THE APPEARANCE OF ACETYLCHOLINE RECEPTORS TRIGGERED BY FUSION OF MYOBLASTS IN VITRO

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1. Introduction

Formation of multinucleated contracting muscle fibers takes place through fusion of post-mitotic mononucleated myoblasts. This process is accompanied by an increase in the level of a number of muscle proteins. The correlation between cell fusion and the appearance of muscle-specific proteins has led some authors to hypothesize that the fusion process might be a prerequisite for the protein formation [1–3]. Recently, however, some authors have suggested that myogenesis, as manifested by the appearance of muscle proteins, is independent of the cell fusion process [4–13].

The following experiments attempt to settle the argument by indicating that the differences seen by previous authors are due to the various systems used. We show here that while in rat muscle the fusion of myoblasts is essential for the appearance of muscle proteins, acetylcholine receptor (AChR), acetylcholinesterase (AChE) and creatine phosphokinase (CPK), these proteins appear in chick muscle even if cell fusion is inhibited.

2. Materials and methods

2.1. *Cell cultures*

Embryonic rat skeletal muscle was grown in cell culture essentially as in [1]. Chick myogenic cells were prepared by mechanical disruption as in [14]. Low calcium growth medium was prepared from Ca^{2+}-free Dulbecco's modified Eagle medium supplemented with 10% horse serum and 2% chick embryo extract. Calcium was removed from the serum and embryo extract by dialysis against Ca^{2+}-free phosphate-buffered saline as in [15]. The concentration of Ca^{2+} was then adjusted to 50 μM for rat cells or 10 μM for chick myoblasts. Higher concentration of Ca^{2+} did not inhibit cell fusion. Similar results were obtained when calcium was reduced by the chelating agent EGTA (1.7 mM) in the normal growth medium. Fusion promoting medium contained 1.7 mM $CaCl_2$.

2.2. *Assay for AChR*

For the determination of AChR levels intact culture dish was exposed to 6×10^{-8} M ^{125}I-labeled α-bungarotoxin (α-BNT) spec. act. 125–50 Ci/mmol [16]. After careful washing the labeled cells were counted directly on a 2 in. diam. flat crystal γ-detector (Elscint, Haifa). Each dish of cells was then frozen in liquid nitrogen for enzyme assays or fixed in formalin for autoradiography [16]. The AChR amounts in homogenates were determined by using the filter assay essentially as in [14].

2.3. *Determination of enzyme activity*

CPK activity was determined by recording ΔA_{340} at 30°C. The amount of ATP formed by interaction of ADP with creatine phosphate was determined by coupling with hexokinase and glucose 1,6-phosphate dehydrogenase, as in [1]. AChE activity at 30°C, was measured using the Ellmann technique [17] in the presence of 0.1 mM iso-OMPA to inhibit non-specific esterase.

3. Results

To examine the correlation between cell fusion and the appearance of muscle proteins, rat myoblasts were grown in Ca^{2+}-deficient medium in which myoblast fusion is inhibited [15]. The levels of AChR, AChE and CPK were found to be very low prior to the onset of cell fusion. However, as soon as Ca^{2+} was added to the culture medium the fusion process started, accompanied by an increase in the activity of these proteins (fig.1).

Similar experiments were carried out on chick muscle cultures. But in this case the development of AChR, AChE and CPK in low Ca^{2+} concentrations proceeded at similar rates to those of the control cultures, although the cells failed to fuse (fig.2). Thus, it seems that in chick muscle, unlike rat muscle, the appearance of these proteins is independent of cell fusion.

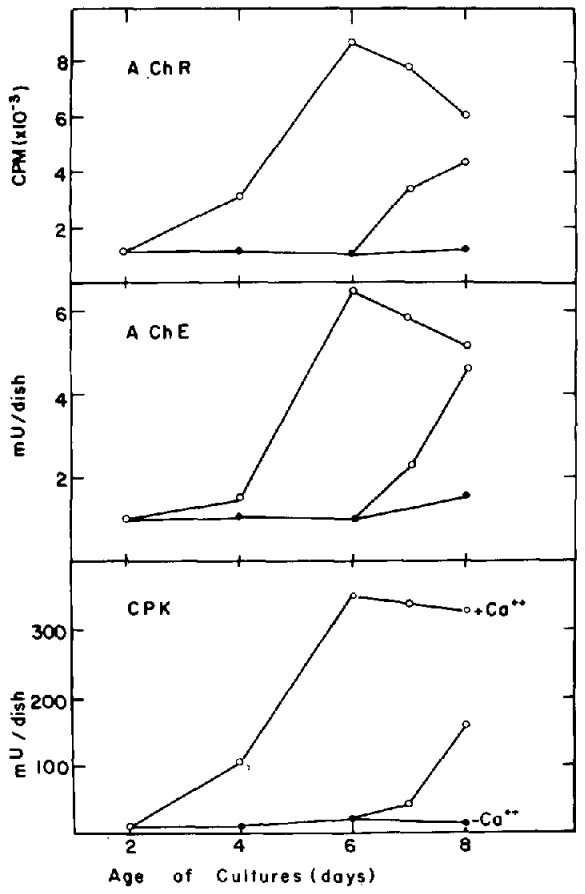

Fig.1. Appearance of muscle proteins in rat muscle cultures. Rat muscle cultures were grown for 2 days in complete medium; then the Ca^{2+} concentration was reduced to 50 μM for one group (●——●), or left in complete medium containing 1700 μM $CaCl_2$ as a control (○——○). On day 6, Ca^{2+} was added to some of the Ca^{2+}-deprived plates.

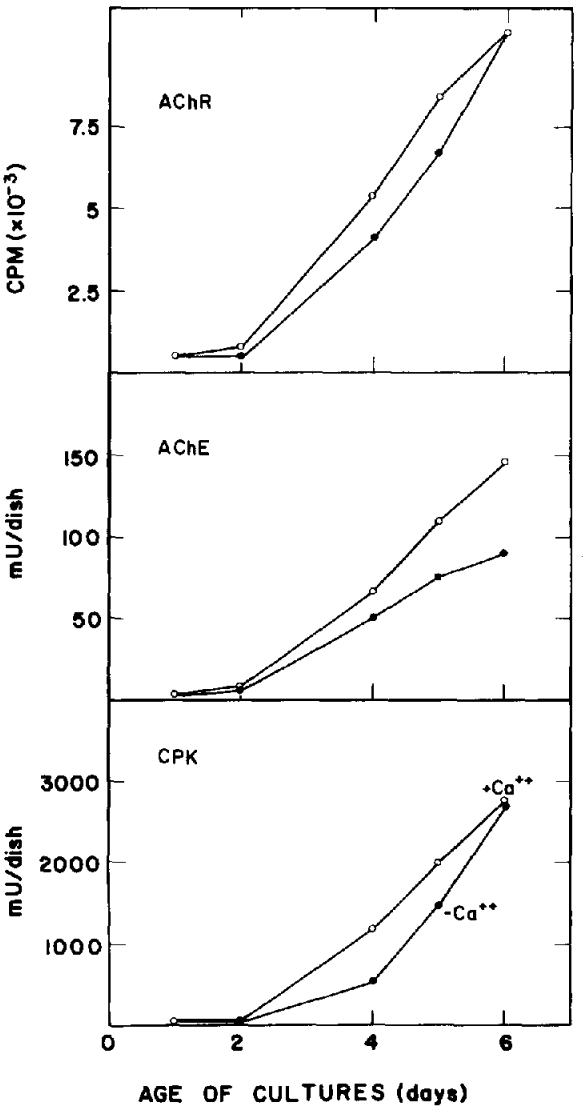

Fig.2. Appearance of muscle proteins in chick muscle cultures. Chick muscle cells were grown for 1 day in complete medium, then were transferred to Ca^{2+}-deprived medium (●——●), or left in complete medium (○——○).

Table 1
. Effect of antimetabolites on cell fusion and AChR appearance

Treatment	α-BNT binding (cpm/dish)	% increase in α-BNT binding	Av. no. nuclei within fibers
Initial conditions	820 ± 29	–	2
Reduced Ca^{2+}	853 ± 86	4	4
Ca^{2+} only	1439 ± 122	75	170
Ca^{2+} + cycloheximide	846 ± 131	3	4
Ca^{2+} + actinomycin D	1405 ± 181	71	162
Actinomycin D only	901 ± 150	10	5

Rat muscle cultures were grown for 1 day in complete medium; then Ca^{2+} was reduced to 50 μM for 2 days until first measurement. Ca^{2+}, 1700 μM, was added to one group of dishes to allow initiation of cell fusion. To test the effect of inhibition of RNA or protein synthesis on AChR level and cell fusion, groups of cultures were exposed to actinomycin D (2 μg/ml) or cycloheximide (3 μg/ml). 10 h after Ca^{2+} addition the amount of AChR was measured. The values represent mean ± SEM. Nuclei within fibers were counted in Giemsa stained dishes. Each value represents the av. no. nuclei within fibers in 5 randomly-selected fields (× 250)

Since it appeared that AChR appearance in rat muscle cultures depended on cell fusion, experiments were devised to test the correlation between myoblast fusion, protein synthesis and the AChR levels in the rat cultures. Cell fusion requires uninterrupted protein synthesis [1]. Here we report that the appearance of AChR is prevented by cycloheximide, a protein synthesis inhibitor, even though Ca^{2+} is available for fusion (table 1). We also found that the rate of AChR degradation was independent of Ca^{2+} (to be published). These observations suggest that the increase in the amount of AChR following fusion represents de novo synthesis of receptors rather than activation of existing latent receptors.

To determine whether the fusion process signals AChR formation by triggering the genes to produce new mRNA, or if fusion activates pre-existing mRNA, an experiment was carried out in which fusion occurred in the presence of RNA synthesis inhibitor. Actinomycin D (AMD) was added to the fusion-arrested cells 30 min before fusion was allowed by addition of Ca^{2+} It was found that in the presence of AMD, fusion occurred and continued for at least 8–10 h, and AChR appeared at a rate and amount similar to those of control (table 1). Autoradiography of rat muscle cultures labeled with ^{125}I-labeled α-BNT after exposure to AMD showed that the main labeling took place on the newly-formed myotubes (fig.3). These fibers formed in the presence of AMD, were also found by iontophoretic methods to be sensitive to acetylcholine (Christian and A. S, to be published).

The hypothesis that AChR, pre-existing inside the myoblasts [14,18] were transferred to the myotube surface as a result of Ca^{2+} addition was also tested. The total amount of AChR (internal and surface) was measured in homogenates of rat myoblasts grown in the presence or absence of Ca^{2+}. The total amount of AChR was significantly lower in fusion-arrested cultures (table 2). These results suggest that AChR that appeared after cell fusion was newly synthesized rather than transferred from pre-existing internal 'pool'.

The total amount of AChR in homogenates of rat myotubes fused in the presence of AMD was similar to that of the control, and was much higher than in the homogenates from fusion-arrested cells (table 2). This rules out the possibility that AMD enhanced the transfer of AChR from pre-existing pool to the cell surface.

4. Discussion

During the normal course of events in the development of muscle fibers in cultures the amounts of AChR, AChE and CPK increase rapidly following cell

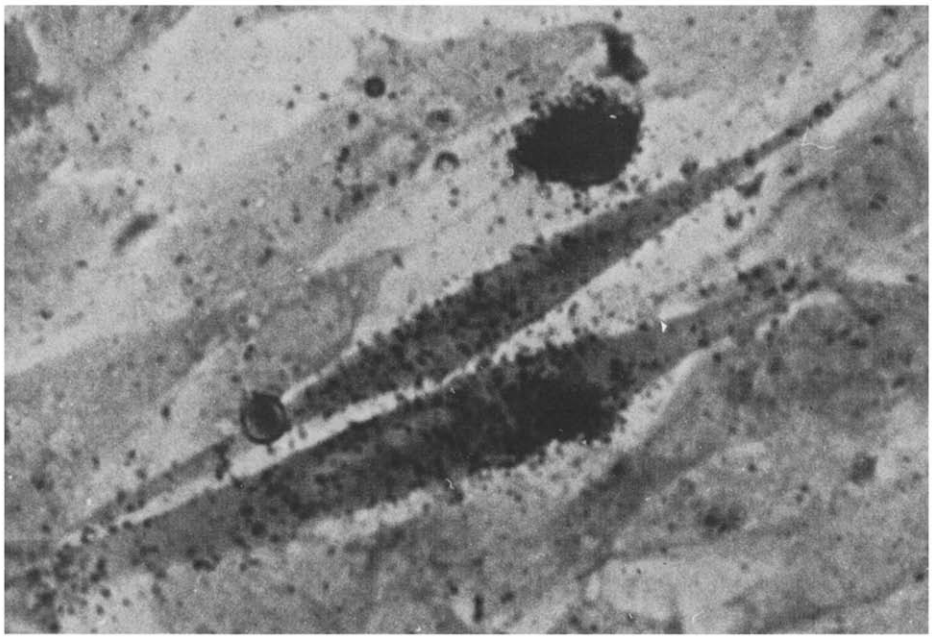

Fig.3. Autoradiography of ^{125}I-labeled α-BNT-labeled muscle cells fused in the presence of actinomycin D (× 400).

fusion. This phenomenon raises the question whether the 2 events, cell fusion and the changes in the amounts of the above mentioned proteins, are causally or only temporarily related. Inhibition of fusion by lowering the Ca^{2+} concentration in the growth medium of rat cell cultures prevents the normal increase in the amount of these proteins. On the other hand, the levels of these proteins in chick muscle cells are hardly affected by the inhibition of fusion due to Ca^{2+} depletion (fig.1,2).

Table 2
Appearance of AChR in cell homogenates

Treatment	cpm/dish (mean ± SEM)
With calcium	2590 ± 241
With calcium + AMD	2138 ± 283
Reduced calcium	1068 ± 118

Rat muscle cultures were grown for 1 day in complete medium, then the Ca^{2+} was reduced to 50 μM for 3 days until the experiment was started. Ca^{2+} (1700 μM) was given to one group of 3 dishes; Ca^{2+} (1700 μM) was given to a second group together with actinomycin D (2 μg/ml); the third group was left with Ca^{2+}-deprived medium. After 10 h the cells were harvested, homogenized and the amount of AChR was measured by the filter paper technique

Similar results were reported [13], working with chick muscle cultures which were fusion-arrested as in [15]. They showed that AChR and AChE developed in the absence of cell fusion resembling the control cultures. However, it was suggested [13] that membrane differentiation and cytoplasmic differentiation are not coupled. Our experiments indicate that the appearance of the membrane proteins (AChR and AChE) and of cytoplasmic protein (CPK) are indeed coupled both in chick and rat muscle cultures. Similarly, it was found that the appearance of AChR and CPK in fetal calf muscle cultures are coupled and furthermore that their elaboration did not depend on cell fusion [10]. The recent reports [6–8,11,12], that cytoplasmic proteins accumulated in fusion-arrested chicken myoblasts, further support the conclusion that cell fusion is not a prerequisite for these manifestations of skeletal muscle differentiation. On the other hand, close association between cell fusion and the synthesis of muscle specific proteins in rat cultures was claimed [19]. Our results indicate that a fundamental difference exists between chick and rat myoblasts

grown in vitro. In rat muscle cells the appearance of AChR, AChE and CPK depends on the occurrence of cell fusion, whereas in chick cells it does not.

To determine the time course of gene expression for AChR during myogenesis, the effect of proteins and RNA synthesis inhibitors on differentiating muscle cultures was studied. It was shown that inhibition of protein synthesis promptly stopped both cell fusion [1] and appearance of new AChR. However, inhibition of RNA synthesis, by AMD, neither prevented cell fusion nor the increase in the amount of AChR. If this increase in the AChR represents receptor synthesis, our results indicate that the mRNA which specify the formation of AChR following cell fusion are already present in the myoblasts, prior to their fusion into multinucleated myotubes. Thus, fusion of rat mononucleated cells signals the translation of AChR from pre-existing mRNA. It is not yet known how the fusion process causes this activation of mRNA.

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