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Acetylcholine Receptors of Muscle Grown *In Vitro*

(α-bungarotoxin/iodination/cholinergic drugs/autoradiography)

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ABSTRACT **[^{125}I]Monoiodo- and [^{125}I]diiodo-α-bungarotoxin were synthesized and shown to bind specifically to the acetylcholine receptor of cultured embryonic chick- and rat-muscle cells. The pharmacologic properties of the receptor of cultured embryonic chick muscle resembled those of the nicotinic acetylcholine receptor of adult vertebrate muscle. Autoradiography of muscle cells labeled with toxin showed that acetyleholine receptors were distributed over the entire cell surface. In addition, discrete areas with a high receptor concentration were found.**

α-Bungarotoxin, a protein of known amino-acid sequence (1) obtained from the venom of the Formosan banded krait, *Bungarus multicinctus*, and similar proteins from cobra and other elapid snake venoms bind with high specificity to acetylcholine receptors of striated muscle (2–8), electroplax (9–11), and brain (12). In this way, neurotoxins such as α-bungarotoxin inhibit the response of certain cells to acetylcholine.

Acetylcholine receptors are distributed over the entire surface of embryonic and neonatal striated muscle cells of vertebrates (7, 13); however, in adult innervated muscle, the acetylcholine receptors are restricted to the area of the synapse (14, 15). Since the binding of α-bungarotoxin to the acetylcholine receptor of muscle is not readily irreversible in the systems examined, labeled α-bungarotoxin can be used to assay acetylcholine-receptor concentration as well as sites of synaptic connections (4–12).

The purpose of this communication is to show that [^{125}I]-α-bungarotoxin binds to the acetylcholine receptor of cultured embryonic chick- and rat-muscle cells, and to describe the properties of this receptor.

METHODS

Reagents. Carrier-free Na^{125}I was obtained from Amersham Searle Co.; acetylcholine chloride, atropine sulfate, carbamylcholine chloride, choline chloride, eserine, pilocarpine hydrochloride, and *d*-tubocurarine chloride from Sigma Chem. Co.; decamethonium iodide and hexamethonium chloride from K & K Lab.; nicotine hydrochloride from J. T. Baker Chem. Co.; 1,1-dimethyl-4-phenylpiperazinium iodide from Aldrich Chem. Co.; gallamine triethiodide (Flaxedil) was the gift of Dr. J. M. Smith, Lederle Laboratories. Crystalline bovine-serum albumin was obtained from Armour Pharm. Co.; crude collagenase (CLS) from Worthington Biochem. Corp.; and Viokase (4× pancreatin) from Grand Island Biological Co.; 3-monoiodo-L-tyrosine was obtained from Aldrich Chem. Co.; 3,5-diiodo-L-tyrosine from Nutritional Biochem. Co.; 2- or 4-monoiodo-L-histidine was the gift of Dr. Jan Wolff.

Purification of α-Bungarotoxin. Lyophilized venom of *Bungarus multicinctus* was obtained from the Miami Serpentarium. α-Bungarotoxin was purified by chromatography on carboxymethyl (CM)-Sephadex (C-25) (1), and appeared homogeneous when subjected to disc-gel electrophoresis. The minimum lethal dose of the purified toxin (intravenous) was 3–4 μg per mouse. The toxin had a curare-like paralytic action on the rat phrenic nerve–diaphragm preparation (3, 16) but had no effect when the diaphragm muscle was stimulated directly.

Labeling of α-Bungarotoxin. Purified α-bungarotoxin was labeled with ^{125}I by a modification of the methods of McFarlane (17) and of Helmkamp *et al.* (18) as follows: 27.5 nmol of ICl were incubated at 0° with 5–7 mCi of carrier-free Na ^{125}I in 75 μl of a solution containing 33 mM HCl and 170 mM NaCl. After 4 min, this solution was injected into 150 μl of a cold, continuously stirred solution containing 13.0 nmol of α-bungarotoxin in 0.4 M NH_4Cl previously adjusted to pH 8.9 with NH_4OH. After 2 min at 0°, the iodination reaction was terminated by the addition of 20 μl of 0.1 M $Na_2S_2O_3$ and then 20 μl of 0.1 M NaI. The volume was adjusted to 1.0 ml with solution A (5 mM sodium phosphate, pH 7.4; 2 mg of albumin per ml). Siliconized glassware was used in subsequent steps since [^{125}I]α-bungarotoxin binds to glass. The iodinated toxin was separated from free ^{125}I by gel filtration on a Sephadex G-25 column with solution A. 50–60% of the radioactivity was incorporated into the toxin fraction.

The biologic activity of the iodinated toxin was tested by incubation of the toxin with a mouse diaphragm for 1 hr, followed by autoradiography (19). Neuromuscular synapses were heavily labeled, as expected. Toxicity of the iodinated toxin to mice was similar to that of unlabeled toxin.

Fractionation of Monoiodo- and Diiodo-α-bungarotoxin. The solution of iodinated toxin was diluted 2-fold with a solution containing 2 mg of albumin per ml of H_2O and adsorbed onto a 1.0-ml column of CM-Sephadex (C-50) equilibrated with solution B (3.3 mM sodium phosphate buffer, pH 7.4; 2 mg of albumin per ml). The column was washed with solution B and the column effluent (peak I, Fig. 1), containing 3–10% of the applied radioactivity, was discarded. Only 20–30% of this material was precipitated by 10% Cl_3CCOOH. The iodinated toxin was eluted (peaks II and III, Fig. 1) with a linear gradient consisting of 40 ml of solution B and 40 ml of solution B containing 80 mM NaCl. More than 90% of the radioactivity of peaks II and III was precipitated by 10% Cl_3CCOOH.

The iodinated toxins of peaks II or III were pooled sepa-

rately and kept at 0°. Each preparation was used for a maximum of 60 days.

When the iodination was performed with a lower ratio of toxin to ICl (6 nmol of toxin per reaction mixture) only one peak of iodinated toxin, peak II, was obtained, with a specific activity that corresponded to 2 atoms of iodide per molecule of toxin. Peak II was therefore identified as diiodo-α-bungarotoxin; its initial specific activity was 320–400 Ci/mmol. The radioactive material of peak III was identified as monoiodo-α-bungarotoxin, with an initial specific activity of 160–200 Ci/mmol (see *Results*).

Portions of the material from peaks II and III were digested with Viokase at 37° for 48 hr. Each 0.2-ml reaction mixture contained: 1.5 mg of Viokase, 50 pmol of iodotoxin, 0.15 mg of albumin, 30 mM Tris buffer (pH 8.0), and 50 mM NaCl. Portions of the hydrolysates (3×10^5 cpm each) were analyzed by paper electrophoresis with 1 M formic acid (20) and by descending paper chromatography with *n*-butyl alcohol saturated with 2 M acetic acid (21). Monoiodotyrosine, diiodotyrosine, monoiodohistidine, and ^{125}I were used as markers. Mono- and diiodotyrosine were detected on the paper by their UV absorption, monoiodohistidine with the Pauli diazo reagent (22), and the radioactivity of 2.5 × 0.5-cm sections of paper with a Beckman liquid scintillation spectrometer.

Muscle Cultures. Chick embryo-muscle cultures were prepared by a modification of the method of Konigsberg (23). Muscle from the hind limb of 10-day-old chick embryos was dissociated with 0.05% collagenase. Myoblasts were selected by flotation for 45 min (24), then 1×10^5 cells were added to each 50-mm, collagen-coated (25) petri dish (Falcon Plastics) containing 2.5 ml of medium composed of 92.5% F-14 (described below), 1.0% fetal-calf serum (Colorado Serum Co.), 5.0% horse serum, and 1.5% chick-embryo extract (Microbiological Asso.). Cultures were incubated at 37° in a humidified atmosphere of 90% air–10% CO_2 (5% CO_2 is now used). Medium was changed every 3–4 days.

F-14 is a modification of Ham's F-12 (26) and the medium described by Coon and Weiss (27). The following changes were made with respect to F-12 (26): concentrations of amino acids and of pyruvate were doubled; ascorbic acid was added to 85 μM; and the concentrations of the following compounds were: 11 mM glucose, 5 mM KCl, 0.85 mM $MgCl_2$, 0.15 mM $MgSO_4$, 2 mM $CaCl_2$, 0.5 μM $ZnSO_4$, and 23.5 mM $NaHCO_3$.

Clonal rat muscle lines L-6 (24) and L-8 (derived by Yaffe) were the gifts of Drs. D. Schubert and D. Yaffe, respectively. They were grown on collagen-coated, 50-mm petri dishes containing 2.5 ml of Dulbecco's Modified Eagle's Medium (28) (Grand Island Biological Co.) with 10% fetal-calf serum.

[^{125}I]α-Bungarotoxin Binding Assay. Duplicate cultures were assayed in a humidified aluminum box gassed with 90% air–10% CO_2 in a 37° water bath. The medium in each petri dish was removed and replaced by 2.0 ml of F-14 medium without choline, containing 2 mg of albumin per ml. Petri dishes were incubated for 20 min, [^{125}I]α-bungarotoxin was added, and incubation was continued for an additional 60 min, unless otherwise specified. Each dish was washed at 24° three times with 3-ml portions of F-14 medium containing 2 mg of albumin per ml, and three additional times with 3-ml portions of F-14 medium.

To determine the amount of radioactive toxin bound to muscle cells after each plate was washed, 1.5 ml of 1% trypsin (Difco, 1:250) dissolved in H_2O and adjusted to pH 7.4 was added, and the dish was rotated for 15 min at 37°. The resulting suspension was transferred to a scintillation vial, and the procedure was repeated three additional times. Separate scintillation vials were used to count each wash. 10 ml of Triton X-100:toluene:Liquifluor solution (29) were added to each vial, and radioactivity was determined.

The amount of iodinated toxin bound to a cell-free petri dish (0.2–0.4 fmol, about 100–200 cpm) was subtracted from all values.

RESULTS

Binding of [^{125}I]α-bungarotoxin to cultured muscle cells

α-Bungarotoxin, purified and iodinated as described under *Methods*, was separated from free ^{125}I and fractionated on a column of CM-Sephadex (C-50) yielding three peaks of radioactive material (Fig. 1). Portions of the radioactive materials from peaks II and III were digested as described in *Methods*. Between 90 and 95% of the radioactive material of peak II hydrolysate was identified as diiodotyrosine; no monoiodotyrosine was detected. In contrast, 90–95% of the radioactive material of peak III hydrolysate was identified as monoiodotyrosine; no diiodotyrosine was found. Two radioactive contaminants (5–10%) were found with each hydrolysate, perhaps the result of incomplete digestion. Monoiodohistidine was not detected in either hydrolysate.

From the calculation of total ^{125}I incorporated into the toxin, the ratio of toxin to ^{125}I in peak III was 1:1. More than 90% of the radioactivity of peak III toxin was shown to be [^{125}I]monoiodotyrosine; therefore, the toxin of peak III was [^{125}I]monoiodo-α-bungarotoxin. The toxin of peak II was [^{125}I]diiodo-α-bungarotoxin (see *Methods*), and >90% of the radioactivity was [^{125}I]diiodotyrosine. Thus, of the two tyrosyl residues present in α-bungarotoxin (1), only one was iodinated.

Both mono- and diiodo-α-bungarotoxin were incubated with chick-embryo muscle cells cultured *in vitro*. In Fig. 2, the relation between the concentration of toxin and the amount of toxin bound to cells is shown. Both mono- and diiodo-toxins bound to the cells; however, at relatively low toxin concentrations, more monoiodo-toxin bound to cells than diiodo-toxin. Maximum binding of monoiodo-toxin was observed at concentrations above 2.5 nM toxin, whereas 7.5 nM diiodo-toxin was required to achieve this level of binding. When higher concentrations of toxin or longer incuba-

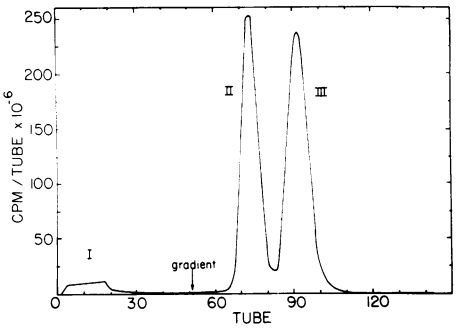

FIG. 1. Fractionation of iodinated toxin. 4.4×10^9 cpm of iodinated α-bungarotoxin was fractionated on a column of CM-Sephadex (C-50) as described in *Methods*. The flow rate was 10 ml/hr; each tube contained 0.87 ml.

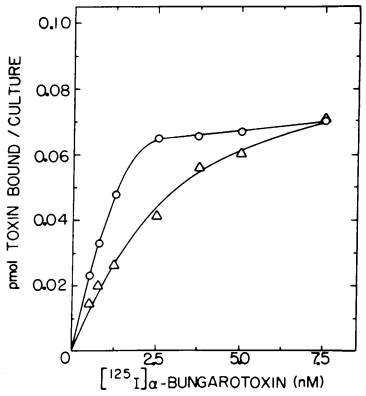

FIG. 2. Effect of [^{125}I]monoiodo-(O——O) and [^{125}I]diiodo-α-bungarotoxin(Δ——Δ) concentrations upon binding to embryonic chick-muscle cells. Chick-muscle cells, in culture for 12 days, were assayed as described in *Methods*.

tion periods were used, equal amounts of mono- and diiodotoxins bound to the cells (data not shown).

The rate of binding of monoiodo-toxin to cultured chick-muscle cells is shown in Fig. 3. Under the conditions used, maximum binding was obtained in about 30 min.

The binding of iodinated α-bungarotoxin to cultured chick muscle was inhibited by compounds known to interact with acetylcholine receptors of the nicotinic type, as shown in Fig. 4 and in Table 1. Binding of monoiodo-α-bungarotoxin (1.25 nM final concentration) was inhibited 50% by about 1.0 μM *d*-tubocurarine chloride, 0.3 μM decamethonium chloride, or 0.3 μM acetylcholine chloride in the presence of 3 μM eserine (an inhibitor of acetylcholinesterase). Eserine alone, at this concentration, had little effect on the toxin binding (Table 1). Bungarotoxin binding was also inhibited by carbamylcholine, nicotine, gallamine, 1,1-dimethyl-4-phenylpiperazinium, and decamethonium. Hexamethonium, a preferential inhibitor of nicotinic acetylcholine receptors of autonomic ganglia rather than muscle (30), had little effect upon binding. Pilocarpine and atropine, inhibitors of muscarininc acetylcholine receptors, had little, if any, effect upon α-bungarotoxin binding to muscle. These results suggest that labeled α-bungarotoxin binds with high specificity to acetylcholine receptors of cultured embryonic chick-muscle cells.

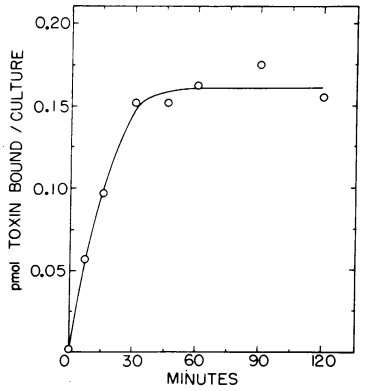

FIG. 3. Rate of [^{125}I]monoiodo-α-bungarotoxin binding to cultures of chick-embryo muscle. Chick-muscle cells, in culture for 9 days, were assayed in the presence of 5 nM [^{125}I]monoiodo-α-bungarotoxin.

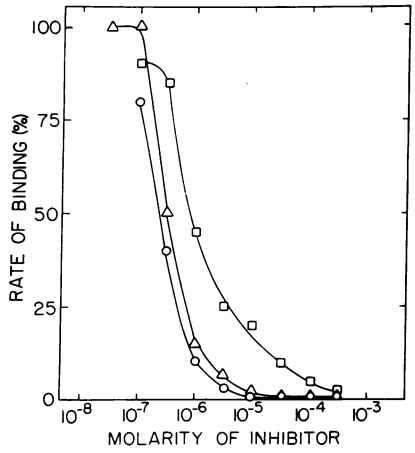

FIG. 4. The effect of inhibitors on the binding of [^{125}I]monoiodo-α-bungarotoxin to cultures of chick-embryo muscle. Muscle cells, in culture for 10 days, were incubated in F-14 medium without choline, containing 2 mg of albumin per ml. At 20 min, inhibitors were added; after an additional 20 min, the cultures were assayed in the presence of 1.25 nM [^{125}I]monoiodo-α-bungarotoxin as described in *Methods*. Symbols represent the following: Δ, acetylcholine chloride in the presence of 3 μM eserine; □, *d*-tubocurarine chloride; O, decamethonium iodide. Binding in the absence of inhibitors (100% on the ordinate scale) corresponds to 172 fmol of [^{125}I]monoiodo-α-bungarotoxin bound per culture. This value was less than half of the amount of bungarotoxin bound at saturation (determined with 5 nM monoiodo-α-bungarotoxin).

Autoradiography

In Fig. 5, photomicrographs are shown of a typical culture of chick-embryo muscle cells grown *in vitro* for 14 days, incubated with [^{125}I]α-bungarotoxin, and autoradiographed. Bright-field illumination was used to show silver grains (panels *A* and *C*) and phase-contrast illumination (panel *B*) was used

TABLE 1. *Inhibition of [^{125}I]monoiodo-α-bungarotoxin binding to cultured chick muscle*

Expt. no.	Addition	Molarity (μM)	Rate of binding (%)
1.	Carbamylcholine chloride	1	100
		10	36
		100	3
2.	Hexamethonium chloride	10	85
	Gallamine triethiodide	10	30
		100	4
3.	Atropine sulfate	10	94
		100	88
	Pilocarpine hydrochloride	10	105
		100	95
	1,1-Dimethyl-4-phenyl-piperazinium iodide	10	5
		100	1
	Nicotine hydrochloride	10	5
		100	1
4.	Eserine	3	97
		30	76

Chick-embryo muscle cells, in culture for 12 days, were assayed as described in the legend of Fig. 4. 100% corresponds to the following (fmol of toxin bound): Exp. 1, 87; Exp. 2, 154; Exp. 3, 270; Exp. 4, 170.

to show cell detail. A heavy deposit of silver grains can be seen overlying the myotubes, whereas adjacent fibroblasts are devoid of label, indicating specificity of α-bungarotoxin binding to muscle. The concentration of acetylcholine receptor varied between individual myotubes in the same culture, and occasionally also varied along the length of a single myotube. In addition, autoradiographs revealed small localized areas with receptor concentrations higher than adjacent areas of the cell.

Specific activity of acetylcholine receptors

Colonies of chick-muscle, as well as cultures of rat-muscle, clonal lines L-6 (24, 31) and L-8, were assayed for acetylcholine receptors. The specific activities are shown in Table 2 and are compared with the receptor concentrations reported for other tissues.

The results show that receptor specific activities of chick and rat muscle grown *in vitro* equal or exceed the values reported for rat diaphragm. The chick and rat muscle cells in each of the cultures assayed were at different stages of maturation and included myoblasts that bind little or no toxin (manuscript in preparation). Therefore, the specific activity of the acetylcholine receptor of mature myotubes is probably higher than the values reported here.

TABLE 2. *Acetylcholine-receptor concentrations of various tissues*

Source of cells	fmol of receptor/mg of protein	Reference
Cultured cells		
Chick-embryo muscle	150–1000	
Rat muscle, clone L-6	52	
Rat muscle, clone L-8	14	
Tissue		
Diaphragm, neonatal rat	12–35*	(7)
Diaphragm, adult rat	0.5–12*	(7)
Electroplax, *Electrophorus*	300–900†	(32)
Electroplax, *Torpedo*	31,000†	(33)
Cerebral cortex, guinea pig	87,500	(12)

Chick-embryo muscle cells, originally 1×10^3 cells per 50-mm petri dish, were cultured 16 days and incubated with 5 nM [^{125}I]diiodo-α-bungarotoxin for 2.5 hr. The plates were washed as described in *Methods*, with two additional washes of 3 ml each of Dulbecco's phosphate-buffered saline (34). 18 Muscle colonies were marked, and 6-mm diameter cloning cylinders were attached to the plates around each colony with silicone grease. Then, 0.1 ml of 0.1 M NaOH was added to each colony within a cylinder, and the plates were incubated for 6 hr at room temperature. The solution was triturated in the cylinder, removed, and a fresh 0.1 ml of 0.1 M NaOH was added. The plates were incubated as before, and the two portions of solution from each clone were pooled. Samples were taken for determination of radioactivity and of protein (35). Range of values obtained is shown.

The clonal rat muscle lines L-6 and L-8 were grown as described in *Methods*. L-6, originally plated at a density of 1×10^4 cells per petri dish, was cultured for 17 days. L-8, originally plated at a density of 5×10^4 cells per petri dish, was cultured for 11 days. The cultures were incubated with 5 nM [^{125}I]diiodo-α-bungarotoxin and washed as above. The cells of each culture were dissolved in 2 ml of 0.1 M NaOH; protein and radioactivity were determined.

* Calculated from values reported, assuming 200 mg of protein per g of diaphragm wet weight. Range given is for nonendplate and endplate regions, respectively.

† Calculated from values reported, assuming 30 mg of protein/g of elecroplax wet weight (36).

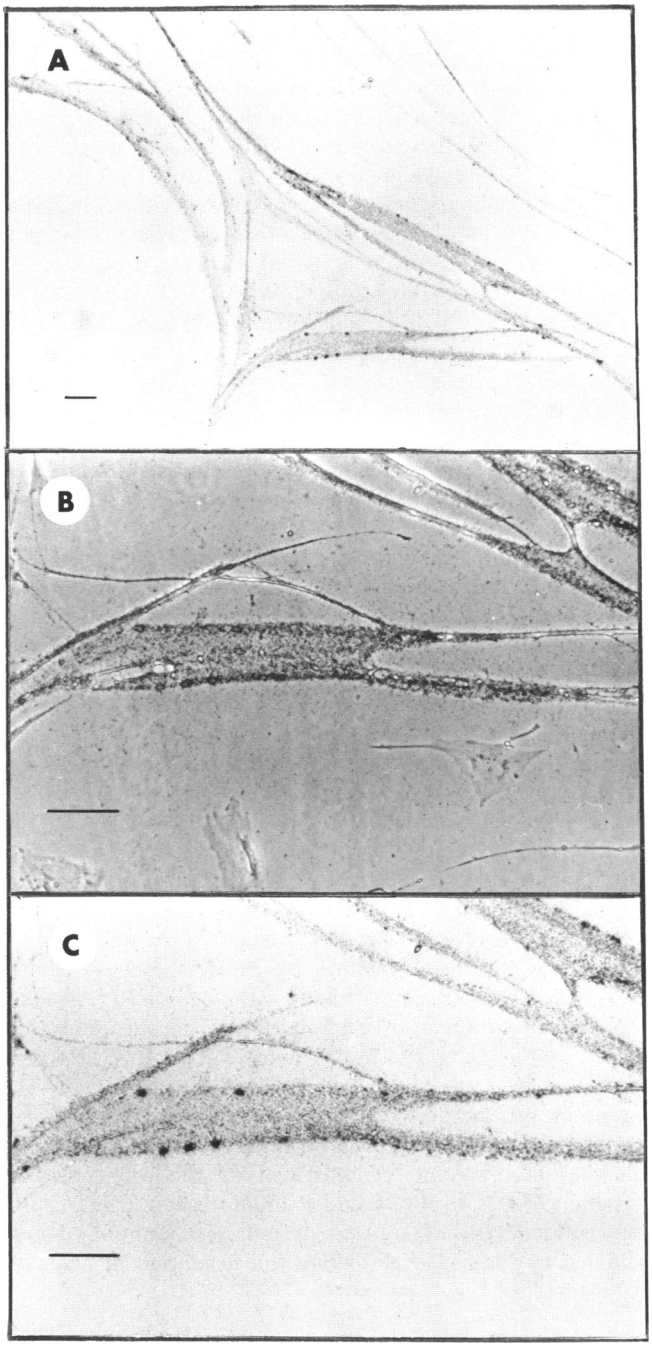

FIG. 5. Autoradiography of chick-embryo muscle cells labeled with [^{125}I]diiodo-α-bungarotoxin. Muscle cells, originally 1×10^3 cells per 50-mm petri dish, were cultured for 14 days. The culture was incubated with 5 nM [^{125}I]diiodo-α-bungarotoxin, washed as described in *Methods*, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2 mM $CaCl_2$, and autoradiographed with Kodak NTB-2 emulsion for 4 days (manuscript in preparation). Bright-field illumination was used to show silver grains of autoradiographs in panels *A* and *C*, and phase contrast illumination was used to show cell detail in panel *B*. Panels *B* and *C* show an area of panel *A* under higher magnification. The *bar* corresponds to 100 μm.

DISCUSSION

It has been demonstrated in several laboratories (37–40) that muscle cells in culture are sensitive to acetylcholine. Hartzell and Fambrough (41) have shown that α-bungarotoxin blocks the response of cultured muscle to acetylcholine. The advantage of the use of tissue culture to study the development and characteristics of the acetylcholine receptor is that such a study can be performed under controlled conditions in the absence of neuronal influence.

We used [^{125}I]α-bungarotoxin to determine the characteristics of the acetylcholine receptor of cultured muscle. The method of iodination used yielded two species of labeled protein, diiodo-α-bungarotoxin and monoiodo-α-bungarotoxin. With both compounds the label appeared to be on one tyrosyl residue. α-Bungarotoxin has two tyrosyl residues (positions 24 and 54) (1). Chang *et al.* (42) have shown that nitration of tyrosine 25 of cobrotoxin requires drastic conditions and results in complete loss of toxicity. Neurotoxins of elapid snake venoms have strikingly similar amino-acid sequences, and all have tyrosyl residues at position 24 or 25 that may be necessary for biological activity (1, 42). Therefore, both mono- and diiodo-α-bungarotoxin were probably labeled at tyrosine 54.

The results of this study show that iodo-α-bungarotoxin bound with high specificity to the acetylcholine receptor of avian and mammalian muscle cells grown *in vitro*. Monoiodo-α-bungarotoxin apparently had a slightly higher affinity for the acetylcholine receptor than did diiodo-α-bungarotoxin, although in the presence of excess toxin, equimolar amounts of mono- and diiodo-α-bungarotoxin were bound. The binding was inhibited by nicotine, carbamylcholine, 1,1-dimethyl-4-phenylpiperazinium, decamethonium, *d*-tubocurarine, gallamine, and by acetylcholine. These data, coupled with the lack of inhibition by atropine, pilocarpine, and hexamethonium, show that the acetylcholine receptor of cultured embryonic chick muscle is similar to the nicotinic acetylcholine receptor of adult vertebrate skeletal muscle.

The autoradiographic data demonstrate that acetylcholine receptors are distributed over the entire cell surface. In addition, localized areas of high receptor concentration were found. Similarly, Cohen and Fischbach, using electrophysiologic methods, have demonstrated "sharp peaks" of acetylcholine sensitivity on the surface of cultured chick-embryo muscle cells (43). In normally innervated muscle, acetylcholine receptors are concentrated at the neuromuscular junction (4, 14, 15, 19). The localized areas of high receptor concentration described here may represent a normal step in the maturation of muscle before the formation of synapses with muscle.

The valuable technical assistance of Terry Gouaux and Judy Mullins is gratefully acknowledged.

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