16S acetylcholinesterase of the extracellular matrix is assembled within mouse muscle cells in culture

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The present paper examines where the extracellular-matrix (ECM) 16S acetylcholinesterase (AChE, EC 3.1.1.7) is assembled in muscle cells in culture. The existence of an internal pool of 16S AChE was detected by using AChE inhibitors of differing membrane permeability. After irreversible inhibition of all cellular esterase, the newly synthesized 16S form appears in an intracellular compartment and is only later detected on the cell surface. Results show that the ECM 16S AChE is assembled within muscle cells.

The physiological role of AChE in synaptic transmission at the neuromuscular junction is well established (Kuffler & Nicholls, 1979). However, the mechanism by which this protein is synthesized and concentrated at the junction is not fully understood. Mouse clonal skeletal-muscle cells cultured *in vitro* can synthesize the endplate form (16S) of AChE in the absence of neural influence (Sugiyama, 1977; Inestrosa *et al.*, 1981a; Inestrosa, 1982a,b). It has been shown that the 16S form is associated with the ECM of C₂ mouse muscle cells in culture (Inestrosa *et al.*, 1982). The extracellular 16S AChE form is a complex macromolecule formed by three tetrameric groups of catalytic subunits and a collagen-like tail (Massoulie & Bon, 1982). Therefore we thought it of interest to find out whether this tailed form is assembled intra- or extra-cellularly. In the present work, the existence of an internal pool of 16S AChE and the reappearance of this form in different cell com-

partments after irreversible inactivation of AChE activity have been investigated.

Materials and methods

Mouse muscle cells

The C₂ cell line was established and initially described by Yaffe & Saxel (1977). In all the experiments, C₂ myotubes were grown for 4 days in a fusion medium, on 60mm-diameter plastic tissue-culture dishes (Falcon) as described elsewhere (Inestrosa *et al.*, 1982; Silberstein *et al.*, 1982).

Muscle extracts, velocity sedimentation and AChE activity

Muscle cultures were rinsed three times with 10mM-Tris/HCl, pH 7.4, mechanically removed from the dishes and hand-homogenized with a Duall-Kontes ground-glass homogenizer in 20mM-Tris buffer (pH 7.4)/1M-NaCl/0.5% Triton X-100/1mM-EDTA/Aprotinin/(20 units/ml)/1mM-benzamidine/bacitracin (0.1 mg/ml) (Inestrosa *et al.*, 1982). The muscle extract was centrifuged for 15 min at 17000g and aliquots of the supernatant (200 µl) were layered on 5–20% (w/v) sucrose density gradients made up in the same Tris buffer (Inestrosa & Méndez, 1983). Gradients were centrifuged at 164500g for 12 h at 4°C, in a SW 50.1 rotor. Fractions were collected and portions assayed for AChE by the procedure of Ellman *et al.* (1961). Sedimentation coefficients for AChE

Abbreviations used: ECM, extracellular matrix; AChE, acetylcholinesterase; BW, BW284c51 dibromide [1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one dibromide]; iPr₂P-F, di-isopropyl phosphorofluoridate; ECHO, (2 mercaptoethyl)trimethylammonium iodide *OO*-diethylphosphorothioate (echothiophate, phospholine); DME, Dulbecco's modified Eagle's medium; phosphate-buffered saline, 10mM-sodium phosphate/150mM-NaCl, pH 7.4.

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forms were determined by comparison with those of β -galactosidase (16.1S) and alkaline phosphatase (6.1S).

AChE inhibitor treatments

To determine which forms of AChE are exposed on the external or internal compartments, the effects of enzyme inhibitors differing in lipid solubility and hence in ability to cross cell membranes (Lazar & Vigny, 1980; Taylor *et al.*, 1981; Inestrosa *et al.*, 1981b) were examined. By using combinations of these inhibitors (BW and iPr_2P-F), it is possible to protect extracellular AChE while inactivating intracellular AChE irreversibly. The enzyme forms that remain active can then be identified after sucrose-density-gradient centrifugation. To selectively inactivate external AChE, ECHO, a cationic phosphorylating agent (Silver, 1974), can be alternatively used.

(a) *Protection of external AChE with BW.* Incubation of cells with iPr_2P-F , which is lipid-soluble, provokes a complete and irreversible inactivation of all of the enzyme activity associated with the cells (McIsaac & Koelle, 1959). Under the above conditions, the presence of an impermeable and reversible inhibitor such as BW will protect from inactivation those enzyme molecules which it can reach (Inestrosa *et al.*, 1981b, 1982).

(b) *Inactivation of external AChE with ECHO.* To inactivate irreversibly the cell-surface enzyme (Lazar & Vigny, 1980), DME containing $5.0\mu M$ -ECHO iodide, a lipid insoluble compound (Brimjoin, 1979), was added to washed myotube cultures at room temperature for 30s. This was followed by several rinses with DME and phosphate-buffered saline to eliminate excess ECHO.

(c) *Inactivation of all cellular AChE with iPr_2P-F .* Exposure of C_2 myotubes to $25\mu M$ - iPr_2P-F in phosphate-buffered saline for 3min, followed by repeated rinses with phosphate-buffered saline,

irreversibly inactivated over 97% of the total AChE activity.

Materials

BW was obtained from Burroughs Wellcome Co.; ECHO iodide was from Ayerst; Aprotinin, benzamidine and iPr_2P-F were from Sigma.

Results

To determine whether there exists an internal pool of the asymmetric 16S AChE, myotube cultures were incubated in ECHO. After washing the cells, it was found that the total AChE activity was inhibited by 27% (Table 1). When the cells were permeabilized with 0.5% Triton X-100 and then incubated for 30s with $5.0\mu M$ -ECHO, almost 96% of the total AChE activity was irreversibly inactivated (Table 1). It follows that, under our experimental conditions, this inhibitor is fully able to block all the available active sites of the enzyme.

The analysis of ECHO-resistant AChE activity by velocity sedimentation in sucrose density gradients showed that about 15–20% of the 16S AChE form remains active (Fig. 1b). It was evident that none of the globular forms of AChE (Inestrosa *et al.*, 1982) was greatly affected by ECHO, suggesting that neither these forms nor a small percentage of the 16S form were accessible to the inhibitor. To rule out differences in the effect of the inhibitor upon the individual forms of AChE, a parallel experimental series was run with broken-cell preparations (Fig. 1c). The results indicate that the ECHO-resistant AChE activity observed after incubation of intact cells with the inhibitor takes place within an intracellular compartment.

On the other hand, it is possible that 16S AChE, instead of representing a true intracellular pool, corresponds to a small percentage of extracellular enzyme previously internalized by the cells. Therefore, to rule out this possibility, the reappearance

Table 1. *Inactivation of cell-surface AChE with ECHO*

Intact C_2 mouse muscle cultures were washed with 2.5ml portions of DME and incubated for 30s at room temperature in 2ml portions of DME alone. A second group was exposed to 2ml of DME containing $5\mu M$ -ECHO, an irreversible inhibitor of AChE. The cultures were subsequently washed with DME for 5min to remove excess ECHO and assayed for total AChE activity after solubilization with Triton X-100. A third group of C_2 myotube cultures were centrifuged, washed with DME containing 0.5% Triton X-100 and then incubated for 30s with $5\mu M$ -ECHO; finally, the muscle extracts were assayed for total AChE activity. The activities shown are means $\pm$ S.E.M. for three cultures. AChE activity is expressed as m-units/dish, where 1 m-unit correspond to 1 nmol of acetylthiocholine hydrolysed/min.

Treatment	AChE activity	
	(m-units/dish)	(% of that in intact cells)
(1) Intact cells	64.3 ± 2.02	100
(2) Intact cells plus ECHO	47.1 ± 1.22	73
(3) Permeabilized cells plus ECHO	3.4 ± 0.57	5

Fig. 1. Intracellular 16S AChE in C_2 myotube cultures. Intact C_2 mouse myotubes were exposed for 30s at room temperature to DME alone. A second group was exposed to DME containing $5\mu\text{M}$ -ECHO. The cultures were subsequently washed with DME for 5min to remove excess ECHO. A third group was washed with DME containing 0.5% Triton X-100 and then incubated for 30s with $5\mu\text{M}$ -ECHO. The cultures were homogenized and centrifuged and the supernatant was mixed with enzyme markers; 200 μl aliquots of the mixture were layered on 5–20% sucrose gradients, sedimented, and AChE activity assayed as described in the Materials and methods section. The arrows indicate the position of (left) β -galactosidase (16.1S), and (right) alkaline phosphatase (6.1S). Velocity-sedimentation profiles are shown of AChE molecular forms from (a) control (○), (b) ECHO-resistant activity (●) and (c) C_2 muscle extracts incubated with ECHO (▲).

of 16S molecules after irreversible inhibition of the total cellular AChE activity by $i\text{Pr}_2\text{P-F}$ was studied. C_2 cultures were incubated for 3min with $25\mu\text{M}$ - $i\text{Pr}_2\text{P-F}$ and 3.5h later the cells were assayed for BW-protected (cell-surface) or ECHO-resistant (intracellular) AChE molecular forms. It was found that, after 3.5h of $i\text{Pr}_2\text{P-F}$ poisoning, about

5% of 16S AChE had been synthesized (Fig. 2b, total extract); practically all the activity was present in an intracellular compartment (Fig. 2b, intracellular), and none was found in the cell surface (Fig. 2b, cell surface). Results also showed that, after $i\text{Pr}_2\text{P-F}$ treatment, the AChE forms with lower sedimentation coefficients recover earlier than the asymmetric forms, as has been reported for rat embryonic muscle cells (Koenig & Vigny, 1978). Our results demonstrated that, at least after $i\text{Pr}_2\text{P-F}$ treatment, the intracellular pool of 16S AChE is a real entity and does not correspond to internalization of surface asymmetric AChE molecules.

To find out whether 16S AChE is assembled within muscle cells, total C_2 AChE activity, cell surface, and intracellular AChE activity were studied at different times after $i\text{Pr}_2\text{P-F}$ treatment. Table 2 indicates the sequence of changes for the 16S AChE form. The newly synthesized 16S form appears in an intracellular compartment and is only later detected on the cell surface. In fact, there is at least a 1h lag between the recovery of ECHO-resistant (intracellular) and BW-protected (cell-surface) pools of 16S AChE activity.

Discussion

Previous studies have demonstrated that the globular forms of AChE are mostly intracellular, whereas the 16S enzyme appears to be extracellular (Inestrosa *et al.*, 1982) and associated with the ECM of the C_2 myotubes (Inestrosa *et al.*, 1981a; Silberstein *et al.*, 1982). In the present study, some 16S AChE remained active after irreversible inactivation of the cell-surface esterase, indicating that a small pool of this collagen-like tail form is localized in an intracellular compartment. The above conclusion supports previous findings showing that a small fraction of 16S AChE was not protected by BW and was resistant to collagenase treatment (Inestrosa *et al.*, 1982). The present report is the first experimental demonstration of an intracellular localization for the muscle 16S AChE form. The results also show that the 16S form of AChE is assembled within some internal compartment for its subsequent deposition on the ECM.

Further evidence of the intracellular assembly of the 16S AChE form is found in studies on peripheral nerve, where the 16S AChE undergoes axoplasmic transport (DiGiamberardino & Couraud, 1978).

The intracellular assembly of the 16S AChE form would imply some co-ordination between synthesis of collagen-like tail subunits and AChE catalytic subunits. Two questions then arise, regarding the biogenesis of 16S AChE. (a) How many genes are needed for synthesizing a complete

Fig. 2. Recovery of AChE molecular forms in different compartments of C_2 myotube cultures after iPr_2P-F inactivation. Intact C_2 mouse myotubes were exposed for 3 min to DME alone (a). A second group was exposed to $25 \mu M$ - iPr_2P-F for 3 min, then repeatedly rinsed with phosphate-buffered saline (b). Both series were returned to the incubator for 3.5 h. After this time, the myotubes were separated into three groups and processed as follows. Total extract: myotubes were homogenized in Tris buffer containing Triton X-100 and NaCl; the homogenate was centrifuged and the AChE molecular forms present in the supernatant were separated by sucrose-gradient sedimentation. Cell surface: C_2 myotubes were exposed for 30 s at room temperature to $750 \mu M$ -BW284c51 dissolved in DME. iPr_2P-F was then added in the presence of BW to a final concentration of $25 \mu M$ for 3 min. The solution containing iPr_2P-F was removed and the myotubes were rinsed, once with $750 \mu M$ -BW284c51 in DME and five times with phosphate-buffered saline (Inestrosa *et al.*, 1982). The cells were then harvested and processed as described for 'Total extract'. Intracellular: C_2 myotubes were exposed for 30 s at room temperature to $5 \mu M$ -ECHO iodide dissolved in DME, and rinsed exhaustively with phosphate-buffered saline. The cells were then harvested and processed as described for 'Total extract'. The Figure shows sedimentation profiles in sucrose gradients (meniscus on right-hand side). The arrow indicates the sedimentation site of β -galactosidase (16.1S).

Table 2. Recovery of 16S AChE activity after iPr_2P-F treatment of cultured C_2 mouse myotubes

Intact C_2 mouse myotubes were exposed to $25 \mu M$ - iPr_2P-F for 3 min and repeatedly washed with phosphate-buffered saline to eliminate unbound iPr_2P-F . At different times thereafter the cells were analysed for AChE molecular forms on sucrose gradients, as described in the Materials and methods section. In similar experiments, after iPr_2P-F treatment, the myotube cultures were either exposed to ECHO or subjected to BW-protection procedures and analysed for AChE forms. The relative activity of 16S AChE in a gradient was obtained by expressing the activity in each fraction as a percentage of the total activity recovered from the gradient (70–90%) and adding the percentages (from minimum to minimum) in the peak of the 16S form. The absolute activity was calculated by multiplying the activity obtained at the time of extraction by the percentage/100 of the gradient activity corresponding to the 16S form. The Table shows two experiments. The percentage of control in the different compartments was calculated by taking as 100% the values of 16S AChE activity in non-treated muscles. Absolute values are: whole myotubes, 25.0 ± 1.8 m-units/dish; cell surface (BW protection), 20.4 ± 2.1 m-units/dish; and intracellular (ECHO treatment), 5.4 ± 0.48 m-units/dish. Values were obtained in four experiments.

Time after iPr_2P-F treatment (h)	16S AChE activity					
	Whole myotube		Cell surface		Intracellular	
	(m-units/dish)	(% of control)	(m-units/dish)	(% of control)	(m-units/dish)	(% of control)
3	1.1–1.3	(5)	0	(0)	0.9–1.2	(20)
4	2.9–3.3	(12)	0.8–0.9	(4)	1.9–2.3	(39)
5	8.3–9.1	(35)	4.8–5.7	(26)	3.0–3.5	(60)
8	17.4–21.2	(77)	13.5–15.6	(71)	4.4–5.1	(88)

16S molecule? (b) Are the genes for the collagen-like tail subunits in the muscle genome close to the genes for the catalytic subunits?

While this work was in progress, M. R. Emmerling & R. L. Rotundo (personal communication) found that quail muscle cultures have an internal pool of asymmetric AChE which is assembled intracellularly after the appearance of the globular forms of the esterase (see also Emmerling & Rotundo, 1981). In turn, our recent studies indicate that the intracellular pool of AChE is present in rat embryo intercostal muscle (Inestrosa *et al.*, 1983).

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