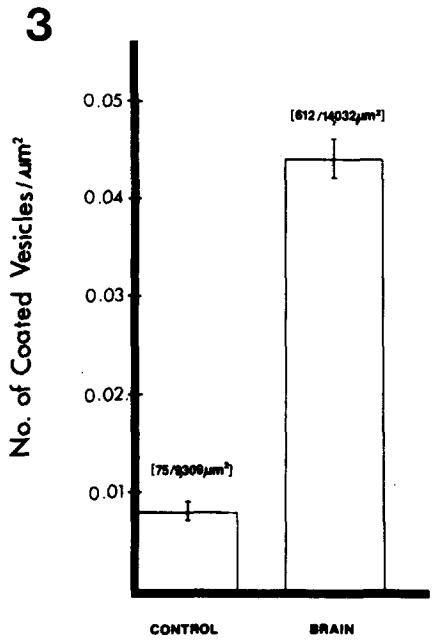


FIGURE 3 The number of coated vesicles is increased in brain-treated myotubes. Three controls and three brain extract-treated cultures were examined, and 18 myotubes were sectioned in each case. Numbers in brackets represent number of vesicles counted and the area surveyed.

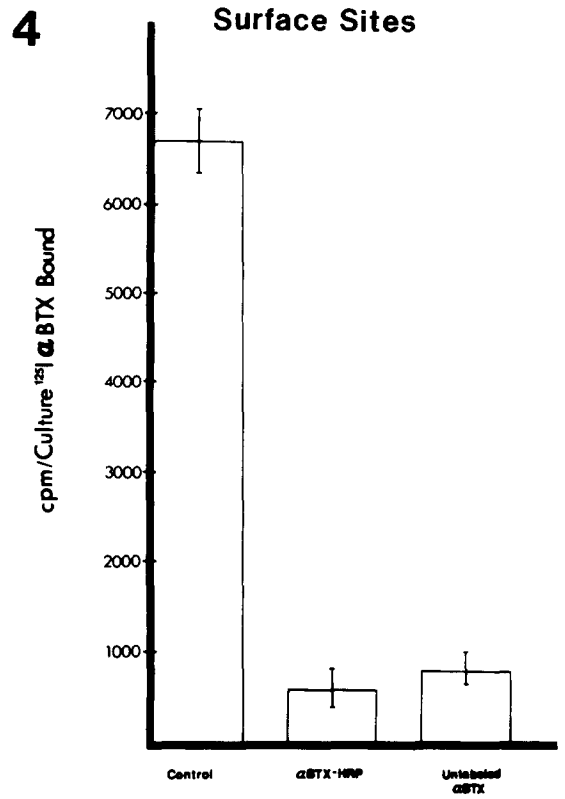


FIGURE 4 Specificity and efficacy of αBTX-HRP conjugates. The inhibition [^{125}I]αBTX binding following preincubation for 1 h at 37°C in 10^{-7} M αBTX-HRP is comparable to the inhibition produced by 10^{-8} M native toxin. Bars represent ±SEM of eight cultures.

of those near the cell surface (60–100 nm). Coated omega shapes were observed in continuity with the surface membrane (Fig. 2*A*, arrowhead), others opened into cisternae of the submembrane tubulovesicular network (Fig. 2*B*), and some appeared to contact other coated vesicles (Fig. 2*C*). Coated vesicles were found in control fibers, but estimates of their number within 2 μm of the surface membrane showed that they were far less numerous than in brain extract-treated muscle cells (Fig. 3).

The fact that brain extract increased both the number of intracellular AChR and the number of coated vesicles is consistent with the possibility that at least some of the receptors are located in the vesicles. Further evidence was obtained with HRP-αBTX conjugates.

Specificity of HRP-αBTX Conjugates

HRP-αBTX was added to intact and to permeabilized cells at several concentrations ranging from 10^{-8} M to 10^{-6} M. Specificity of binding is indicated by the following observations. (*a*) HRP-αBTX revealed the expected receptor distribution. On the light microscopic level, receptor aggregates appeared as dark brown streaks or small patches against a light tan background. On the electron microscopic level, reaction product was restricted to short segments of surface membrane. The lack of staining elsewhere presumably reflects the relatively low AChR density. (*b*) The conjugates apparently saturated all toxin-binding sites after 2 h in that they blocked the binding of [^{125}I]BTX as completely as did excess native toxin (Fig. 4). (*c*) No HRP reaction product was observed when 10^{-7} M HRP-αBTX was added in the presence of 10^{-7} M αBTX or 10^{-4} g/ml D-tubocurare. (*d*) In spinal

cord–muscle cocultures, 10^{-7} M HRP-αBTX drastically reduced stimulus-evoked synaptic responses, so we conclude that the binding sites are indeed functional AChR.

Intracellular AChR

Myotubes labeled after brief exposure to saponin appeared darker than labeled intact cells, and the stain was most intense at hot spots. Therefore, on a qualitative level it seemed clear that HRP-αBTX entered detergent-treated cells. The extent of HRP-αBTX entry was quantitated by measuring inhibition of (^{125}I)αBTX binding to intracellular sites (Fig. 5). By this criterion, HRP-αBTX gained access to an intracellular or "hidden" pool that was comparable to that "seen" by unconjugated αBTX.

Coated vesicles could be recognized in saponin-treated myotubes even though the sections were not stained with heavy metals. Many, but not all of them, contained a thin rim of HRP reaction product (Fig. 6, *A–C*). Several omega-shaped indentations of the surface membrane associated with tufted coats on their cytoplasmic surface were also labeled (Fig. 6*D*). No vesicles were labeled in control cultures preincubated in 10^{-8} M αBTX before 10^{-7} M HRP-αBTX (Fig.7).

Data collected from 18 brain extract-treated myotubes are summarized in Table I. Coated vesicles were three times more common beneath surface hot spots than elsewhere, and a higher percentage of the sub–hot spot vesicles contained HRP reaction product.

HRP-αBTX binding was not restricted to coated vesicles. In some sections, small spots of reaction product were diffusely distributed throughout the cytoplasm. Reaction product was also observed along short segments of Golgi membrane, which is consistent with the localization of [^{125}I]αBTX described by Fambrough and Devreotes (16). Finally, many cisternae of the subsurface tubulo-vesicular network were labeled (Fig. 8). Using unconjugated HRP, we repeated earlier demonstrations that most of these cisternae communicate with the extracellular space (15, 23). However, under our incubation conditions, they were more heavily labeled with HRP-αBTX following brief exposure to saponin. Subsurface membranes near surface hot spots (cluster of AChR) were

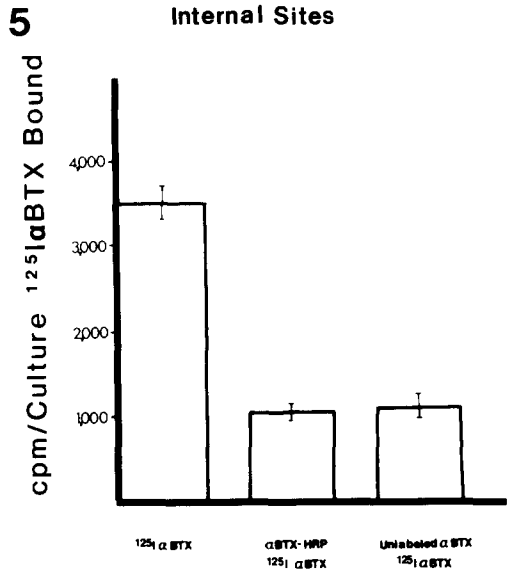


FIGURE 5 Myotubes incubated with HRP-αBTX (10^{-7} M) conjugate for 1 h at 37°C, fixed, and permeabilized with saponin show that the binding of [^{125}I]αBTX (5×10^{-9} M, incubation time 1 h at 37°C) to the internal sites is blocked. The block is as complete as that observed with native αBTX. Internal sites were assayed after all surface receptors were blocked with unlabeled toxin.

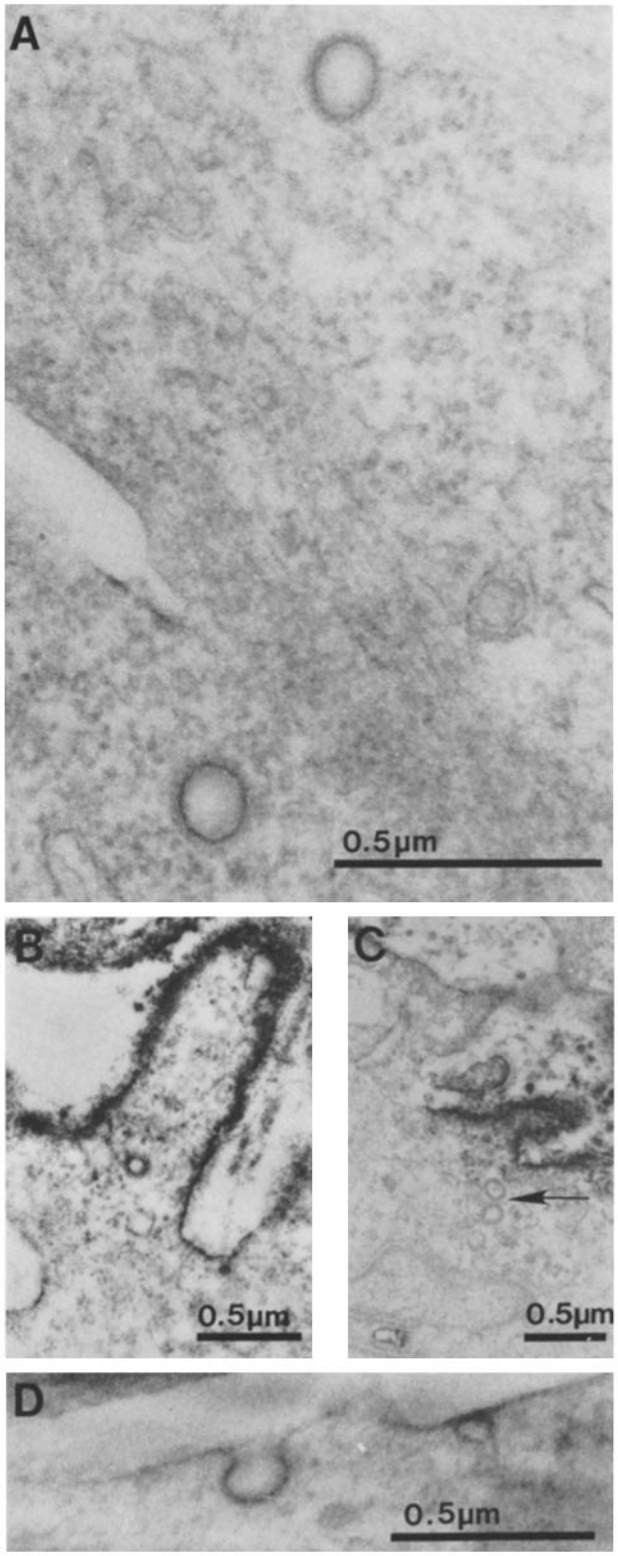


FIGURE 6 Coated vesicles that contain rim of reaction product following exposure to HRP-αBTX for 2 h are shown in (*A*). Many vesicles beneath surface membrane that contain a high receptor density have HRP-αBTX binding sites (*B*), but some coated vesicles beneath surface hot spots are not labeled (*C*, arrow): a labeled pit opening at the surface membrane at the edge of a hot spot is shown in *D*. (*A* and *D*) × 55,000; (*B*) × 27,500; (*C*) × 22,000.

much more extensively labeled than membranes beneath nearby, non-hot spot surface membrane (Table I).

Direction of Coated Vesicle Movement

Several tests were performed to determine whether sub-hot spot coated vesicles were involved in exocytosis or endocytosis.

Fambrough and Devreotes (16) have shown that 2–3 h is sufficient time to allow the pool of newly synthesized AChR to travel from the site of synthesis to the cell surface. If coated vesicles carry receptors to the surface membrane, then the number of labeled vesicles should be greatly diminished a few hours after inhibition of protein synthesis. Brain-treated myotubes incubated in 20 μg of puromycin for 6 h did show a significant decrease in the number of coated vesicles near the surface membrane and a decrease in the percentage labeled with HRP-αBTX (Table I). Puromycin treatment also reduced the extent of cisternal membrane that was labeled. Hot spots in the surface membrane, on the other hand, stained intensely after inhibition of protein synthesis for 6 h (Fig. 9).

Cultures were incubated in the HRP-αBTX at 37°C for periods of 1–6 h. After 1 h of incubation, only 10% ± 3.0 of the coated vesicles contained reaction product (Fig. 10*A*). In contrast, fibroblasts in the same cultures took up a great deal of the conjugate after 1 hour. Numerous vesicles and large vacuoles were filled with reaction product (Fig. 10*B*). After 6 h of incubation with HRP-αBTX 17.6% ± 3.6 of the coated vesicles were labeled.

In another experiment several cultures were incubated with unlabeled toxin for 2 h followed by fixation, treatment with 0.5% saponin, and incubation with HRP-αBTX conjugates. In cultures whose surface receptors were not blocked, 44% ± 3.0 coated vesicles were labeled, whereas in surface-blocked cultures 38% ± 4.5 coated vesicles were labeled.

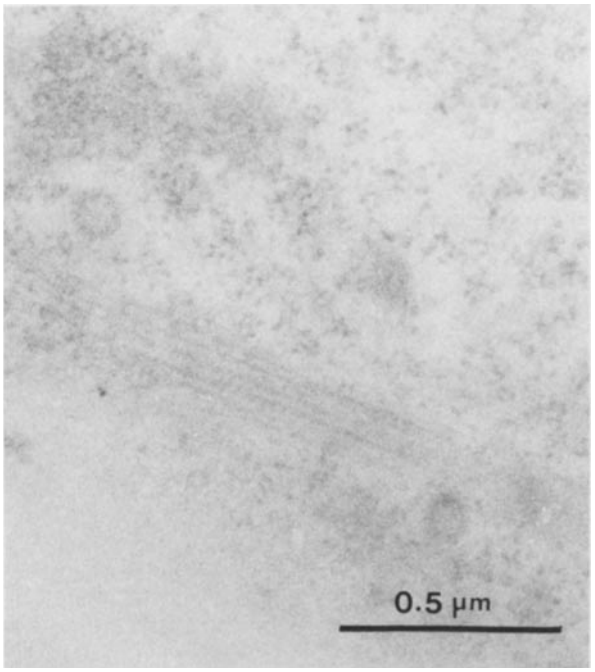


FIGURE 7 Specificity of HRP-αBTX controls. The cells were fixed, treated with saponin, incubated in 10^{-8} M αBTX and finally in 10^{-7} M HRP-αBTX. Note the absence of the reaction product over coated vesicles. × 45,000.

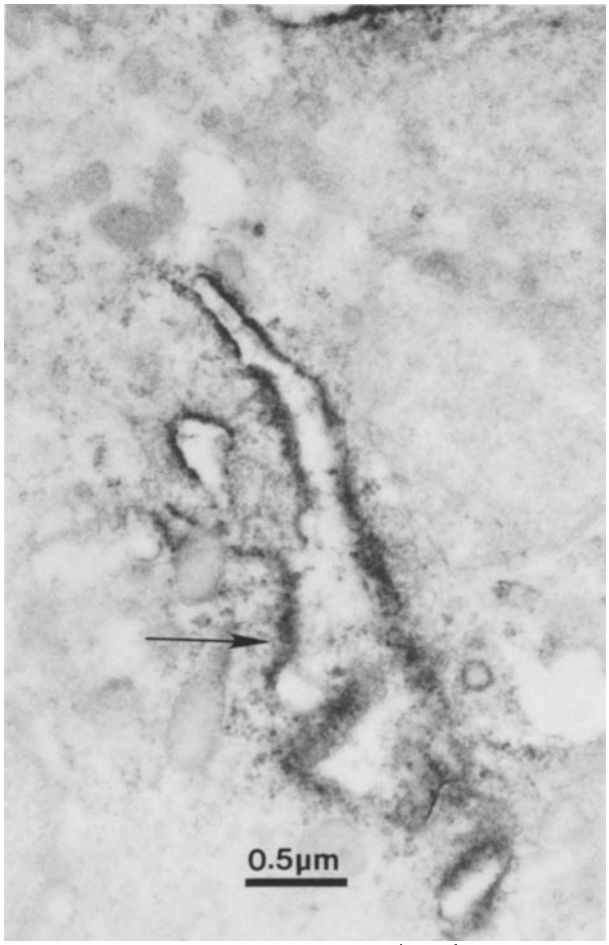


FIGURE 8 Dense HRP-αBTX reaction product along a cisterna of the tubulovesicular network (arrow). Note the dense reaction product (hot spot) on the near-by surface membrane. × 41,000.

TABLE I

Coated Vesicles and Subsurface Membranes Labeled with HRP-αBTX

	Extract-treated		Extract-treated + puromycin	
	Hot spot	Non-hot spot	Hot spot	Non-hot spot
No. of coated vesicles counted	248	203	205	103
Coated vesicles/100 μm^2	12.0 ± 0.5	4.0 ± 0.8	1.06 ± 0.07	1.2 ± 0.1
% Coated vesicles labeled	54.8 ± 3.2	20.2 ± 2.8	22.9 ± 2.9	2.0 ± 1.4
% Cisternal membrane labeled	76.0 ± 1.5	7.0 ± 1.0	17.0 ± 1.3	4.0 ± 1.1

Myotubes were fixed with phosphate-buffered formaldehyde, permeabilized with saponin, and incubated with αBTX-HRP for 2 h at room temperature as described in Methods. The data are based on sections cut through 36 hot spots on 18 myotubes in three extract-treated cultures and through 30 hot spots on 21 myotubes in three extract plus puromycin cultures. Puromycin was added at 20 μg/ml for 6 h before labeling. Each entry = mean ± SE. Hot spots were defined as patches of surface membrane covered with HRP-αBTX reaction product. Coated vesicles and cisternae within 2 μm of the surface were counted.

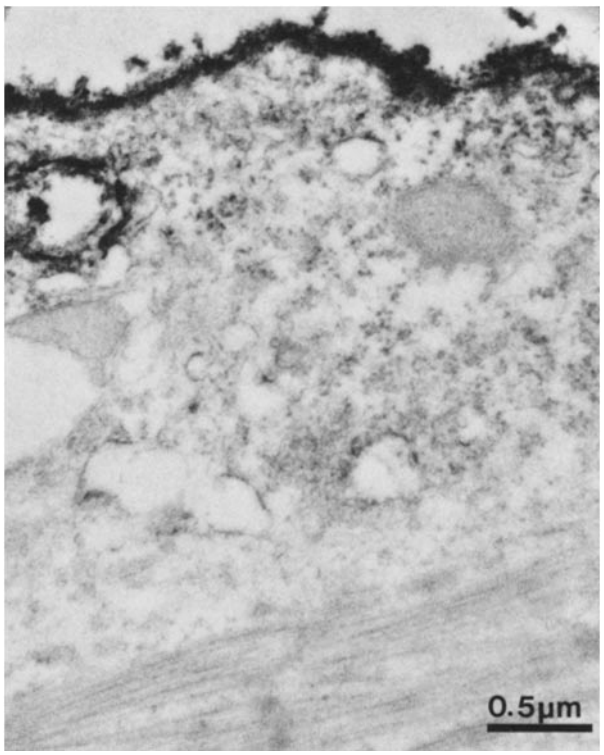


FIGURE 9 Unstained cisternae and vesicles following exposure to 20 mg/ml puromycin for 6 h. Receptor aggregates in the surface membrane remain intensely stained. × 27,500.

DISCUSSION

Coated vesicles that contain AChR are present in the cytoplasm of embryonic chick myotubes grown in culture. This conclusion is based on the appearance of a thin rim of HRP reaction product following incubation of permeabilized cells in HRP-αBTX. Other workers have shown that HRP-αBTX binds to the same surface sites as αBTX (11, 27, 41). We confirmed this observation with our own preparation of conjugates, and we have shown that the same selectivity holds for intracellular sites. Thus, the appearance of label in coated vesicles is related to the presence of AChR. Most of the vesicles examined were located immediately beneath patches of the surface membrane that contain a high density of HRP-αBTX binding sites, so the possibility exists that label within them simply reflects diffusion of reaction product from surface sites (30). This seems unlikely, however, because many unlabeled coated vesicles were observed beneath intensely stained surface patches near other vesicles that were labeled. Moreover, approximately the same number of vesicles were labeled whether or not surface receptors were blocked with αBTX before permeabilization. Finally, very few vesicles contained reaction product after puromycin treatment even though the surface hot-spot label appeared as intense as controls. Our finding of AChR-rich coated vesicles is consistent with the recent cell fractionation studies of Porter-Jordan et al. (34). They demonstrated specific [^{125}I]αBTX-binding sites in highly purified coated vesicles prepared from homogenates of 17-d embryonic chick limb muscle.

We estimate that ~50% of the coated vesicles contain AChR but this figure must be considered only approximate. It may be an overestimate because it is difficult to identify coated vesicles unambiguously in sections that are not counterstained with heavy metals. It may be an underestimate because "unlabeled" vesicles may contain AChR at a density that is too low to detect with HRP-αBTX.

AChR degradation involves internalization (12, 13), so it is perhaps not surprising that a few coated vesicles were labeled following addition of HRP-αBTX to the medium bathing intact cells for 1 h. However, the number of vesicles labeled in this situation was <20% of the total labeled by HRP-αBTX in saponin-permeabilized cells, which suggests that the majority of vesicles are involved in exocytosis of receptors rather than in endocytosis. The degree of coated vesicle–mediated endocytosis may be underestimated in this experiment. Devreotes and Fambrough (12) described a population of "hidden" receptors in intact cells, distinct from the precursor pool, that was not labeled after a 1-h exposure to a saturating concentration of αBTX, but that was labeled after 24 h. Hidden receptors may be located in a membrane compartment that is continuous with the surface membrane,[2] and some of the unlabeled vesicles may have budded off from this compartment. However, two other findings indicate that the majority of labeled coated vesicles are on route to the cell surface. First, the number of HRP-αBTX–labeled vesicles was markedly increased following chronic exposure to brain extract. Brain extract induces a three- to fourfold increase in receptor synthesis and incorporation without a significant change in receptor degradation (7, 24). Second, exposure to 20 μg of puromycin for a period of time sufficient to deplete the intracellular, precursor pool (12–14) resulted in more than 20-fold decrease in the number of HRP-αBTX-stained vesicles beneath hot spots. In chick myotubes, inhibition of protein synthesis does not increase receptor degradation (12).

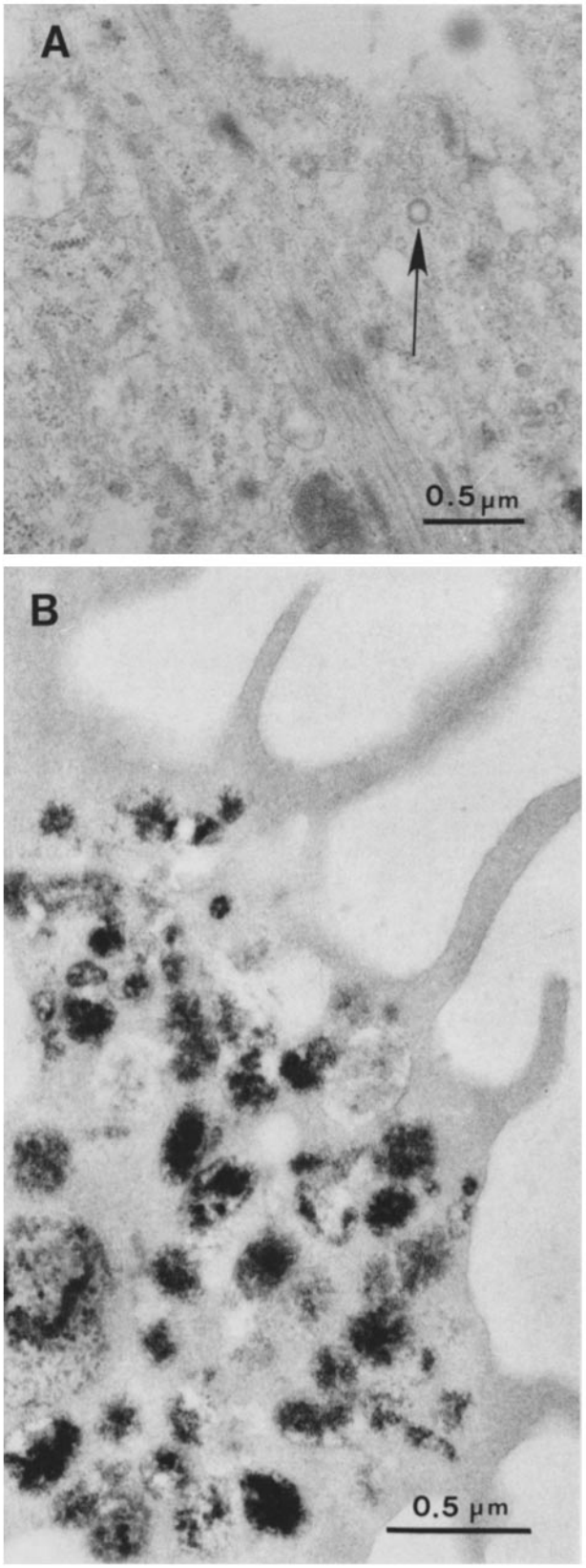
Direct evidence for coated-vesicle–mediated exocytosis has been obtained in vesicular-stomatitis-virus–infected Chinese hamster ovary cells (37, 38), where the kinetics of movement of the viral coat glycoprotein via coated vesicles from the rough endoplasmic through the Golgi system to the cell surface have been accurately charted. This example is somewhat far afield from AChR in chick myotubes, but it serves to emphasize the plausibility of the proposed sequence.

Insertion of AChR in small packets may account for the presence of many microaggregates of receptors that appear as bright "speckles" when myotubes are labeled with rhodamine-α-BTX (3, 6, 7). Speckles are prominent within hot spots, but they also are evident over the remainder of the cell surface. Insertion of receptors into packets may also account for the appearance of small islands of intramembrane particles observed in replicas of freeze-fractured myotube membranes (10, 32, 33, 44). The size of the particle islands is approximately equal to the area of an opened coated vesicle. It will be important, in this regard, to determine whether the density of receptors (or particles) within coated vesicles is the same as that within the surface micropatches.

Surface hot spots in uninnervated chick myotubes are stable in that they remain in approximately the same position (21), even though individual receptors within them turn over with a half-life of ~24 h (40). Axelrod et al. (4) obtained the same result in rat myotubes, and they also showed that, in this species, hot spot and extra-hot spot receptors do not exchange rapidly. If the same result holds in chick cells stimulated by

[2] Our results indicate that membrane invaginations in the immediate vicinity of surface receptor clusters contain AChR that are not labeled after 1–2 h exposure to HRP-αBTX (see Fig. 8 and Table I).

brain extract, then hot spots must be maintained by local insertion of newly synthesized receptors. The presence of a relatively large vesicle pool beneath hot spots suggests that the rate constant which, presumably, is determined by the basic mechanisms governing vesicle-membrane fusion, need not be greater at hot spots than elsewhere.



In addition to inducing the synthesis of AChR, brain extract also promotes the aggregation of AChR already present in the surface membrane (7). Therefore, insertion of new receptors probably does not account for the formation of all new hot spots. However, this does not invalidate the argument presented above, that local insertion of new receptors is necessary for the maintenance of all hot spots once they appear.

It is unlikely that coated vesicles contain the entire intracellular precursor pool of AChR. We confirmed Fambrough and Devreotes's (16) finding of αBTX-binding sites in Golgi membranes. In addition, since the labeling over subsurface membranes was abolished after 6 h in puromycin, these membranes probably contain newly synthesized receptors. It is significant in this regard that subsurface cisternae in the immediate vicinity of hot spots were more extensively labeled than cisternae located beneath other regions of the surface membrane that contain a relatively low density of AChR. We observed coated vesicles in contact with subsurface cisternae, so this membrane system may be on the AChR route to the cell surface. It is probably not an obligatory path, however, because we also found coated vesicles opening on the cell surface adjacent to existing hot spots.

Although this paper has focused on receptor clusters in uninnervated myotubes treated with brain extract, our long-range goal is to describe the assembly of the postsynaptic membrane at nerve-muscle synapses. The notion that coated vesicles contribute to new postsynaptic sites was first suggested by Altman (1) in his studies of embryonic rat cerebellar Purkinje cell dendrites, and similar observations were made in the embryonic chick spinal cord by Stelzner et al. (41). Rees et al. (35) described coated vesicles in dissociated rat embryonic sympathetic ganglion neurons maintained in culture. They found two- to threefold more coated vesicles in ganglion neurons that were contacted by spinal cord nerve processes than nonuncontacted cells. In young cultures, coated vesicles were especially prominent at close growth cone–cell body contacts (7–10 nm gap). In older cultures, they were present beneath morphologically differentiated synapses marked by a wide cleft (20 nm), increased membrane density, and clusters of synaptic vesicles in the presynaptic element. In a subsequent brief note, Rees (36) reported that antibodies raised against *Torpedo* AChR bind to ganglion cell coated vesicles.

Anderson and Cohen (2) obtained evidence that, in *Xenopus*, myocyte AChR migrate to nerve-muscle contacts by labeling exposed receptors with rhodamine-αBTX before plating neurons, but they did not determine whether mobile receptors account for the entire postsynaptic population. In chick cultures, prelabeled, exposed receptors account for only a small fraction (~10%) of the population in receptor clusters beneath cholinergic neurites observed 15–24 h after contact (J. Matthews-Bellinger and G. D. Fischbach, unpublished observations). Our preliminary experiments have shown that coated vesicles are more common at newly formed nerve-muscle synapses in vitro (identified by focal, extracellular recording of synaptic currents) than elsewhere in the myoplasm (9). We do not yet know whether these subsynaptic

FIGURE 10 Intact myotubes incubated with HRP-αBTX for 1 h take up little of the label (*A*). The arrow points to an unlabeled coated vesicle. In contrast, fibroblasts present in the same culture (*B*) show a great deal of endocytosis as numerous vacuoles and vesicles are filled with the reaction product. × 41,000.

vesicles contain AChR or whether they are transporting membrane to the cell surface. We assume that brain extract mimics the inductive action of cholinergic neurons, but in the absence of additional data this remains simply an attractive hypothesis.

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