MULTIPLE FORMS OF ACETYLCHOLINESTERASE IN CLONAL MUSCLE CELLS

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1. Introduction

Acetylcholinesterase (AChE; EC 3.7.1.1) in mammalian tissues and cells is heterogeneous in molecular form. Three major forms have been identified with sedimentation coefficients of 4 S, 10 S and 16 S [1–4]. The 16 S form has been found only at the site of the synapse between neuron and skeletal muscles [1,3]. Moreover, the presence of this form of AChE is closely correlated with the presence of motor innervation of the muscles and seems to be regulated by the influence of innervating nerves [1,3]. However, the precise mechanism of this regulation is largely unknown.

In the present study, AChE of a clonal line of mouse striated muscle cells, G8-1, a subclone of the clonal line, G8 [5,6] was analyzed. These cells form myotubes with nicotinic ACh receptors (AChR), which actively contract. G8 cells form functional synapses with a neuroblastoma X glioma hybrid cell line [5]. This paper reports that the G8-1 cells synthesize 16 S AChE, that this ability is clonally inherited and is expressed in the absence of neural influence when myoblasts fuse and form myotubes.

2. Materials and methods

The G8-1 cell line was obtained by recloning a mouse myogenic clonal line G8 [5,6] for increased ability to form myotubes under the conditions used. The G8-1 cells were plated into Falcon 100 mm plastic plates coated with collagen (Calbiochem) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and incubated at 36°C in a humidified atmosphere of 10% CO_2–90% air. When the cultures were nearly confluent the medium was changed to DMEM, 0.5% FCS, 5% heat-inactivated horse serum (HS) to promote fusion. Usually cultures were treated with 10^{-5} M cytosine arabinoside (AraC; Sigma) for 2–3 days after most of the cells had fused into myotubes, to prevent overgrowth of myoblasts. Cultures were washed with a balanced salts solution [7] and stored frozen in a liquid nitrogen freezer until use.

AChE was extracted by homogenizing the cells in 0.5–1.0 ml solution A/plate (1.0 M NaCl, 50 mM $MgCl_2$, 1% Triton X-100, 10 mM Tris–HCl, pH 7.4) and centrifuging at 20 000 × *g* for 30 min [3]. Most of the AChE activity in the crude homogenates was recovered in the supernatant fractions (110%). The supernatant fractions were immediately applied to linear sucrose gradients (5–20% in solution A), and centrifuged at 40 000 rev/min in a Beckman SW41 rotor for 17 h at 4°C.

AChE activity was assayed using [*acetyl*-^{3}H]-choline chloride (Amersham/Searle) as substrate [8]. The reaction was initiated by combining 10 μl [*acetyl*-^{3}H]choline 12.0 mM (0.80 Ci/mol) in 50 mM Na-phosphate, pH 7.4, with 40 μl solution A containing the extracted enzyme. After the incubation at 37°C, the reaction mixture was diluted with 2 ml 100 μM eserine, at 0°C, and immediately passed over a column (0.8 × 3.2 cm) of cation exchange resin AG 50WX8 (Bio-Rad Lab.). The reaction tube was washed with 2 ml H_2O, and the wash was also passed through the column. The effluent was collected in a glass scintillation vial, mixed with 10 ml Aquasol (New England Nuclear), and counted in a liquid scintillation counter. Under these conditions,

enzymatic degradation of [^{3}H]ACh was almost completely (>99%) blocked by an AChE specific inhibitor, BW284C51 (Burroughs-Wellcome) at 5 μM. Thus essentially all the cholinesterase activity detected was due to specific AChE. One unit of AChE is defined as the activity corresponding to the hydrolysis of 1 μmol ACh/h at 37°C.

[^{125}I]Diiodo-α-bungarotoxin ([^{125}I]α-BT) was prepared according to the method [9]. The cell homogenates in solution A were assayed for the toxin binding by a Millipore filtration assay [10]: homogenates were incubated with 8 nM [^{125}I]α-BT at 37°C for 60 min, diluted with detergent-free saline solution more than 300-fold, filtered and washed through Millipore EGWP filters, and counted in a Packard Gamma Scintillation Spectrometer 5375.

β-Galactosidase (Worthington) was assayed by a spectrophotometric method using *O*-nitrophenyl-β-D-galactopyranoside as substrate [11].

3. Results

In fig.1 the time course of the appearance of AChR and AChE is shown as a function of development of G8-1 myoblasts into myotubes when myoblasts were plated at a density of 8 X 10^4/100 mm plate. After the culture reached the confluency, the cells started to fuse and form multinucleated myotubes (at day 6–7). A few days after the initiation of fusion, G8-1 myotubes started to develop AChR and AChE. The development of AChE follows very closely that of AChR. The precise time course of AChR and AChE development varied to some extent depending on the culture conditions. However, the close relationship between AChR and AChE was relatively unaffected. The specific activities increased to maximal values of 1.51 AChE units and 0.46 pmol α-BT binding sites/mg protein at day 17, and then decreased. During this later period, the cultures consisted predominantly of multinucleated myotubes with few myoblasts because of the treatment with Ara C. Although some cell degeneration was noticed, the majority of myotubes appeared to be very healthy and contracted very actively. The total amount of protein was 6.4 mg/plate at day 17, and had decreased by less than 20% at day 26 (5.3 mg/plate), suggesting that there was no serious cell loss or damage. Thus,

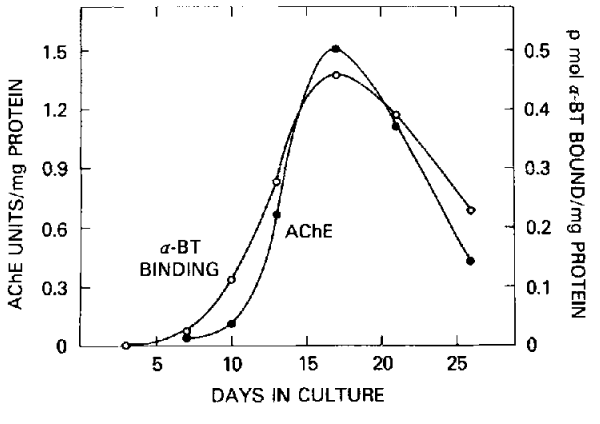

Fig.1. Development of AChE and AChR in G8-1 cells. AChR was measured by α-bungarotoxin (α-BT) binding. The cells were plated at a density of 8 X 10^4/100 mm plate. The cultures were treated with 10^{-5} M Ara C from day 11–13.

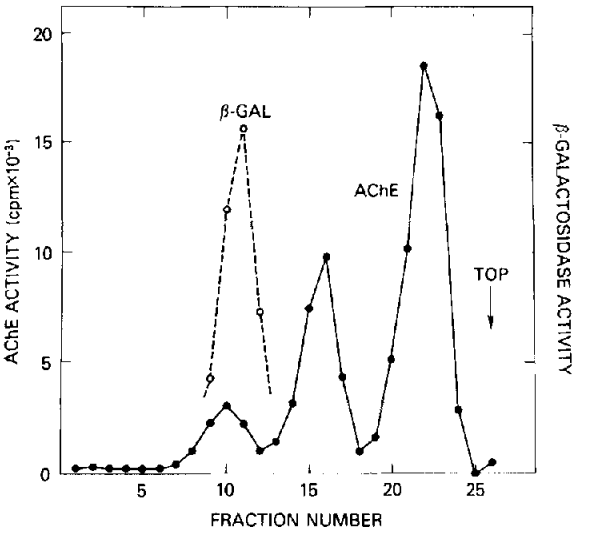

Fig.2. Sedimentation profile of AChE activity of G8-1 cell extract. Fractions were collected from the bottom of the tube. Myoblasts were plated at a density of 1.4 X 10^5/plate and cultivated for 23 days. From the day 14–17, Ara C was added to a concentration of 10^{-5} M. The sedimentation profile was not affected by the treatment with Ara C. β-Galactosidase (β-GAL) activity is given in arbitrary units. Observed sedimentation coefficient values of '16 S' AChE ranged from 15.9–16.8 S (in the case of the figure) with the mean value of 16.4 S.

the decrease in AChR and AChE activity may reflect more likely a mechanism by which neurons regulate the amounts of AChR and AChE of muscles which decrease in the absence of neural influence. Similar disappearance of AChR has been reported in the case of chick primary muscle cells [12].

The molecular forms of AChE were analyzed by sucrose density gradient centrifugation. A typical result is shown in fig.2. Three distinct peaks were observed, with sedimentation coefficient of 4 S, 10 S and 16 S, respectively, estimated from the position of the internal marker enzyme β-galactosidase (16 S). The proportion of these forms is approx. 16 S : 10 S : 4 S ≃ 1 : 2 : 4 in activity units and was not drastically changed during the course of muscle cell differentiation (table 1); as soon as an appreciable amount of AChE appeared, the 16 S form was found in similar proportion to those found in the cells incubated for longer times, and little, if any, further increase in its proportion was evidently observed. However, the proportion of 10 S AChE increased slightly with a concomitant decrease in 4 S AChE. Further study is required to determine the significance of this point.

The presence of the 16 S form of AChE is not a special property of the clonal muscle cell line. As shown in table 1, myotubes grown in culture from dissociated newborn rat muscle also have the three forms in proportions similar to those found in G8-1 cells.

4. Discussion

The muscle cells studied in the present work have all three forms of AChE that have been identified in the rat muscle in vivo [1,3]. Of these three forms, the 16 S form has been found exclusively in the end-plate regions where muscles are innervated by motor nerves [1,3]. In the embryonic muscles, the 16 S form of the enzyme appears only after the muscles are innervated [3]. It disappears from previously innervated muscles when they are denervated [1,3], and reappears upon re-innervation of these muscles [3]. Thus, the presence of 16 S AChE seems to be closely controlled by the influence of innervating nerves.

The cell line G8 originated from myoblasts obtained from mouse embryo hind limbs at 17–18 days gestation [6]. In mouse embryos of this age, it is known [13] that the hind limb muscles are already innervated by the motor nerves. However, the cell from which the cell line was derived was probably a myoblast, and it seems unlikely that a myoblast would be innervated. Thus, it is unlikely that the myoblast which gave rise to the G8-1 clonal line was innervated. The presence of 16 S AChE in these cells strongly suggests that the synthesis of 16 S AChE is not dependent on innervation. Moreover, the results showed that after subculturing cells in the absence of neural influence more than 20 times, G8-1 cells had 16 S AChE in a proportion

Table 1
Relative proportion of different forms of AChE in muscle cells

Muscle cell type	Days after plating cells	Relative proportion of each form of AChE (% of total activity)		
		16 S	10 S	4 S
G8-1	13	12	24	64
	17	15	24	61
	21	17	31	52
	26	14	39	47
Newborn rat muscle	7	19	26	55

Cultures of G8-1 cells were from the same preparation as that of fig.1. Rat muscle cells were dissociated from newborn Wistar rat, plated at a density of 6.1×10^6/100 mm plate, and cultivated as described [15]. From day 4–7, Ara C was added to a concentration of 10^{-5} M

similar to that of rat primary muscle cells. Thus, we conclude that the G8-1 cells have the ability to synthesize 16 S AChE, that this ability is clonally inherited, and is expressed upon cell fusion in the absence of neural influence.

On the other hand, after myotubes were formed and 16 S AChE was synthesized, the specific activity of AChE decreased with the time in culture in the absence of neural influence. Denervated muscles show a similar decrease in AChE [1,3,14]. The decrease observed in vitro may reflect a mechanism by which innervating nerves regulate the AChE activity in muscles and its localization at synaptic regions.

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