Subcellular distribution of acetylcholinesterase asymmetric forms during postnatal development of mammalian skeletal muscle

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This study describes the changes which occur in intra- and extracellular asymmetric acetylcholinesterase (AChE) forms in rat gracilis muscle during postnatal development. Initially (day 7) these forms (12.5 S and 16 S AChE) were evenly distributed along the muscle and only present intracellularly. With advancing age (days 7–28) they gradually became concentrated in endplate (*vs* non-endplate) muscle regions where a certain proportion of them was subsequently externalized. In contrast, no externalization was observed in the non-endplate regions. Our results support the view that AChE asymmetric forms are assembled within the muscle cell prior to their deposition on the extracellular synaptic compartment.

Acetylcholinesterase *Postnatal development* *Subcellular distribution* *Neuromuscular junction* *Cholinesterase inhibitor* *Skeletal muscle*

1. INTRODUCTION

Mammalian skeletal muscles contain multiple molecular forms of AChE (acetylcholine acetylhydrolase, EC 3.1.1.7) [11], one of which, with a 16 S sedimentation coefficient, has attracted much attention partly because its activity is regulated by the motor nerve [2,3]. In adult rat muscle most of the 16 S AChE form, which is composed of three catalytic tetramers and a collagen-like tail structure [4], is highly concentrated at the motor endplates (+EP), although small amounts are also detected in non-endplate (−EP) regions [5,6]. This well defined localization is not present in neonate rats; the enzyme is initially distributed evenly along muscle fibers and progressively accumulates in the +EP regions until the adult pattern is reached at approximately 3–4 weeks of age [5,7–9]. At this age, a large proportion of the 16 S AChE exists extracellularly in association with the synaptic basal lamina [4]; however, the possible developmental changes in subcellular distribution leading to such localization have yet to be determined.

Recent studies have shown that the subcellular distribution of AChE forms in both embryonic [10,11] and adult [6] rat muscles can be determined by using cholinesterase inhibitors which differ in lipid solubility and hence in their ability to penetrate cell membranes. We have previously used similar methods to distinguish intra- from extracellular AChE forms in adult rat anterior gracilis muscles [12]. This report documents the changes in subcellular distribution of asymmetric AChE forms which occur during postnatal development.

2. MATERIALS AND METHODS

Experiments were performed on obturator nerve-anterior gracilis muscle preparations [3] from littermate Sprague-Dawley rats (Sasco, Omaha, NE), 7–60 days old, anesthetized with Na-pentobarbital (36 mg/kg body wt; i.p., injection).

2.1. *Experimental treatments and extraction of AChE activity*

To distinguish between intra- and extracellular pools of AChE activity muscles were exposed in situ to one or a combination of AChE inhibitors.

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To assay intracellular AChE the muscle's surface enzyme was irreversibly inactivated by 2.5 μM echothiophate iodide (ECHO; water-soluble) applied for 3 min, followed by six 30-s rinses with phosphate-buffered saline (PBS) which eliminated excess ECHO [12]. Extracellular AChE was assayed after: (i) exposure of the muscle to 70 μM BW284C51 dibromide (BW; water-soluble) in PBS for 30 s to reversibly inactivate the surface enzyme; (ii) With the muscle still exposed to BW the remaining AChE was irreversibly inhibited by 0.4 mM di-isopropylfluorophosphate (DFP; lipid-soluble) in PBS for 1 min, followed by three 15-s rinses with 70 μM BW to remove unbound DFP; (iii) Subsequently, the surface enzyme was reactivated by eight 1-min rinses with PBS to remove BW [12]. Prior to the aforementioned treatments contralateral control muscles were removed and rinsed with PBS for a total exposure time identical to that of the experimental muscles.

Next, the muscles were separated into endplate (+EP) and non-endplate (−EP) regions (whole muscle weight = [+EP] + [−EP]) which were rapidly rinsed with PBS and weighed [3]. For 7-, 10-, 14-, 21-, 28-, 45- and 60-day-old rats the wet weights of +EP samples were, respectively, 1.2 ± 0.2, 2.2 ± 0.4, 3.1 ± 0.7, 3.7 ± 0.4, 8.3 ± 1.8, 15.2 ± 0.8 and 18.9 ± 0.6, whereas those of the corresponding −EP samples were 1.2 ± 0.2, 2.9 ± 0.3, 4.1 ± 0.2, 5.2 ± 0.4, 10.5 ± 2.2, 23.3 ± 1.1 and 35.8 ± 1.2 mg (mean ± SD, n = 12). Samples were then homogenized (1:35, w/v) in low ionic strength buffer (0.05 M Tris–HCl, 5 mM EDTA, 1% lubrol-wx; pH 7.3; 4°C). After two sequential centrifugations (20000 × g, 30 min, 4°C) the pellets were pooled and resuspended in high ionic strength buffer (same as above, but with 1 M NaCl) to extract the asymmetric AChE forms [13].

2.2. *Velocity sedimentation*

AChE forms were separated in linear sucrose gradients (5–20%) containing high ionic strength buffer. Sedimentation was performed at 4°C and 260000 × g_{max} (Beckman L8-70 ultracentrifuge; SW41Ti rotor) to an w^2 value of 1.06×10^{12} rad^2/s (roughly 18 h 45 min). Approximately 60 fractions (200 μl each) were collected from each gradient and assayed for AChE activity. Sedimentation markers were β-galactosidase (16.1 S) and catalase (11.3 S). AChE forms were quantified by adding the enzymatic activities under each of the sedimentation profile peaks [13].

2.3. *AChE assay*

AChE activity was measured as in [13], using [^{3}H]acetylcholine iodide (90 mCi/mmol; 0.03 mM final concentration; New England Nuclear) as the substrate and 0.1 mM *iso*-OMPA (Sigma) as an inhibitor of cholinesterase (ChE, EC 3.1.1.8). One unit of AChE activity is defined as 1 μmol of [^{3}H]acetate formed per min.

3. RESULTS

At 7 days postnatal age, whole muscle AChE activity was 77.5 ± 2.1 munits/mg wet wt (mean ± SD, n = 6; 2.4 ± 0.2 mg wet wt; 151.2 ± 3.2 munits/mg protein), whereas at 60 days it was 10.6 ± 0.2 munits/mg wet wt (56.7 ± 3.6 mg wet wt; 83.1 ± 2.0 munits/mg protein). Table 1 shows that this decay was less dramatic for the +EP (3.75-fold) than for the −EP (13.33-fold) muscle regions. Thus, with progressing age the relative proportion of whole muscle AChE activity contained in +EP regions became greater. For example, at day 7 both +EP and −EP regions contained approximately 50% of whole muscle AChE, while at day 60 this proportion had changed to approximately 78% +EP and 22% −EP.

Developmental changes in 16 S AChE activity were qualitatively similar to those described for total AChE. At day 7, whole muscle 16 S activity

Table 1

Total AChE activity in rat anterior gracilis muscles during postnatal development

Age (days)	AChE activity (munits/mg wet wt)			
	+EP	%	−EP	%
7	77.6 ± 3.3	50.1	77.3 ± 1.9	49.9
10	76.4 ± 4.2	59.2	52.7 ± 4.7	40.8
14	75.3 ± 6.7	72.3	28.9 ± 6.6	27.7
21	60.2 ± 3.7	80.4	14.7 ± 2.1	19.6
28	21.2 ± 0.9	76.0	5.9 ± 0.7	24.0
45	21.1 ± 1.0	78.7	5.8 ± 0.7	21.3
60	20.7 ± 1.1	78.1	5.8 ± 0.2	21.9

Values are the means ± SD of 6 experiments each run in triplicate. One munit of AChE activity is defined as 1 μmol [^{3}H]acetate formed per min × 10^{-3}

was 54.8 ± 3.2 munits/mg wet wt (n = 6) and declined to 5.0 ± 0.8 munits/mg wet wt by the 60th day. Over the same period, +EP region 16 S AChE decreased from 27.7 ± 2.3 munits/mg wet wt to 4.2 ± 0.7 munits/mg wet wt, whereas that in −EP regions decayed from 27.1 ± 2.1 munits/mg wet wt to 0.8 ± 0.2 munits/mg wet wt. Fig.1 illustrates the temporal course of the changes in 16 S AChE activity from +EP and −EP regions. At day 7 this form's activity was evenly distributed between both muscle regions. With advancing age the proportion of whole muscle 16 S AChE in +EP regions increased (80% at day 28), while that proportion in −EP regions decreased (20% at day 28). Beyond the 28th day, the above proportions did not change significantly, at least through day 60 (+EP, 84%; −EP, 16%).

As previously described for the gracilis muscle preparation [12], under optimum inhibitor treatment conditions, the ECHO-inaccessible (intracellular) and the BW-protected (extracellular) AChE pools were for the most part mutually exclusive; i.e., the activity spared by ECHO-inactivation was completely inhibited by sequential treatment with BW and vice versa. As shown in fig.2, at day 7 the 12.5 S and 16 S form activities in +EP regions were localized entirely intracellularly. With advancing age, this intracellular pool of +EP asymmetric forms decreased, while there was a concomitant increase in the corresponding extracellular pool. Although the absolute activities of intracellular asymmetric forms in −EP regions also decreased as a function of age, there was no corresponding increase in the extracellular pool. In fact, no asymmetric form activity was detected extracellularly in −EP regions throughout the postnatal ages studied. Fig.3 shows that there was a clear correspondence between the

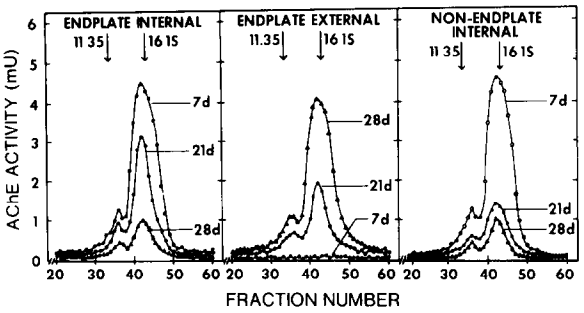

Fig.2. Velocity sedimentation profiles of AChE asymmetric forms (12.5 S and 16 S AChE) in +EP and −EP regions of rat anterior gracilis muscle. Internal (ECHO-inaccessible) and external (BW-protected) enzymes were evaluated at postnatal ages of 7 (○—○), 21 (●—●), and 28 (□—□) days. AChE inhibitor treatments, extraction, and sedimentation analysis, were performed as described in section 2. Sedimentation markers (arrows) are catalase (11.3 S) and β-galactosidase (16.1 S). Enzymatic activity (munits) represents that portion of the sample-dependent AChE hydrolysis that is insensitive to 0.1 M *iso*-OMPA and 1 munit corresponds to 1 μmol ^{3}H-labeled acetate formed per min $\times 10^{-3}$.

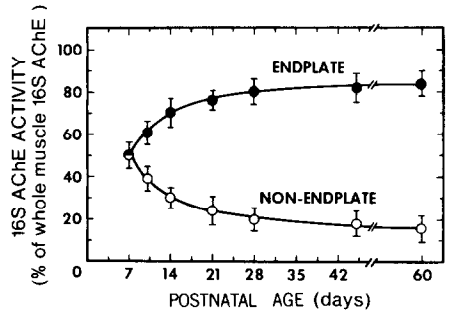

Fig.1. Distribution of 16 S AChE in +EP and −EP regions of rat anterior gracilis muscle as a function of postnatal age (days). The enzyme was separated in sucrose gradients as described in section 2 and its activity is expressed in terms of total 16 S AChE obtained by adding that in the two muscle regions analyzed (% of whole muscle 16 S AChE). Each point represents the mean ± SD of 6 experiments.

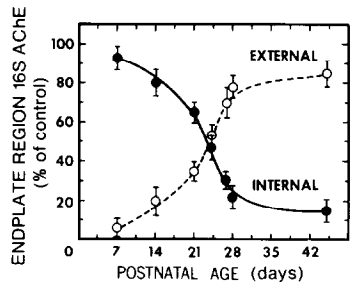

Fig.3. Distribution of internal (ECHO-inaccessible) and external (BW-protected) 16 S AChE activity (% of control) in +EP regions of rat anterior gracilis muscle as a function of postnatal age (days). AChE inhibitor treatments (ECHO and BW) and sedimentation analysis are as described in section 2. Activity of 16 S AChE in treated muscles is expressed in terms of that measured in the contralateral untreated control muscle. Each point is the mean ± SD of 6 experiments.

decrease of intracellular 16 S AChE in +EP regions and the increase of its extracellular counterpart. In turn, the intracellular pool of 16 S AChE in −EP regions, expressed as a percentage of total −EP 16 S AChE (intra- plus extracellular), did not change significantly.

4. DISCUSSION

Our results show that rat anterior gracilis muscle AChE activity, expressed in terms of either tissue wet wt or total protein content, decreased as a function of postnatal age with a temporal course similar to that reported for other rat muscles [14]. Taking advantage of the fact that the gracilis innervation pattern permits an easy separation of +EP from −EP regions [3,13], we also found that such an AChE decrease extended to both muscle regions. While in +EP the decay of enzymatic activity was not apparent until day 21, that in −EP was more dramatic and was detected as early as 10 days after birth (table 1). Whether there is a causal relationship between the aforementioned enzymatic changes and the disappearance of polyinnervation [15], cannot be resolved by our experiments. Nonetheless, the important point to be stressed is that the AChE activity detected in +EP regions, expressed as a percentage of that in whole muscle, significantly increased during muscle maturation whereas that found in −EP actually decreased (table 1).

Consistent with such changes in total AChE activity, during early postnatal development the asymmetric 16 S form was also evenly distributed along the gracilis muscle and with age its relative proportions in +EP *vs* −EP regions progressively increased (fig.1). Analogous results have been previously reported for changes in the developmental distribution of 16 S AChE activity in rat diaphragm [5,7,9]. These findings may now be ascribed to the more prominent disappearance of 16 S AChE from −EP as opposed to +EP regions. While the possibility of a spatial developmental shift in the localization of the enzyme cannot be discarded, further studies are necessary to resolve this point. In turn, our experiments also show that despite the noticeable localization of 16 S AChE activity in +EP regions observed by the end of the first month after birth, a small amount of this activity still persisted in the −EP regions (fig.2). Other investigators have claimed that 16 S AChE is completely absent from −EP regions of adult rat diaphragm [5,7,9]; however, our results are in agreement with a more recent report showing detectable amounts of the enzyme in the −EP regions of this muscle [6]. Accordingly, as has been suggested for human skeletal muscle [16], the 16 S AChE form in rat muscle cannot be entirely considered as an endplate specific enzyme.

The most important aspect of this study involves the developmental changes in the subcellular distribution of asymmetric AChE activities, particularly the 16 S form. As mentioned earlier, the ECHO-inaccessible and BW-protected asymmetric AChE activities can be considered to represent, for the most part, intra- and extracellularly located enzymes, respectively [6,10,11]. This conclusion is strongly supported by recent experiments showing that denervation of adult gracilis muscle causes intracellular 16 S AChE activity to decay earlier than its extracellular counterpart [12]. The present results not only support the existence of such subcellular pools of asymmetric AChE forms, but more importantly they demonstrate that the activity of these forms in intra- and extracellular compartments undergo distinct changes during postnatal development. For example, there was a clear temporal correspondence between the observed developmental decrease of intracellular 16 S AChE and its extracellular increase at muscle +EP regions (fig.3). We conclude, then, that with muscle maturation there is a gradual externalization of 16 S AChE in +EP regions until the adult distribution (15% internal; 85% external) is attained approximately 1 month after birth. This +EP enzymatic externalization process does not extend to the −EP regions which, within the limits of resolution of our methods, did not show any detectable amounts of extracellular asymmetric AChE throughout the muscle's postnatal development. In this context, the extracellular 16 S AChE in rat gracilis muscle may be considered as an endplate specific enzyme, whereas that located intracellularly is present in both +EP and −EP regions. Our present and previous [12] findings, together with those showing that muscle cells are capable of producing 16 S AChE even in the absence of innervation [17], are consistent with the view that 16 S AChE (as well as the 12.5 S form) is assembled

within muscle cells prior to its deposition on the synaptic basal lamina.

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