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APPEARANCE OF ACETYLCHOLINE RECEPTORS IN CULTURED MYOBLASTS PRIOR TO FUSION

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The development of the acetylcholine receptors in chick embryo myoblasts from 11-day old embryos was studied in vitro. Using the purified α-bungarotoxin labeled with radioactive iodide, a high concentration of acetylcholine receptors was found in the prefusing myoblasts; most of these receptors were located in the interior of the myoblasts. However, upon the completion of myoblast fusion, the majority of the acetylcholine receptors appeared on the external cell surface of the myotubes.

INTRODUCTION

It has been shown that embryonic muscle cells as well as established myogenic cell lines are able to manifest acetylcholine (ACh) receptors on the cell surface during myogenesis in vitro (1–4). Prefusion myoblasts have been shown to have few if any ACh receptors on the external cell surface (1, 5), but postfusion myotubes possess a high level of ACh receptors.

In this communication, we report the detection of ACh receptors in prefusion myoblasts, using ^{125}I-labeled α-bungarotoxin, a neurotoxin obtained from the venom of a krait, Bungarus multicinctus. α-Bungarotoxin has been shown to bind ACh receptors in striated muscle (1–6) and of electric tissues (7, 8) with high specificity and affinity. We will show that significant levels of ACh receptors can be detected in myoblasts well before the onset of fusion, and, furthermore, that most of these receptors are found inside the cells.

MATERIALS AND METHODS

Cell Culture

Chick embryo myoblasts were prepared by mechanical dispersion without the use of proteolytic enzyme from thigh muscle of 11-day embroys, according to a previously described method (9). They were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 2% chick embryonic extract, and plated at $6–8 \times 10^5$ cells per 60 mm Falcon culture dish or $2–3 \times 10^6$ cells per 100 mm dish. Cultures prepared in this fashion generally proliferate two to three generations and proceed to fuse at approximately 40–42 hr after plating. The fusion usually is 90% completed after 55–60 hr in culture.

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^{125}I-α-Bungarotoxin Preparation

α-Bungarotoxin was purified and iodinated from venom of the snake Bungarus multicinctus (Miami Serpentarium, Miami, Fla.) according to a previously described method (10). The purified α-bungarotoxin migrated as a single component electrophoretically on an SDS-acrylamide gel. The ^{125}I-labeled α-bungarotoxin had a specific activity of approximately 40–50 Ci/mmol.

^{125}I-α-Bungarotoxin Binding Assay

Whole cells. Dishes were rinsed three times with phosphate-buffered saline (PBS) and covered with DMEM equilibrated with 10% CO_2. Cultures were incubated for 20 min at room temperature with or without nonradioactive α-bungarotoxin (10^{-7} M). ^{125}I-α-Bungarotoxin was added to a final concentration of 5×10^{-9} M and the dishes were sealed with parafilm and incubated for 30–60 min at room temperature. At the end of incubation, the cultures were rinsed five times with phosphate-buffered saline and the cells were removed with 1 ml of 0.1 N NaOH. The radioactivity was determined in a Nuclear Chicago gamma counter.

The nonspecific background was determined from the extent of binding in the presence of excess amounts of unlabeled toxin or of 10^{-5} M decamethonium (K & K Laboratory, Inc.) or 10^{-4} M d-tubocurarine (K & K Laboratory, Inc.). The data are reported as specific counts bound per culture and were calculated as the diffference between total and nonspecific counts bound.

Cell lysates. Cells, washed five times with PBS, were lysed in 1 ml of 10 mM phosphate buffer, pH 7.4, containing 0.1% Triton X-100 and 2 mM phenyl methyl sulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) to inhibit endogenous proteolytic activity. The lysates were incubated at room temperature with or without the other cholinergic agents used above for 20 min. ^{125}I-α-Bungarotoxin was then added and incubated with the cell lysate for 30–60 min at room temperature.

At the end of the incubation, the lysates were applied to DEAE cellulose paper filter (DE 81, Whatman) presoaked in lysis buffer and filtered at a flow rate of one drop per second (11). The filters were washed three times with 10 ml of lysis buffer and the radioactivity was determined as above. Data are reported as described.

Autoradiography

The cultures were incubated with ^{125}I-α-bungarotoxin as described. At the end of the incubation, the cultures were washed five times with PBS and fixed with 2.5% glutaraldehyde in 0.1 M s-collidine buffer at pH 7.1 for 30 min at 37°C. The culture dishes were coated with NTB-3 Kodak nuclear emulsion at 1:1 dilution. The exposure was at 4°C for 1–2 weeks.

RESULTS

Appearance of the External ACh Receptors

Figure 1 shows the autoradiograms of prefusion myoblasts and postfusion myotubes labeled with ^{125}I-α-bungarotoxin. Panels A and C are phase-contrast micrographs of an 18-

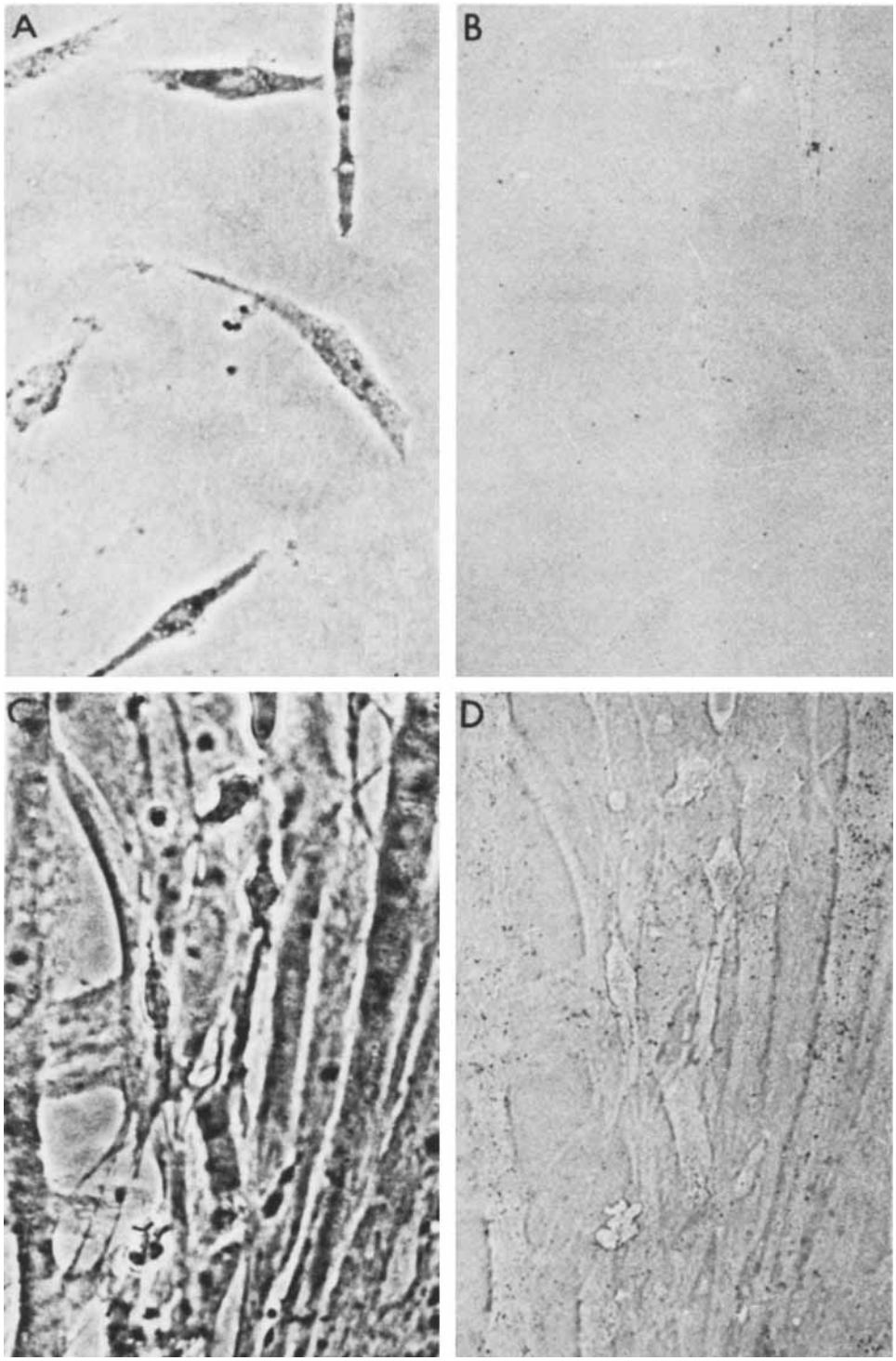

Fig. 1. Light micrographs of autoradiograms showing binding of ^{125}I-α-bungarotoxin to myogenic cells. Myoblasts, 18 hr in culture, phase-contrast (A), bright-field (B). Myotubes, 7 days in culture, phase-contrast (C), bright-field (D).

hr culture of myoblasts consisting of over 95% mononucleated cells, and a 7-day culture of mature myotubes. The completion of fusion is evident in C. Panels B and D are the bright-field micrographs of the same cultures A and D, respectively. The mononucleated cells, whether fibroblasts or myoblasts, show little binding. A distribution of grain counts on different types of cells is given in Table I. A significant increase of labeling occurs when myotubes possess more than five nuclei. There is a small population of cells or vesicles, less than 2% of the total population, which are not identifiable, but they do bind ^{125}I-α-bungarotoxin. These vesicles often do not have nuclei and possibly are fragments of pre-existing myotubes or are damaged myoblasts.

Appearance of the Internal ACh Receptor

The result of the filter assay technique, as described in Materials and Methods, is illustrated in Fig. 2. Good linearity was obtained in the range of our experimental conditions and all binding assays were carried out within the linear region.

Table II shows the result of a typical experiment. Cell cultures 18 hr after plating were labeled with ^{125}I-α-bungarotoxin in different ways. There was little binding to the prefusing myoblasts when the labeled toxin was incubated with intact whole cells. When the cell lysate of the equivalent culture was labeled, however, a significant increase of specific binding was detected. This finding implies the existence of internal toxin binding components which are not accessible to the toxin in the intact whole cells. Most interestingly, bindings to cell lysates in postfusion myotubes were the same as those to whole myotubes.

The toxin-binding results are not affected by the methods of lysis used. Similar results were obtained in the two experiments presented in Table II where different procedures of cell lysis were used. The quantitative difference observed in the specific counts between the two experiments is due to the difference in the number of myogenic cells used.

As another control, fibroblast cultures of same cell densities were used in the binding assays. No specific binding was ever obtained in either cell lysates or intact whole cells.

The specificity of ^{125}I-α-bungarotoxin binding to the internal ACh receptor was tested further with other cholinergic compounds. Cell lysates were preincubated with decamethonium and d-tubocurarine before the addition of labeled toxin. Complete inhibition of toxin binding was observed with decamethonium at a concentration of 10^{-5}

TABLE I. Distribution of ^{125}I-α-Bungarotoxin Labels on Different Cell Types

Cell Type	No. of nuclei per cell	No. of radioactive grains per cell
Fibroblast	1	0.65 (150)[1]
Myoblast	1	0.75 (85)
Unidentified[2]	0–1	10.5 (16)
Myotube	2–3	9.0 (11)
	4–5	>15.0 (13)
	>5	>200

[1] Numbers in parentheses are the number of cells counted in each case.
[2] Cells or vesicles not identified. These cells often are ghost vesicles without nuclei, possibly remnants of pre-existing myotubes or damaged myoblasts.

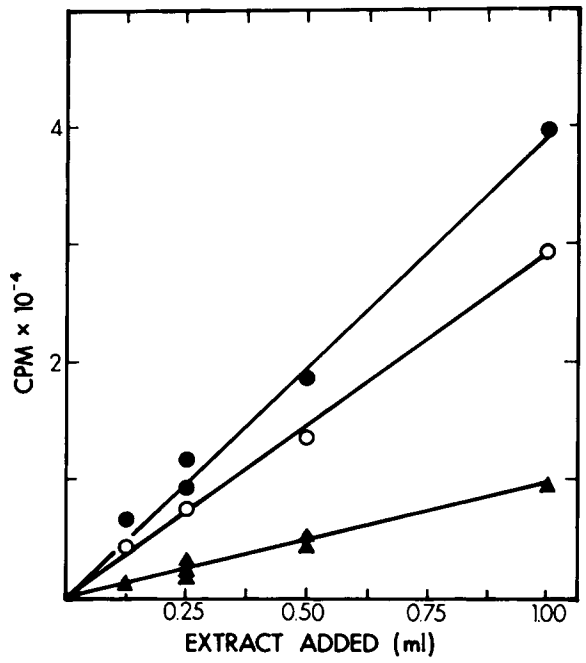

Fig. 2. Binding of ^{125}I-α-bungarotoxin to solubilized acetylcholine receptors. Triton X-100 solubilized cell lysates of 7-day myotubes from a confluent 100-mm petri dish were incubated with ^{125}I-α-bungarotoxin in a final volume of 1 ml. At the end of the incubation, various amounts of lysate were filtered through DEAE-cellulose filters. Nonspecific binding in the presence of 10^{-7} M unlabeled toxin and 5×10^{-9} M ^{125}I-α-bungarotoxin (solid triangles), total binding in the presence of the radioactive toxin (5×10^{-9} M) only (solid circles), specific binding obtained by the difference between the total binding and nonspecific binding (open circles).

M and d-tubocurarine at 10^{-4} M, whereas atropine sulfate at 10^{-4} M has a minimal effect on the binding of labeled α-bungarotoxin.

DISCUSSION

The development of ACh receptors has been of great interest in studying the differentiation of muscle cell surface properties (1–5). We have presented evidence of detecting a singificant concentration of ACh receptors in myoblasts well before the onset of fusion, and most of these receptors are found to remain either inside the cell or "masked" on the cell surface, and therefore, are not accessible to α-bungarotoxin. The specificity of our detection of the internal ACh receptor is supported by the fact that nicotinic cholinergic ligands are capable of preventing toxin binding, while the muscarinic agent, atropine sulfate cannot and furthermore fibroblasts, which have been shown to lack ACh sensitivity (1), do not exhibit toxin binding to either cell lysate or whole cell. Similar binding results were obtained whether cells were lysed by detergent, Triton X-100, or hypotonic shock followed by Dounce homogenization. We think the observation of the appearance of ACh receptors inside myoblasts cannot be due to a change of affinity of ACh receptor for α-bungarotoxin caused by the presence of detergent.

TABLE II. Specific Binding of ^{125}I-α-Bungarotoxin to External and Internal Receptors

Cell Type		Hr in culture	^{125}IαBt binding per culture (cpm)	
			Cell lysate	Whole cell
Experiment I. Cells lysed by 0.1% Triton X-100				
Myoblast	(5)[1]	18	5,200	−200
Myotube	(90)	144	52,700	50,900
Fibroblast		18	−900	−900
Experiment II. Cells lysed by Dounce homogenization				
Myoblast	(3)	24	2,400	240
Myoblast	(30)	43	5,400	1,700
Myotube	(70)	70	11,800	10,500
Fibroblast		40	192	935

^{125}I-α-Bungarotoxin binding is expressed as specific counts as described under materials and methods per culture dish. The cell number usually increases twofold prior to fusion which begins at approximately 40–45 hr. 100-nm plastic culture dishes were used in Experiment I and 60-nm plastic culture dishes were used in Experiment II. Cells were plated at 2×10^6 cells/100-mm dish and 0.5×10^6 cells/60-mm dish respectively.

[1] Numbers in parenthesis are the index of fusion scored as the percentage of nuclei in fused myotubes.

It is interesting to observe that immediately after fusion, binulceated young myotubes are capable of binding toxin on the external cell surface (Fig. 1A, B, upper right corner, and Table I). This is in agreement with previously observed ACh sensitivity in freshly fused myotubes with very few nuclei (1). It is somewhat surprising that the appearance of ACh receptors should be so early in myogenesis, considering that their sole function is to participate in synaptic transmission. It is also interesting that after fusion most of the ACh receptors are found on the external cell surface.

Fambrough and Rash (1) and Patrick et al. (3) have observed ACh sensitivities in some mononucleated myoblasts. Their observations may suggest that the internal ACh receptors that we have detected in myoblasts are already functional. Alternatively, these internal ACh receptors are immature. Only after the completion of fusion, do these receptors become fully functional and express both ACh sensitivity and binding affinity for α-bungarotoxin. Work is in progress to characterize these receptors further.

NOTE ADDED IN PRESS

It has come to our notice that Devreotes and Fambrough (12) have observed an internal pool of ACh receptor containing about 40% as many binding sites as does the surface in developing myotubes. This figure is much higher than the percentage which we have observed in mature myotubes; however, this figure is similar to our results in cultures which are in the midst of the fusion process and which have accumulated approximately 50% of the plateau level of external ACh receptors (unpublished result).

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REFERENCES

1. Fambrough, D., and Rash, J. E., Dev. Biol. 26:55 (1971).
2. Vogel, Z., Sytkowski, A. J., and Nirenberg, M. W., Proc. Natl. Acad. Sci. U.S.A. 69:3180 (1972).
3. Patrick, J., Heinemann, S. F., Lindstrom, J., Schubert, D., and Steinbach, J. H., Proc. Natl. Acad. Sci. U.S.A. 69:2762 (1972).
4. Paterson, B., and Prives, J., J. Cell Biol. 59:241 (1972).
5. Sytkowski, A. J., Vogel, A., and Nirenberg, M. W., Proc. Natl. Acad. Sci. U.S.A. 70:270 (1973).
6. Chang, C. C., and Lee, C. Y., Arch. Int. Pharmacodyn. 144:241 (1963).
7. Changeux, J. -P., Kasai, M., and Lee, C. Y., Proc. Natl. Acad. Sci. U.S.A. 67:1241 (1970).
8. Miledi, R., Molinoff, P., Potter, L. T., Nature 229:554 (1971).
9. Konigsberg, J. R., McElvain, N., Tootle, M., and Herrmann, H., J. Biophys. Biochem. Cytol. 8:333 (1960).
10. Berg, D. K., Kelly, R. B., Sargent, P. B., Williamson, P., and Hall, Z. W., Proc. Natl. Acad. Sci. U.S.A. 69:147 (1972).
11. Schmidt, J., and Raftery, M. A., Anal. Biochem. 52:349 (1973).
12. Devreotes, P. N., and Fambrough, D. M., J. Cell Biol. 65:335 (1975).