APPEARANCE OF ACETYLCHOLINE RECEPTOR
IN DIFFERENTIATING CULTURES OF
EMBRYONIC CHICK BREAST MUSCLE

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It is now well established that primary cultures of embryonic muscle tissue (1, 2) and muscle cell lines (2, 3) elaborate acetylcholine receptor during the process of muscle formation. However, the precise timing of the appearance of receptor with regard to the cessation of DNA synthesis, cell fusion, and the synthesis of the cytoplasmic and contractile proteins, major events that mark the course of muscle differentiation, has yet to be elucidated. Previous studies (1–3) have been hampered by the lack of rapid fusion kinetics. We have measured the appearance of acetylcholine receptor in rapidly differentiating cultures of embryonic chick skeletal muscle, under normal and fusion-arrested conditions (4–6), using iodine-125–labeled neurotoxins from elapid snake venoms as specific markers (8). This method has been used to assay receptor in adult muscle (9–11), electrogenic tissue (12), and cultures of embryonic muscle

(1–3). This paper demonstrates that the elaboration of receptor in differentiating muscle, unlike other muscle proteins which have been previously assayed during differentiation (4–7), is not dependent upon the fusion process and can proceed in the absence of fiber formation.

METHODS

Cultures

Primary cultures of 12-day embryonic chick breast muscle were prepared as described earlier and were plated on 50-mm plastic Petri dishes (Nunc) coated with collagen (5, 14). Cells were plated at initial densities of 0.5–2.5 × 10^6 cells per dish. Growth conditions and fusion kinetics were the same as previously reported (5, 13, 14). Over this range of initial plating densities the rate of cell fusion is constant and the onset of cell fusion commences from 32 to 38 h in vitro (14).

Preparation of Toxins

Neurotoxins were purified from the venoms of *Naja naja siamensis* (α-neurotoxin) (15) and *Bungarus multicinctus* (α-bungarotoxin) (19). 100-μg portions of the purified toxins were iodinated with 2.50 mCi of carrier-free iodine-125 by the chloramine-T method (16) and separated from unincorporated iodine by gel filtration through a 0.9×25 cm column of Sephadex G-25 (fine) in column buffer containing 0.05 M potassium phosphate buffer pH 7.5 and 2 mg/ml bovine serum albumin (BSA). Aliquots of 0.50 ml of the pooled peak fractions containing approximately 5 μg of toxin were frozen at $-20°C$ in column buffer. Under these conditions approximately 50–70% of the labeled iodine in the reaction mixture was incorporated into the toxin as judged by the radioactivity traveling with the toxin peak. The toxins used in these experiments had initial specific activities of approximately 40–50 Ci/mmol for α-neurotoxin and 100–120 Ci/mmol for α-bungarotoxin. Higher specific activities could be obtained with the α-bungarotoxin as it has an additional histidine and tyrosine residue (8). These are believed to be the reactive groups in the iodination procedure used here (2, 16).

Toxin-Binding Assay

The binding assay was performed in the tissue culture dishes (2). Dishes were rinsed three times with 2.5–3.0 ml of saline (0.9% sodium chloride wt/vol) at room temperature and then covered with 0.9 ml of Eagle's minimal essential medium (MEM) minus calcium, containing 2 mg/ml of BSA. Cultures were equilibrated for 20 min at 37°C with or without inhibitors. Iodine-125 toxin was then added to a final concentration 1×10^{-8} M and the dishes were kept at 37°C. Although specific toxin binding reached saturation levels within 20 min, incubations were routinely carried out for 1 h. Specific toxin binding did not increase for either toxin with a tenfold increase in toxin concentration (10^{-7} M), suggesting little toxin inactivation by excess iodination (2). The incubation medium was removed by aspiration, the cultures were rinsed three times with 3.0 ml of room temperature saline and the cells were removed from the culture dish with the application of 1.5 ml of trypsin solution (0.3% in saline) followed by two additional 1 ml trypsin rinses. These were pooled and counted in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The efficiency of counting was not determined. Nonspecific background binding was determined from the extent of labeling in the presence of 10^{-5} M decamethonium or 10^{-4} M d-tubocurarine and toxin bound to empty collagen-coated culture dishes. Data are reported as specific counts bound per culture and were calculated as the difference between total and nonspecific counts bound. Counts were not normalized to the slight variation in the number of cells per culture as this was less than 10%.

Fusion Arrest

Calcium-free horse serum and embryo extract were prepared with Chelex-100. Serum and embryo extract were mixed 5:1 and made into a 10% slurry with washed, sterile Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). This mixture was stirred slowly overnight in the cold at 4°C. The resin was removed from solution by a 10 min centrifugation at 2,000 rpm in a tabletop Sorvall GLC-1 (Ivan Sorvall, Inc., Newtown, Conn.), decanted under sterile conditions into 6-ml aliquots, and stored frozen at $-80°C$. To prepare sterile Chelex-100, the resin was allowed to stand overnight in 95% ethanol. The ethanol was decanted and the resin was equilibrated with sterile, calcium-free MEM, containing no phenol red, by three centrifugation cycles with 5 vol of medium at 1,200 rpm in the tabletop centrifuge. Cultures to be fusion blocked were plated in normal culture medium for the first 24 h, were rinsed three times with 3 ml of saline at 37°C, and covered with 2.50 ml of low calcium medium containing 25 μM calcium. Low calcium medium was prepared by mixing 12 ml of the calcium-free embryo extract mixture, 88 ml of calcium-free MEM, and the appropriate amount of $CaCl_2$ from a 100 mM stock solution per 100 ml of medium. Under these conditions fusion arrest and fusion kinetics, upon the addition of calcium to the medium, were identical to those described earlier (5) for EGTA fusion-blocked cultures.

Cell Counts and Fusion Index

Cell counts to determine rates of fusion and cell densities were carried out as described previously (5, 13, 14). Saline-rinsed cultures were fixed with 100% methanol, stained with a 10% aqueous solution of Giemsa's stain for 30 min, rinsed once with water to remove excess stain, and air dried before addition of cover slips. Giemsa staining was found to be superior to hematoxylin as cytoplasmic background was much lower, obviating RNase treatment.

RESULTS

Specificity of Toxin Binding

As shown in Table I, the presence of either 10^{-4} M d-tubocurarine or 10^{-5} M decamethonium resulted in a significant decrease in the binding of the labeled neurotoxins in the cultures. These compounds are known specific ligands for the acetylcholine receptor and bind to the same subsite as the neurotoxins (17, 18). The binding elim-

TABLE I

Iodine-125 Toxin Counts per Minute Bound Per Culture in the Presence of:

Toxin	No additions	Decame-thonium	d-Tubocurarine	No cells	Specific labeling
(A) α-Neurotoxin	(1) 1,268	104	190	111	1,164
	(2) 1,252	120	206	109	1,132
	(3) 1,355	132	205	120	1,223
(B) α-Bungarotoxin	(1) 10,827	750	1,306	850	10,077
	(2) 11,294	765	1,275	790	10,529

Values were obtained from different control cultures in separate experiments (A, B) after toxin binding had reached a maximum (60–75 h). For incubation conditions see Methods. Concentrations of decamethonium and d-tubocurarine were 10^{-5} M and 10^{-4} M, respectively. Specific labeling is the difference between counts per minute bound with no additions minus counts per minute obtained with decamethonium. Plating densities: (A) 0.5×10^6 cells per 50-mm dish; (B) 2.5×10^6 cells per 50-mm dish.

inated with these agents was taken as the specific component of labeling. Empty culture plates coated with collagen bound amounts of toxin equivalent to the nonspecific component of labeling established with these inhibitors. Decamethonium resulted in a greater inhibition of labeling than did d-tubocurarine, at a tenfold lower concentration, in agreement with Vogel et al. (2), and was routinely used to establish specificity of labeling.

Appearance of Receptor in Culture

The time-course of appearance of the toxin binding component in differentiating cultures is shown in Figs. 1 and 2. Under standard culture conditions both labeled neurotoxins gave similar kinetic patterns for the elaboration of acetylcholine receptor. Although both neurotoxins gave the same relative increase in labeling during differentiation, approximately eight- to tenfold, α-bungarotoxin yielded the greatest net increase in specific counts due to its higher specific activity. As shown, the amount of receptor per culture does not begin to increase substantially until 55–60% of the myoblasts have fused to form multinucleated fibers. Near the end of the main fusion period, around 50 h in culture, toxin binding begins to increase dramatically. By 65 h, receptor has increased 5–6 times above the 50 h level with little increase in the percentage of fusion. Under standard culture conditions, therefore, the appearance of acetylcholine receptor in the plasma membrane of differentiating embryonic chick muscle follows the main period of cell fusion.
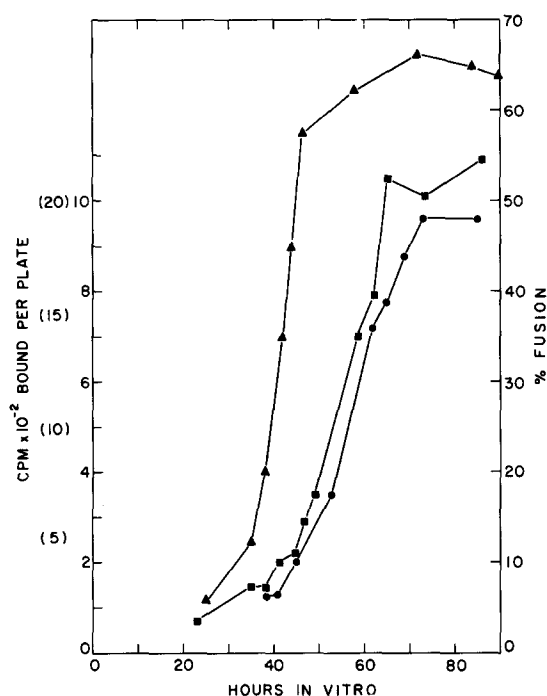

FIGURE 1 Kinetics of receptor appearance and cell fusion in standard cultures assayed with α-neurotoxin. Plating densities: 0.5×10^6 cells per dish (●—●); 1.0×10^6 cells per dish (■—■); percent fusion (▲—▲). Counts per minute in parentheses refer to plating densities of 1×10^6 cells per dish. Cells scored for fusion had an initial plating density of 1×10^6 cells per dish.

Fusion Arrest and the Elaboration of Acetylcholine Receptor

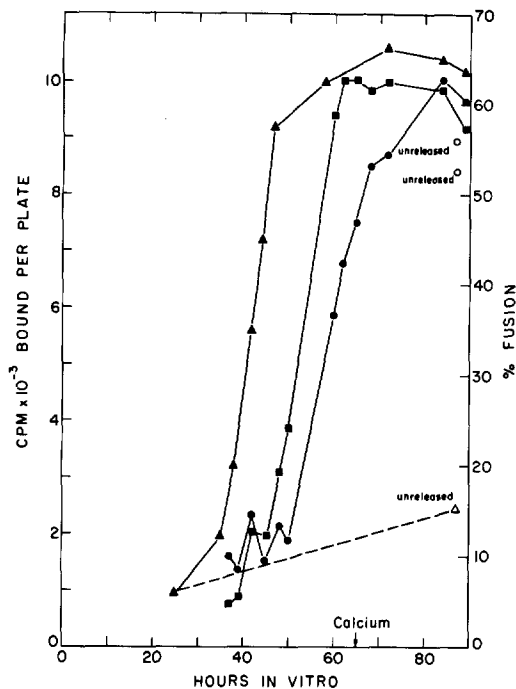In order to determine if receptor elaboration was dependent upon cell fusion, cultures of fusion-

FIGURE 2 Kinetics of receptor appearance and cell fusion in control and fusion-arrested cultures assayed with α-bungarotoxin. Plating density: 2.5 × 10⁶ cells per dish. Toxin binding in control cultures (■—■) and in fusion-arrested cultures (●—●); fusion in control (▲—▲) and fusion-arrested cultures (△—△); toxin binding in cultures maintained under fusion arrest in low calcium medium (unreleased) (○). Time at which calcium concentration was restored to control levels in fusion arrested cultures is indicated by arrow.

arrested myoblasts were prepared by growing the cells in medium containing 25 μM calcium. Such cells are arrested in a postmitotic state and do not divide before cell fusion (5). Under these culture conditions less than 15% of the myoblasts had fused during the entire culture period whereas control cultures had fused to 65% (Fig. 2). Nevertheless, receptor elaboration proceeded at rates similar to those of control cultures and reached comparable levels in the absence of overt cell fusion. Addition of calcium to the fusion-arrested cultures at 65 h, a procedure which resulted in rapid cell fusion to control levels in less than 7 h (5), had no apparent effect on the quantity of specific toxin binding as compared to cultures maintained in low calcium medium. Thus, the elaboration of acetylcholine receptor does not depend upon cell fusion per se, but can take place in the postmitotic, fusion-arrested cell population.

DISCUSSION

The results reported here show that receptor appearance follows cell fusion under normal culture conditions. Under low calcium conditions, however, acetylcholine receptor is elaborated in the absence of cell fusion. In contrast, the majority of cytoplasmic and structural proteins characteristic of differentiated muscle are apparently dependent upon the fusion process for their elaboration (4–7). These findings suggest a scheme in which the regulatory pathways in the development of chemical sensitivity (membrane differentiation) and contractility (cytoplasmic differentiation) are not coupled.

Assuming that acetylcholine sensitivity is a reflection of the presence of acetylcholine receptor at levels detectable by labeling with neurotoxin, our results with fusion-blocked cells support the conclusion of Fambrough and Rash (1), using rat myoblasts, that differentiating cells can become sensitive to acetylcholine in the absence of cell fusion. However, our findings do not support the notion that acetylcholine sensitivity precedes or is coincident with myotube formation and the synthesis of the contractile machinery as has been proposed (1, 3). Acetylcholine receptor does not increase above prefusion levels until several hours after fusion in control cultures. There is recent evidence suggesting that this discrepancy is not attributable to a species difference in the mode of differentiation: myoblasts in differentiating cultures of both chick and rat embryonic muscle have been found to bind little or no labeled neurotoxin (2). In addition, it is now established that the synthesis of proteins associated with contraction is dependent upon previous fusion in both chick and rat muscle cultures (4–7). Therefore, the sequence of events in myogenesis for both species appears to be the same. Under standard culture conditions both the elaboration of acetylcholine receptor and the synthesis of the contractile machinery in chick skeletal muscle follow cell fusion for the majority of cells in culture.

SUMMARY

Differentiating cultures of embryonic chick breast muscle with rapid fusion kinetics have been used to determine the relationship between cell fusion, a primary event in muscle differentiation, and the appearance of acetylcholine receptor as measured by the binding of two neurotoxins from elapid snake venoms. Under normal culture conditions

the amount of acetylcholine receptor begins to increase at significant rates only after the main fusion period has been completed. Postmitotic myoblasts prevented from fusing by calcium deprivation elaborate acetylcholine receptor at comparable rates to control levels even though cytoplasmic differentiation is repressed under these conditions. It is suggested that the differentiation of the excitation and contractile systems in skeletal muscle can proceed independently of one another.

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