REGULATION OF ACETYLCHOLINESTERASE IN CULTURED CHICK EMBRYO SPINAL CORD NEURONS

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1. Introduction

Primary nerve cell cultures contain acetylcholinesterase (AChE: EC 3.1.1.7) and other special macromolecules associated with cholinergic transmission [1–4]. Cultured chick nerve cells, like cultured muscle, have been shown to release AChE into their medium [5,6]. The experiments presented here examined the levels of activity and release of AChE in nerve cell cultures after treatment with di-isopropylphosphofluoridate (DFP) and acetylcholine analogs. The results confirm that dissociated chick embryo spinal cord cells release AChE and demonstrate that the cells regulate their level of AChE activity in response to chemical treatments.

2. Methods

2.1. *Nerve preparations*

The spinal cords from day 7–8 White Leghorn chick embryos were cut into small fragments and incubated with 0.1% trypsin in Hank's salt solution for 30 min at 37°C and dissociated by suction. Cells were plated at 1×10^6 cells/dish onto 35 mm Falcon plastic tissue culture dishes treated with 0.1% collagen.

Cortex and brainstem of day 21 Sprague-Dawley rat embryos were dissected out separately. Cells were plated at 4.5×10^6 cells/dish onto 60 mm Falcon plastic tissue culture dishes as in [7].

Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and air. The medium was changed every 2 days.

The medium was 85% basal medium (Eagles) (BME) with 0.1% glutamine, 600 mg% final conc. glucose, and 15% fetal calf serum in which cholinesterase activity was inhibited. Chick spinal cord cells grown in a medium consisting of 90% nutrient mixture F-12 with Hank's salt solution plus 1.5 g bicarbonate and 10% fetal calf serum yielded similar results. Cultures were maintained up to 4 weeks in vitro and were routinely observed in the living state with an inverted phase contrast microscope.

Fetal calf serum was incubated with 0.1 mM DFP, an irreversible inhibitor of cholinesterase enzymes [8] to inhibit endogenous cholinesterase activity. DFP was added 3 times and after each addition, the serum was incubated for 8 h at 37°C. Cholinesterase activity was 95% inhibited and there was no detectable DFP activity. Acrylamide gel electrophoresis was based on the methods in [9].

2.2. *Enzyme analysis*

Localization of AChE was studied cytochemically by the technique in [10], after 5 min fixation in 4% formaldehyde (v/v). AChE and nonspecific cholinesterase (BChE) activities were determined on rinsed and sonicated cultures using the spectrophotometric assay in [11]. AChE activities are expressed as units of activity (μmol thiocholine ester hydrolyzed/min/culture dish).

Samples were assayed with acetylthiocholine iodide (ACTC) and butyrylthiocholine iodide (BUTC) as the substrates, with the specific AChE inhibitor 284c51 (10 μM) and with the non-specific cholinesterase inhibitor iso-OMPA (100 μM). There was only trace enzyme activity with BUTC as substrate or with ACTC in the presence of 284c51. Samples run with and

without iso-OMPA had virtually the same activity, indicating that over 95% of the esterase activity in these experiments was AChE.

2.3. *DFP treatment*

Cell cultures were rinsed 4 times at 37°C with saline and incubated with a saline solution of 0.1 mM DFP for 10 min at room temperature, rinsed 4 times with saline and returned to the complete medium at 37°C. At this time, in some dishes, cycloheximide at final conc. 10 μM, acetylcholine, choline or acetylbeta-methylcholine (AβMC) at final conc. 5 mM, or 10 Ci [^{3}H]leucine were added and samples were taken at various time intervals.

2.4. *Drugs*

ACTC, BUTC, DTNB, iso-OMPA, DFP, cycloheximide, acetylcholine, choline and AβMC were purchased from Sigma Chemicals, 1:250 trypsin from Difco Lab, F-12 and Hank's powdered medium from Gibco, fetal calf serum from Irvine Scientific, Grade A collagen from Calbiochem, and [^{3}H]leucine from ICN Corp.

Chick cultures were prepared and examined at the University of California, Davis. Rat cultures were prepared at UCLA, sampled, and sent frozen to UC Davis for analysis and interpretation.

3. Results

Initially, cells from chick spinal cord appeared round and morphologically undifferentiated. Within 2–3 days in vitro many cells developed long axon-like processes. After 5–7 days these neural cells formed a network of interconnecting processes with groups of round cells between them. Between 7–20 days some of the nerve cells became larger and exhibited thickened axons while others stayed small and extended thin processes. Flattened, morphologically undifferentiated cells appeared in the cultures and increased greatly in number with time. Only the neural cells showed any activity when these various cells were stained for AChE.

AChE activity of the cells was low at first; it increased during the first week in culture and then remained relatively constant for at least 24 days. The AChE activities found in a typical experiment, shown in fig.1, rose to ~7 X 10^{-3} units/min/dish in the first week.

Figure 1 also shows the amounts of AChE that appeared in the medium during the 2-day intervals between medium changes. In this, and in 3 other experiments not shown here, the total AChE activity released by the cells was greater than that which they retained. In the experiment shown here, the mean ratio of AChE released to that retained by the cells over 48 h averaged 4.5 : 1 with a range of 2.5 : 1 to 11 : 1. Cells continued to produce AChE at a high rate for as long as the cultures were maintained, and most of this AChE was released into the medium. Acrylamide electrophoresis showed 3 bands of AChE activity in both the cells and medium.

A series of 5 experiments was performed to examine the recovery of nerve-cell AChE from acute treatment with DFP. The I_{50} of DFP for AChE from 14-day

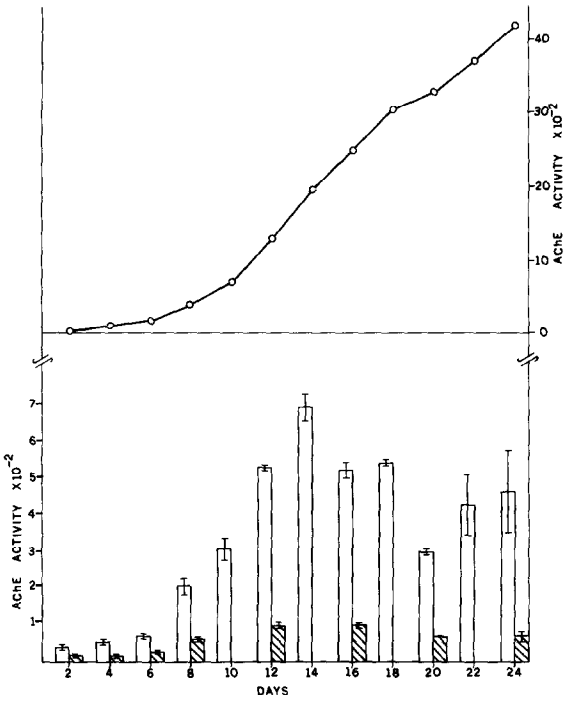

Fig.1. AChE production in spinal cord cells. The lower bar graph compares AChE activity measured in spinal cord cells (hatched bars) to the activity released into the medium (clean bars) every 2 days. The upper line graph represents cumulative AChE activity for the 24 day period. Values are the averages of 3 dishes.

Table 1
Recovery of AChE of DFP-treated nerve cells (10–14 day old cultures)

% Initial activity[a]					
0 h	1 h	2 h	4 h	8 h	24 h
8.25 ±1.26 (4)	14.5 (2)	44.0 +12.0 (4)	65.0 (2)	80.5 (2)	79.5 ±15.3 (4)

[a] Values are means ± standard deviations when appropriate

Number of experiments given in parentheses. Duplicate or triplicate samples were taken for each experiment. Activities were μmol ACTC hydrolyzed/min/dish

nerve cultures was ~1×10^{-7} M, and concentrations of 1×10^{-5} M DFP and above inhibited greater than 90% of the AChE activity. The results of 4 separate experiments are compiled in table 1. AChE rapidly returned to 44% of its initial activity in 2 h and then increased more slowly to ~80% of its original activity within 8 h. Release of AChE into the medium was not detected 8 h or 12 h after DFP treatment. The amount of AChE released into the medium at 24 h averaged 0.5-times and in one experiment at 48 h was 2.5-times the activity of the cells.

Addition of 10 μM cycloheximide to the cells immediately after treatment with DFP resulted in a reduction of 92–95% in the AChE found at 24 h (2 experiments), and a 97% decrease in incorporation of [^{3}H]leucine into protein.

DFP-treated cells were used to examine the effects of AβMC, ACh, and choline on the AChE activity of nerve cell cultures. The results of an experiment in which 5 mM AβMC was added to day 10 nerve cultures are shown in table 2. Total AChE activity increased 1.5-times by the presence of AβMC during the 48 h experiment. Cell AChE activity increased 1.4-times during the same interval.

A similar experiment conducted with ACh and choline is shown in tables 3 and 4. There was a linear increase in AChE activity for at least 48 h after DFP treatment (correlation coefficients were 0.97 and above for all groups), and the presence of ACh resulted in ~ 1.4-times more activity than the controls. (Increased values of AChE with choline were not statistically significant.)

Table 2
Effect of AβMC on DFP-treated nerve cells

Time (h)	Treatment	Activity ($\times 10^{-4}$)[a] Cell	Medium	Total
0 (2)	No DFP	20.8	0	20.8
	DFP	7.0	0	7.0
24 (3)	No DFP	21.4 ± 3.5	8.1	29.8 ± 6.0
	DFP	22.6 ± 5.9	8.6	31.2 ± 12.4
	AβMC	23.5 ± 6.0	22.5	46.0 ± 12.7
48 (3)	No DFP	21.0 ± 3.3	43.1	64.1 ± 5.3
	DFP	26.8 ± 2.0	35.5	62.3 ± 9.6
	AβMC	36.4 ± 7.1	63.0	99.3 ± 18.6[b]

[a] Values are means ± standard deviations when appropriate. Number of samples are given in parentheses. Activities are μmol ACTC hydrolyzed/min/dish

[b] Significantly different from DFP values at $P < .05$

Table 3
Effects of AChE and choline on DFP-treated nerve cells

Time (h)	Treatment	Activity ($\times 10^{-4}$) Cell	Medium	Total
0	No DFP	42.7	0	42.7
	DFP	3.16	0	3.16
8	DFP	38.7	0	38.7
	ACh	39.4	0	39.4
	Choline	34.7	0	34.7
12	DFP	23.6	0	23.6
	ACh	34.1	21.3	55.4
	Choline	47.6	25.7	73.3
24	DFP	37.6	25.7	63.3
	ACh	44.1	55.9	100
	Choline	49.1	30.1	79.2
48	DFP	51.5	136	188
	ACh	92.8	169	262
	Choline	61.9	178	240

(5×10^{-3} M substrates; duplicate samples; activity in μmol ACTC hydrolysed/min/dish)

Table 4
Rate of increase of AChE activity of DFP treated cells

Treatment	Rate/h
DFP	3.86
ACh	5.37[a]
Choline	4.77

[a] Significantly different from DFP-treated cells, $P < 0.5$, $F = 166$ [1,3]

(Data from same cultures as in Table 3. Linear regressions calculated for 0–48 h)

Release of AChE into the medium was detected earlier (12 h) for ACh- and choline-treated cells than with the DFP-treated controls (24 h).

To see if other nerve cells released AChE in culture, AChE activity was measured in cells from the cortex and brain stem of embryonic rats grown in the laboratory of Dr Jean de Vellis. Week 4 cultures developed morphologically differentiated neurons, but produced virtually no measureable AChE or BChE in either cells or medium.

4. Discussion

The results reported here, some of which were presented in preliminary form [5], and the report [6], demonstrate that AChE is released in quantity from cultured chick-nerve cells. The AChE activity retained by the cells studied here was only a relatively small portion of the total AChE produced.

Studies of embryonic muscle cultures, denervated muscle and dystrophic muscle of the chicken show that AChE appears in the culture medium or in tissue fluids whenever its activity in muscle is high [12]. AChE has been demonstrated in chick-embryo serum [13], and in the cerebrospinal fluid of mammals [14]. AChE has been proposed to be [15,16] actively secreted by cells in the adrenals and in the central nervous system of mammals. A 10 S form of AChE was released from rat sympathetic ganglia explants [17].

The physiological significance of the release of AChE from nerve and muscle systems may lie in the fact that the enzyme is probably in the basement membrane material at the synaptic cleft, outside of the muscle and nerve cells themselves [18,19]. It is possible that its release from nerve provides at least some of the enzyme at the motor end plate.

The DFP experiments demonstrate that cultured nerve rapidly recovers much of its AChE after it has been inhibited by an irreversible organophosphate agent. The lack of recovery of enzyme activity in the presence of cycloheximide suggests that the increase is due to synthesis of new enzyme. Approximately 50% of the AChE recovered in 2 h. In situ, AChE of mammalian brain recovered with a half-life of 7–16.5 days with DFP [20]. The activity of a low molecular weight form of AChE rapidly reappeared in mammalian retina after DFP treatment [21], and complete recovery of AChE to control values in 4 h using subacute doses of parathion was shown [22].

Brief treatments of cultured muscle with DFP or with paraoxon are also followed by rapid recovery of enzyme activity [23,24]. The kinetics of the recovery suggest that AChE is rapidly synthesized and degraded by the cells [23] and that the rapidity of the recovery is dependent on the extent of inhibition, i.e., the more enzyme inhibited, the more rapid the recovery [24].

AβMC and ACh dramatically increased the AChE activity of the nerve cultures studies here, particularly after 48 h incubation. ACh protected cultured chick spinal cord explants from loss of AChE [25], but the values of AChE in the medium were not determined. AChE increased when ACh was perfused into retinas following DFP treatment [26].

AβMC and ACh increased the AChE activity of chick-embryo muscle cell cultures [27,28]. The effect was not an 'induction', i.e., it was not inhibited by actinomycin D. However, it was blocked by the addition of curare, as if it were mediated by ACh receptor. The modulation of ACh receptor and AChE activities in muscle cultures has been associated with 'activity' of the cultures [27,29,30]. Whether or not contraction itself is necessary to regulate AChE and ACh receptor of muscle is not known. The results presented here for nerve cultures show that regulation of AChE levels by ACh and ACh analogs need not be contraction-mediated. One possibility is that movement of ions plays a regulatory role in controlling levels of AChE and ACh receptor. These findings with cultured muscle and nerve raise the possibility that release of ACh at synapses and at the neuromuscular junction plays a role in maintaining the levels of AChE and ACh receptor.

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