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Differentiation of Cell Membranes in Cultures of Embryonic Chick Breast Muscle

(acetylcholine receptor/acetylcholinesterase/adenylate cyclase/cell fusion)

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ABSTRACT **Three components of differentiated muscle membrane, the acetylcholine receptor, acetylcholinesterase (EC 3.1.1.7; acetylcholine hydrolase), and adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)], appear simultaneously during myogenesis in cultures of embryonic chick muscle, after the main period of rapid cell fusion. However, unlike the cytoplasmic proteins of differentiated muscle, the elaboration of these membrane components is unaltered when fusion is blocked by lowering the calcium concentration in the medium. These results suggest that membrane differentiation and cytoplasmic differentiation are regulated independently during muscle development.**

The differentiation of muscle *in vitro* involves the elaboration of several specialized membrane components, including the acetylcholine receptor (1–3) and acetylcholinesterase (EC 3.1.1.7; acetylcholine hydrolase) (4, 5), in the absence of direct neuronal influence. We previously reported that the appearance of the acetylcholine receptor occurs subsequent to cell fusion in differentiating cultures of chick skeletal muscle; however, if fusion is prevented by lowering the calcium concentration in the medium, the rate and extent of receptor appearance are unaffected (6). In contrast, the cytoplasmic proteins characteristic of differentiated muscle, such as myosin (7) and creatine phosphokinase (8, 9), are not synthesized in fusion-arrested cells.

We now present evidence that two other components of differentiated muscle cell membranes, acetylcholinesterase and adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)], appear concurrently with the acetylcholine receptor in both control and fusion-arrested cultures. The simultaneous appearance of these constituents suggests that the differentiation of skeletal muscle cell membranes involves the coordinated synthesis of discrete membrane components and is regulated independently of cytoplasmic differentiation.

METHODS

Cultures. Primary cultures of embryonic chick breast muscle were prepared as described (6) and plated at initial densities of 2×10^6 cells per 60-mm dish and 6×10^6 cells per 100-mm dish. The cultures were grown in 88% (v/v) Dulbecco's modified Eagle's medium (10) with 10% (v/v) horse serum (Gibco) and 2% (v/v) chick embryo extract.

Where specified, growth medium was replaced by low calcium medium (25 μM calcium) prepared with Chelex 100 (6). Cell fusion was scored as described (6).

Abbreviations: BW284C51, 1,5-bis-(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide; iso-OMPA, tetraisopropylpyrophosphoramide.

Assays. The acetylcholine receptor was measured by the binding of ^{125}I-labeled α-bungarotoxin and is expressed as specific labeling (6).

Acetylcholinesterase was assayed by monitoring the hydrolysis of [1-^{14}C]acetylcholine (Amersham) as described by Nirenberg and coworkers (11, 12). For each determination, tissue was removed by scraping from two 60-mm diameter culture dishes after three washes with Dulbecco's phosphate-buffered saline (13) and was stored as a pellet in 0.5 ml of phosphate-buffered saline at −80°. Thawed samples were dispersed by sonication before assay. The final reaction mixture (11) contained 0–200 μg of protein in a volume of 0.05 ml.

Adenylate cyclase was assayed (14) with a reaction mixture containing 40 mM Tris·HCl (pH 7.4), 4 mM $MgCl_2$, 2.2 mM theophylline, 2.7 mM [α-^{32}P]ATP (1 mCi/mmol, Amersham), 2.2 mM creatine phosphate, 30 μg of creatine kinase, and 10 mM NaF. For each determination, four culture dishes 100-mm in diameter were rinsed with saline and scraped; the tissue was stored at −80° in 1 ml of 50 mM Tris·HCl (pH 7.6) until use. Aliquots (50 μl each; 500–800 μg of protein) of tissue were added per tube to initiate the reaction, which was performed in a final volume of 0.15 ml. Samples were incubated at 37° for 20 min, and the reaction was terminated by boiling for 3 min. To each tube were added 10^3 cpm of [^{3}H]cyclic AMP (New England Nuclear Corp.) in 0.1 ml of 50 mM Tris·HCl (pH 7.6) to allow correction for loss of cyclic AMP in the subsequent procedure. Samples were diluted with 50 mM Tris·HCl (pH 7.6) to a final volume of 1 ml and passed through columns of alumina (15) (0.5 × 2 cm) that had been equilibrated with the same buffer. The columns were washed with 2.5 ml of the Tris buffer and the combined eluate (3.5 ml) was collected directly into vials containing 15 ml of Triton–toluene scintillant [5.5 g of diphenyloxazole (PPO), 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP), 667 ml of toluene, and 333 ml of Triton X-100].

RESULTS

Appearance of Acetylcholine Receptor and Acetylcholinesterase in Fusing and Fusion-Arrested Cells. The acetylcholine receptor appears 5–10 hr after the main period of fusion in control cultures, and the time and extent of receptor elaboration are unaffected when fusion is blocked by lowering the Ca^{++} concentration in the medium (Fig. 1).

Cholinesterase activity was demonstrated in avian muscle by Nachmansohn (16), and this activity has been recently reported to increase in parallel with receptor in embryonic chick muscle during development *in ovo* (17). The rapid kinetics shown by the chick muscle cultures with respect to cell fusion

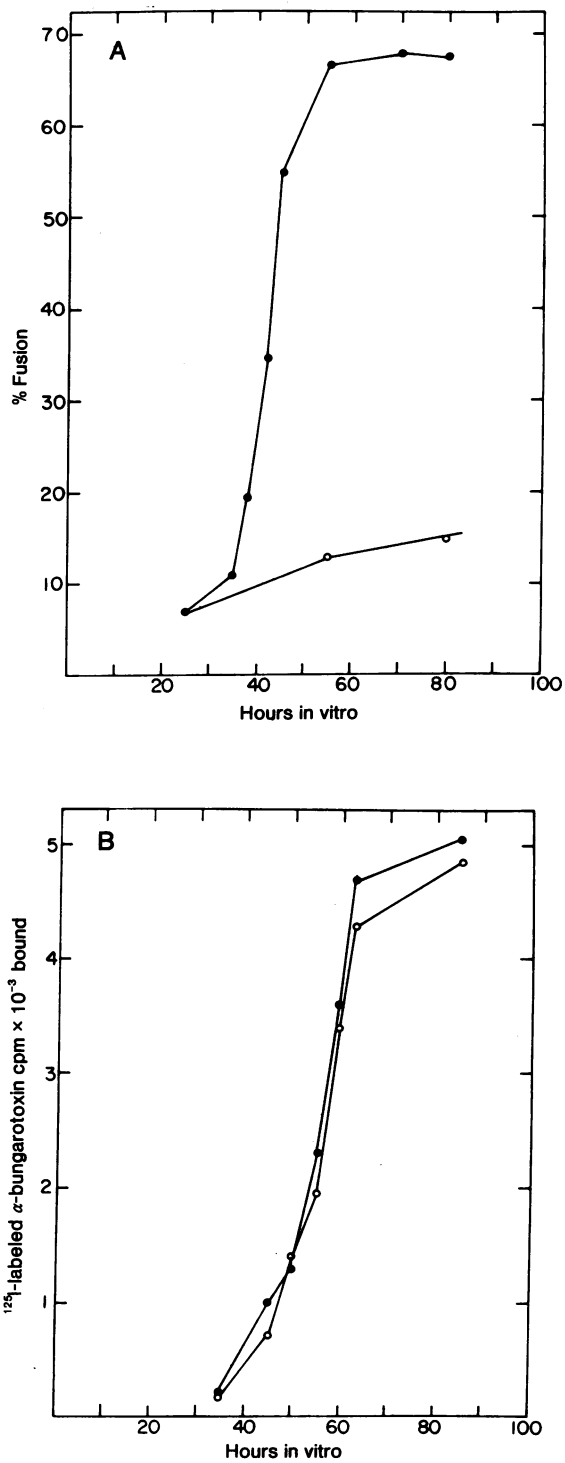

FIG. 1. Kinetics of cell fusion and receptor appearance in control and fusion-arrested cultures. Plating density, 2×10^6 cells per dish. (*A*) Fusion in control cultures (●) and in cultures changed to low calcium medium (25 μM Ca^{++}) 24 hr after plating (○). Fusion is expressed as the percentage of nuclei found in multinucleated cells. (*B*) Appearance of acetylcholine receptor measured by specific binding of ^{125}I-labeled α-bungarotoxin to control (●) and fusion-arrested (○) cultures. Cells were incubated with 12 nM ^{125}I-labeled α-bungarotoxin as described (6). The specific activity of the toxin used in these determinations was about 10^{10} cpm/μmole. Toxin bound at 85 hr corresponded to about 5×10^{-13} moles per culture or 4.5×10^{-13} moles/mg of protein.

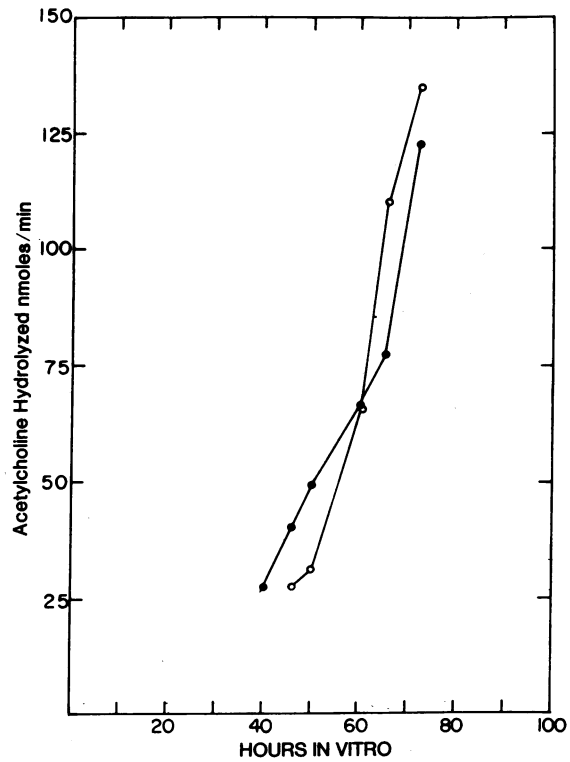

FIG. 2. Cholinesterase activity in control and fusion-arrested cultures. Results are expressed as the rates of hydrolysis of [^{14}C]-acetylcholinesterase by control (●) and fusion-arrested (○) cultures.

and receptor elaboration allow a precise comparison of changes in activity patterns of acetylcholine receptor and acetylcholinesterase with differentiation. In both control cultures and those in which fusion has been blocked, levels of cholinesterase activity increase rapidly and with similar kinetics between 40 and 70 hr after plating (Fig. 2).

To determine the proportion of acetylcholine hydrolysis due to acetylcholinesterase, we assayed 50-, 60-, and 70-hr cultures in the presence of one of two inhibitors, 10 μM 1,5-bis-(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide (BW-284C51, Burroughs, Wellcome and Co.), which specifically blocks acetylcholinesterase (18), and 10 μM tetraisopropyl-pyrophosphoramide (iso-OMPA, Koch-Light Labs.), a specific inhibitor of cholinesterase (EC 3.1.1.8) (18). As shown in Fig. 3, BW284C51 inhibited acetylcholine hydrolysis by more than 75% in all cases, while iso-OMPA inhibited hydrolysis by less than 20%. These data indicate that the major proportion of acetylcholine hydrolysis, about 80%, is specifically due to acetylcholinesterase, in agreement with earlier work (5), and that this proportion does not vary during the period of rapid elaboration of acetylcholinesterase.

Acetylcholinesterase appears in parallel with the acetylcholine receptor in both control and fusion-arrested cultures. This result is emphasized when the relative rates of appearance of these two components are measured in the same cultures (Fig. 4), and are seen to be almost identical, suggesting that these functionally related proteins are synthesized in a coordinated manner.

Appearance of Adenylate Cyclase. It was of interest to contrast the kinetics of appearance of acetylcholine receptor and

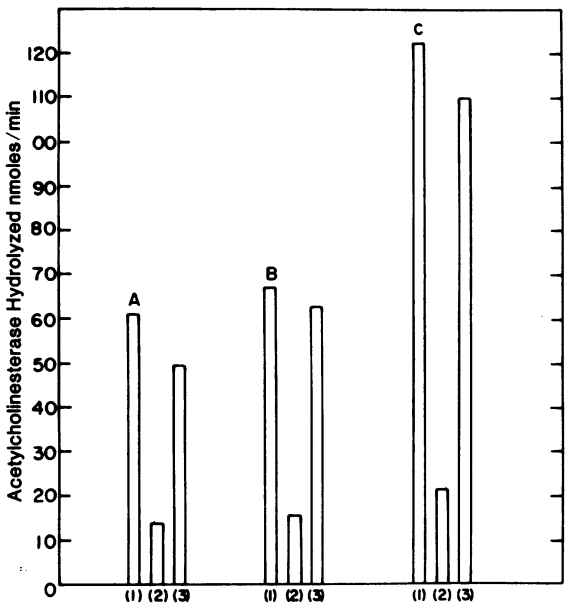

FIG. 3. Effects of esterase inhibitors on rates of acetylcholinesterase hydrolysis by muscle cultures at (*A*) 50 hr, (*B*) 60 hr, and (*C*) 70 hr after plating. Hydrolysis was measured in control cultures (*1*) and in the presence of 10 μM BW284C51 (*2*), and 10 μM iso-OMPA (*3*).

acetylcholinesterase with those of a membrane protein not involved in the response to acetylcholine. Adenylate cyclase activity was found to increase in the period after fusion (Fig. 5) at a similar rate to that seen with acetylcholinesterase and receptor. As with the two proteins mediating the action of acetylcholine, this rate of increase in adenylate cyclase activity was unchanged in cultures in which cell fusion was prevented by reducing the calcium concentration.

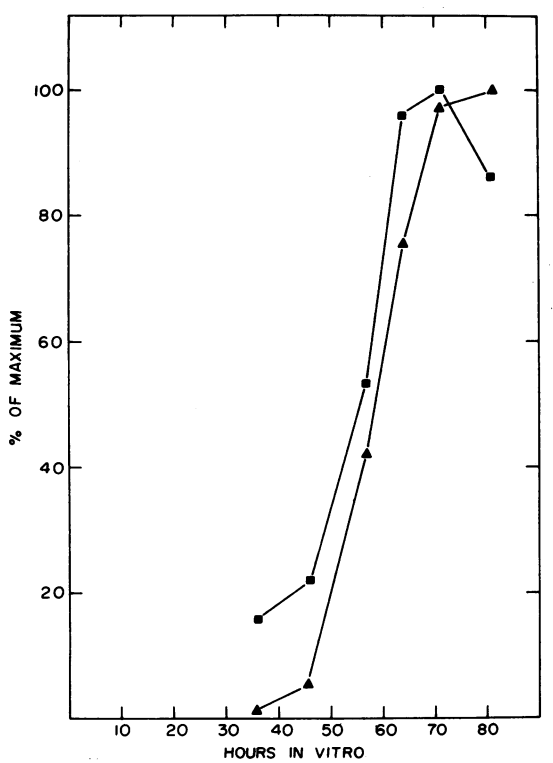

FIG. 4. Relative rates of appearance of acetylcholine receptor and acetylcholinesterase during differentiation *in vitro*. Acetylcholine receptor (▲) and acetylcholinesterase (■) were determined on companion cultures in the same experiment. The maximum value obtained for toxin binding was 4000 cpm (3.6×10^{-13} moles) per culture, and the value for acetylcholine hydrolysis was 180 nmoles/min per culture.

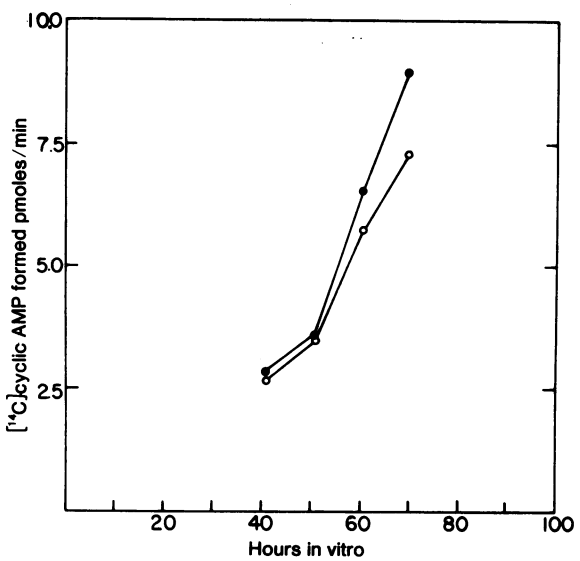

FIG. 5. Appearance of adenylate cyclase activity in control (●) and fusion-arrested cultures (○). Cells were plated at a density of 6×10^6 cells per 100-mm culture dish, and tissue from four dishes was pooled for each determination.

DISCUSSION

The acetylcholine receptor and acetylcholinesterase, specialized components of the cholinergic system which function in mediating transient increases in membrane permeability to Na^+ and K^+ in response to acetylcholine, are elaborated simultaneously and at similar rates, independently of cell fusion. This process of elaboration does not rely on direct neuronal influence or the formation of synaptic junctions.

Maximal binding of ^{125}I-labeled α-bungarotoxin corresponds to 3 to 5 $\times 10^{-13}$ moles of acetylcholine receptor per mg of protein (Figs. 1*B* and 4). This value closely agrees with levels of receptor reported for embryonic chick muscle in culture (3) and *in ovo* (17) and is comparable to the amounts of receptor per mg of protein in electrolplax of electric eel, estimated by affinity labeling (19).

The acetylcholinesterase activity observed in the muscle cultures, about 180 nmoles/min per mg of protein (Fig. 4), is similar to the peak specific activity for acetylcholinesterase in skeletal muscle dissected from 12-day-old chick embryos (17). If it is assumed that the acetylcholinesterase of embryonic chick muscle is similar to purified acetylcholinesterase from electric eel with regard to turnover number and molecular weight per active site (20, 21), then the rate of acetylcholine hydrolysis observed in the muscle cultures corresponds to an amount of enzyme in the range of 1 to 5 $\times 10^{-13}$ moles per mg of protein. Taken together with the values for receptor, it appears that binding sites of acetylcholinesterase and acetylcholine receptor are present in differentiated skeletal muscle membrane in approximately equivalent amounts. The parallel rates of synthesis resulting in equivalent amounts of the two acetylcholine-binding components support the notion that these elements may act in close spatial association in the differentiated plasma membrane (22).

Adenylate cyclase, a constituent of skeletal muscle cell membranes functionally associated with surface receptors (23), but apparently not directly involved in the membrane response to acetylcholine, increased in activity simultaneously with acetylcholinesterase and acetylcholine receptor under control conditions and when fusion had been blocked. If the turnover number recently reported for adenylate cyclase in erythrocyte membranes (24) is applicable to the muscle enzyme, the adenylate cyclase activity of this tissue corresponds to about 10^{-13} moles/mg of protein and is comparable to the levels of acetylcholinesterase and receptor.

A general interpretation of these findings suggests that the specialized components of differentiated muscle membrane are elaborated in concert in response to a developmental program which, unlike cytoplasmic differentiation, is not dependent upon cell fusion.

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