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USE AND DISUSE AND THE CONTROL OF ACETYLCHOLINESTERASE ACTIVITY IN FAST AND SLOW TWITCH MUSCLE OF RAT.

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Although the role of acetylcholinesterase (AChE) in neuromuscular transmission is relatively well established, little is known of the mechanisms that regulate its synthesis and control its specific distribution in fast and slow muscle. Innervation plays an important role in the regulation of AChE and elimination of the influence of the nerve by surgical denervation results in a loss of AChE (1). The question of how these influences of the nerve are mediated has been a matter of continuing controversy. Muscle usage as well as other factors such as materials carried by axonal transport may participate in the regulation of this enzyme.

Nerve transection has two effects: by preventing nerve impulses from reaching the nerve terminal it produces disuse, and by interrupting axonal transport it eliminates the release of trophic materials from nerve to muscle. These two effects of denervation--disuse and loss of neurotrophic factors--are not necessarily independent.

Changes in impulse traffic along the peripheral nerve may

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alter the rate, type or amount of materials that are axonally released. In addition, differences may be found in the muscles themselves which may control their characteristics by substances whose availability may depend on the level of contractile and metabolic activity. It is clear from recent reports that muscular activity, either neurally invoked (2,3,4) or generated in culture via electrical stimulation (3,4), plays a major role in the control of AChE. Studies using the interventions of limb immobilization (5,6), models of neuromuscular overload such as compensatory hypertrophy (6), and endurance training attest to the mutability of this component of neuromuscular function in the face of chronically altered neuromuscular demands. Previous reports on the effects of reduced activity by limb immobilization or tetrodotoxin (TTX) induced block of axonal conduction (7) have not effectively differentiated the response of molecular forms of AChE (8,9,10). An attempt to determine disuse on these distinct enzyme forms is essential, especially in light of evidence that a polymorphism of AChE exists which relates to function. In addition, the reports that these forms are differentially affected by nerve stump length after denervation (11,12) suggest variation among these AChE forms in their control by neuromuscular activity.

More recent observations of the effects of disuse in the regulation of the different forms of AChE do not agree with each other. When cordotomy (13) was used AChE of the non-endplate region was reduced while the enzyme activity of the endplate (16S) was unchanged. With limb immobilization (14) the opposite results were found such as a decrease in endplate AChE and increase in non-endplate AChE. These investigations differ, however, in regard to disuse technique, as well as to the muscle used.

Disuse as previously produced; by tenotomy (15), denervation

(16,17,18), spinal cord section, and/or dorsal root section involves either drastic changes in resting muscle tension, interruption of the reflex arc, transection of suprasequential inputs to the arc or manipulations that might cause damage to nerve and muscle. Results may be due, in large measure, to modification of intercellular relations which are unrelated to nerve impulses, or to cell injury of either muscle or nerve (19,20,21).

Disuse produced by injecting drugs such as TTX (7) beneath the perineurium of small nerves might mechanically damage an unspecifiable number of fibers. It seemed important, therefore, to use a new model for producing disuse, a procedure that does not involve manipulations in the vicinity of the lower motor neuron and nerve, such as the hindlimb suspension model of hypokinesia.

Although the rate of loss of (AChE) activity that follows denervation is similar in rat fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles, the return of AChE activity during reinnervation showed a distinct difference between these two muscles (1). This observation raised interesting questions regarding differences in mechanisms that regulate the patterns of individual AChE forms in fast and slow mammalian skeletal muscle.

We report here on studies undertaken in our laboratory in an attempt to understand the mechanisms that regulate AChE and its molecular forms in two functionally different muscles.

First, we will describe the development of the AChE molecular forms during differentiation in fast and slow muscle. A developmental study of AChE and its molecular forms in EDL and SOL muscles may be helpful in explaining the distinct difference in activity and control of AChE in EDL and SOL and

elucidate the mechanisms that control the physiological and biochemical properties of muscle. Although information is available on both morphological and biochemical changes and AChE activity during prenatal development, there have been no direct comparisons in the same series of animals between molecular forms of AChE during the postnatal development of EDL and SOL.

Second, experiments are presented which investigate the effect of denervation and reinnervation on AChE activity in SOL and EDL. This approach was chosen when previous data indicated that these muscles differed significantly in their AChE activity, their twitch speed and their histochemistry. Furthermore, recovery of AChE activity during reinnervation differed between these muscles. It was thought that the changes induced by loss and re-establishment of functional connection between nerve and muscle are a suitable model for studying the mechanisms that control AChE. A difference between these muscles in the rate of loss or recovery of this enzyme may help in the elucidation of the regulating mechanisms.

Third, we have investigated whether functional activity has a role in the control of AChE and its molecular forms. We will present experiments in which hindlimb suspension was used for reducing muscle activity. The differences found in AChE activity in SOL and EDL may have its origin in the different stimulation patterns these muscles normally receive. By removing the work load from both of these muscles the differences between them should be reduced and we can determine the regulatory effect of muscle work on AChE activity.

1. Developmental Alterations in the Molecular Forms of Acetylcholinesterase in Fast and Slow Twitch Muscle of Rat.

Termed pregnant Sprague-Dawley rats weighing 180-250 g were used for this study. Litter size was controlled at eight pups per mother. The pups were killed at 1 day, 1, 2, 3, 4, 5, 6 and 14 weeks after birth by decapitation and exsanguinated. Pups were sampled from different litters in order to minimize differences between mothers. The SOL and EDL muscles were removed, cleaned of the connective tissue, weighed and stored on ice. For very young rats (less than 2 weeks) a dissecting microscope was used during the isolation of muscles.

AChE activity as calculated per gram muscle or mg protein are shown in Fig. 1. At one day after birth, both muscles show similar AChE activity, and between the first and second postnatal week the SOL exhibited a rapid 2.5-fold increase in enzyme activity, followed by an equally rapid decline. Enzyme levels approached the lower adult values by 4 weeks. In the EDL, AChE activity also rose rapidly between the first and second postnatal week and remained at this level, however, never reaching the peak activity of the SOL. Within four weeks the mature level of enzyme activity was reached in both muscles.

Rapid changes in the pattern of AChE molecular forms were particularly apparent during the first four postnatal weeks. Representative velocity sedimentation experiments are shown for 1 day, 1 week, and 14 weeks post partum in Figs. 2 and 3. One day after birth, the intermediate (10S and 12S) and heavy (16S) molecular forms account for approximately 60% of the total enzyme activity and both muscles (Fig. 2A), exhibit similar patterns of molecular forms of AChE not unlike that of the mature SOL.

Fig. 1. Developmental changes in acetylcholinesterase(AChE) activity as a function of muscle wet weight (A) and protein (B) in extensor digitorum longus (EDL) and soleus (SOL) muscles. Values represent the mean ± SEM of 3 separate determinations for each age.

One week after birth, a relative increase of the light 4S AChE molecular form was observed in the EDL muscle (Fig. 2B). Whereas an increase of the 4S form did not occur in SOL, the relative activity of the 12S and 16S forms increased, however.

Four weeks after birth most of the postnatal alterations in AChE molecular forms in EDL and SOL muscles have occurred (not shown here) and resembled the profiles of mature muscles (Fig. 3).

As evident from Fig. 1, the most rapid alterations in total

Fig 2. Developmental changes in the velocity sedimentation gradients of AChE molecular forms from: A, 1 day and B, 1 week postnatal extensor digitorum longus (EDL) and soleus (SOL) muscle. Gradients contained two hundred micrograms of a high speed supernatant from the muscles which was layered on a 5 to 20% sucrose gradient and centrifuged at 35,000 rpm for 18 hr. Estimated sedimentation constants are indicated for the separate forms. Each gradient was independently calibrated with B-galactosidase, catalase, and alkaline phosphatase. Approximately 30 fractions were collected and 0.03 ml was incubated for 4 hr in the presence of ^{3}(H)-acetylcholine and iso-OMPA (10^{-5}uM).

AChE activity occurred during the first 4 postnatal weeks. The relative percent contribution of the different AChE molecular forms, which were quantitated by comparing enzyme peak areas to the area of the total enzyme activity, are shown in Table I. One day following birth both EDL and SOL muscles exhibit similar levels of 4S, 10S and 16S AChE (27%, 43% and 30% for EDL and 22%, 54% (12S) and 24% for SOL, respectively). The marked differences of AChE molecular form patterns seen between the mature SOL and EDL muscles become

Table 1. Activities of AChE Forms During Postnatal Maturation of EDL and SOL.

Postnatal Period	EDL			SOL			
	4S	10S	16S	4S	10S	12S	16S
1 Day	27.0	43.0	30.0	22.0		54.0	24.0
1 Week	56.0	24.0	20.0	36.0		40.0	24.0
2 Weeks	45.0	27.0	28.0	33.0	17.0	14.0	36.0
3 Weeks	49.0	23.0	28.0	29.0		35.0	36.0
4 Weeks	51.0	33.0	16.0	21.0		62.0	17.0
14 Weeks	50.0	35.0	15.0	22.0	18.0	34.0	26.0

Activities of AChE molecular forms from EDL and SOL. AChE molecular forms were recovered from sucrose density gradients during postnatal development. Activities are expressed for each form as a percentage of the total recovered activity. Data are from three separate preparations of high speed supernatants (100,000 x g) isolated from rat muscle. Values are expressed as the mean $\pm$ SEM.

Fig. 3. Velocity sedimentation gradient separation of AChE molecular forms from EDL and SOL muscle of 14 week old rat. Exp. Neurol. 79: 519-531, 1983.

already apparent by the first postnatal week (Fig. 2B). In the EDL the 4S form accounts for approximately 50% of the total activity and remains at this level for the remaining developmental period. At no time does the EDL muscle exhibit the relative increases in the 16S AChE or decreases in 4S AChE as observed in SOL.

In the SOL muscle, an increase of the 16S molecular form is evident between 1 day and the 3rd week following birth. As the 4th postnatal week is reached the 16S has decreased from the 2 week level of activity. The decline is accompanied by an increase in the activity of the 12S molecular form of AChE.

2. Distinct Difference Between Slow and Fast Muscle in Recovery of AChE Activity After Denervation in Rats.

Within 3 days following nerve crush, AChE activity was reduced to about 40% of control in both muscles and by the end of the second week the remaining activity was 15% of control in both muscles (Fig. 4).

Following the second week, enzyme activity began to recover in both muscles; however, the rate of recovery in the SOL was much faster than in the EDL. Three weeks after crush, AChE activity in the SOL had risen to 250% of control while the activity in the EDL was only 40% of control. SOL enzyme activity returned to normal, while EDL had regained only 50% of control activity at the end of the fifth week.

The changes of total AChE activity were also studied in endplate and non endplate regions of the SOL. There was no qualitative difference in the response of AChE activity to crush and reinnervation between endplate and non-endplate regions of the SOL.

Molecular forms in the EDL, compared with their contralateral controls, exhibited marked decreases. Despite the proportional redistribution in some forms, such as in the 10S which now contributed 60% of the total activity compared with 35% in a contralateral control muscle, there remained a comparative loss in activity compared with its contralateral

Fig. 4. Changes in muscle wet weight and AChE activity after denervation in the EDL and SOL muscles. A-EDL and SOL muscles were removed and trimmed of excess fascia before weighing. B-total AChE activity from EDL and SOL muscles after nerve crush. Twenty-microliter samples from 5% (w/v) homogenates were assayed in the presence of ^{3}H-acetylcholine for 20 min. AChE activity is expressed as the release of ^{3}H-acetate (micromoles) per gram wet weight per hour. Both wet weight and AChE activity from denervated muscles were compared with contralateral muscles and expressed as a percentage of control. Exp. Neurol. 79: 519-531, 1983.

control. Decreases in AChE molecular form activity in the SOL also were evident compared with their contralateral controls, except in the 4S form which increased above contralateral activity (Table 2).

In the SOL, 2 weeks after nerve crush all molecular forms, especially the 4S, had higher activity than those of the contralateral control muscle (Fig. 5A). Four weeks after nerve crush, as the reinnervation progressed, the molecular form pattern favored the heavier molecules in addition to the 4S form (Fig. 5B). Total AChE activity remained

TABLE 2. Activities of Molecular Forms of Acetylcholinesterase One Week After Denervation[a]

Molecular Forms	SOL		EDL	
	Control	Denervated	Control	Denervated
4S	25	44	50	22
10S	20	25	35	58
12S	25	17	0	0
16S	30	14	14	20

[a]Activities in AChE molecular forms 1 week after nerve crush, expressed as a percentage of the total distribution. Peaks from the distribution were weighed and divided by the sum of the peaks to give the relative amounts of AChE. Recoveries were obtained from three different preparations, each containing three muscles.

Fig. 5. Velocity sedimentation gradient separation of AChE molecular forms in the SOL muscle and contralateral control muscle after nerve crush. All gradients contained 3.5 mg protein from the SOL high-speed supernatant. A-2 weeks, B-4 weeks, after nerve crush. Exp. Neurol. 79: 519-531, 1983.

significantly higher than control. In EDL muscle, however, 2 weeks after nerve crush (Fig. 6A), the activity of individual molecular forms of AChE was reduced. In the fourth week, the 4S and 16S molecular forms had recovered (Fig. 6B), whereas the activity of the 10S AChE molecular form remained significantly below control values even at the end of the sixth week.

Fig. 6. Velocity sedimentation gradient separation of AChE molecular forms in the EDL muscle and contralateral control muscle after nerve crush. Gradients for the 2-week preparation contained 5 mg protein, and gradients for the 4- and 6-week preparations contained 3.5 mg protein. A-2 weeks, B-4 weeks, after nerve crush. Exp. Neurol. 79: 519-531, 1983.

As demonstrated in Table 3, two entirely different profiles for the AChE molecular forms were evident in fast and slow twitch muscle after denervation and during reinnervation. The EDL exhibited decreases in all three forms, followed by slow increases. On the other hand, the SOL muscle exhibited selective increases in the AChE activity associated with the 4S and 10S forms and decreases in the 12S and 16S forms. After reinnervation, only a gradual increase was evident in the EDL muscle. However, transient increases to several times the contralateral controls were apparent for all four

TABLE 3. Activities of the Molecular Forms of Acetylcholinesterase Expressed as a Percent of the Contralateral Controls[a]

Time after crush (weeks)	N	4S	10S	12S	16S
		Extensor Digitorum Longus			
1	3	11.0± 2.3	39.5± 9.6	0	29.6±16.3
2	5	29.9±12.2	20.8± 7.9	0	22.0±10.6
3	3	45.6± 6.7	21.6± 2.2	0	30.5± 1.3
4	5	83.1± 5.7	37.9± 9.9	0	177.8±37.3
5	3	33.8± 5.0	49.8±16.8	0	94.1±27.6
6	5	114.2± 8.1	50.1±10.8	0	96.4±10.0
		SOL			
		4S	10S	12S	16S
1	3	140± 30	85± 47	36± 10	28± 18
2	5	222± 47	157± 10	55± 17	81± 32
3	3	687±173	310± 10	248± 24	439± 39
4	5	419±101	257± 57	504±146	165±101
5	3	155± 85	262±158	219± 94	165± 23

[a]Comparisons of the AChE activity from EDL and SOL muscles after denervation for each molecular form. Peaks from the distributions between experimental muscles were compared with the peaks from their contralateral controls, to give the percentage of contralateral controls. Between three to five determinations (N) were made for each postoperative period, and there were three muscles for each group. Values are the mean ± SE. Exp. Neurol. 79: 519-531, 1984.

molecular forms of AChE in the SOL muscle. The presumed endplate form (16S) increased by more than 500% of contralateral control values, and was maximal between 3 and 4 weeks after nerve crush. The remaining AChE molecular forms exhibited a similar transient increase with respect to the contralateral control muscles between the second and fourth weeks after nerve crush.

3. Decreased Loadbearing and Its Effects on AChE and Its Molecular Forms in Slow and Fast Muscle.

Reduced loadbearing causing decreased activation of muscle units was used to examine the role of muscle activity in the regulation of AChE. This was accomplished by suspension of rat hindlimbs off the floor of animal cages for prolonged period (3 weeks). The hindlimbs so treated are not in a position to bear weight. Therefore, patterned motor unit activity producing movement of the limbs may occur, but since there is minimal load on the muscles the level of activity required to produce each movement is decreased.

There was a significant weight loss in both muscles. The weight of the EDL was reduced to 71% and that of the SOL to 40% of control weight. AChE activity in EDL showed no significant reduction when calculated as activity per g muscle or per mg protein. In SOL an increase by 420% and 537%, respectively, was seen when enzyme activity was calculated on the basis of gram per muscle or mg protein (Table 4).

These changes in enzyme activity were also reflected in the molecular forms of AChE. In SOL, hypokinesia increased

TABLE 4. Effect of Hindlimb Suspension Hypokinesia on Acetylcholinesterase Activity in Rat Extensor Digitorum Longus and Soleus Muscle.

	EDL		SOL	
	umol ACh/g/h	nmol ACh/mg protein/h	umol ACh/g/h	nmol ACh/mg protein/h
Control	109.61± 8.77 (100)	533±41 (100)	56.12± 5.91 (100)	295± 75 (100)
1 Week	93.72±11.49 (85)	470±51 (88)	71.10± 5.47 (127)	426± 69 (144)
Control	101.42± 3.89 (100)	474±18 (100)	50.54± 2.40 (100)	243± 10 (100)
2 Weeks	97.83± 3.99 (96)	440±32 (93)	102.60± 6.50[a] (206)	661± 70[a] (272)
Control	94.66± 5.97 (100)	447±35 (100)	48.28± 2.60 (100)	247± 20 (100)
3 Weeks	74.88± 5.13[a] (79)	361±20[a] (81)	126.21±10.54[a] (262)	892±100[a] (361)

Each value is the mean ± SEM of acetylcholinesterase activity, obtained from 5-10 animals, numbers in parenthesis represent percent of controls.

[a]indicate significant difference ($p < 0.05$) compared to control value at each corresponding time interval.

significantly the activity of all four major forms - 16S, 12S, 10S and 4S. In the EDL no significant change ($p < 0.05$) was observed in the 4S and 16S forms while the 10S was slightly increased.

Fig. 7. Velocity sedimentation gradient separation of the acetylcholinesterase (AChE) forms from soleus (SOL) and extensor digitorum longus (EDL) muscles. A. Effects of three weeks of disuse on the SOL Δ——Δ control muscle, o——o hypokinetic muscle. B. Effects of three weeks of disuse on the EDL Δ——Δ control muscle, o——o hypokinetic muscle. Gradients contained two hundred micrograms of a high speed supernatant from the muscles. Arrows indicate position of markers, G = β-galactosidase (16.0S), C = catalase (11.1S) and P = alkaline phosphatase (6.1S).

Discussion

Adult fast EDL and slow SOL muscles have different AChE activities as well as different contributions of molecular

forms to the overall AChE activities. This may be the result of innervation by distinct classes of motor neurons that transmit impulse patterns at different rates. At birth, however, AChE activity as well as the contribution of molecular forms are similar and closely approximate the characteristics of the mature SOL.

During the initial two weeks of postnatal development both muscles continue to increase AChE activity due to an endogenous program that determines synthesis of AChE. After functional innervation is fully established the fast EDL continues to synthesize AChE at the same rate, while the slow SOL synthesizes AChE at a reduced rate. During reinnervation of adult SOL, the early postnatal experience is repeated, i.e. with a reduction of functional input, SOL synthesizes AChE temporarily at a high rate, not unlike that seen during the first weeks of postnatal development (22). If the difference in AChE activity between SOL and EDL is the product of functional innervation, experiments that alter neural input should cause changes. The reduced work load placed on both muscles in the hindlimb suspension model suggests that in the absence of continuous neural stimulation and work the SOL reverts to the characteristics of the early postnatal development when innervation is established, but functional activity is not fully developed and thus synthesis of AChE is increased. AChE activity of the mature EDL muscle appears to be less affected by changes in the functional innervation (22).

Both muscles have the ability to synthesize AChE at a high rate and this may be considered a programmed event in the development of fast and slow muscle. This commitment is probably regulated by motor neuronal input through variation in impulse pattern. Thus, in SOL full functional innervation

by slow motor neurons with a rate of continuous impulse frequency reduces the rate of synthesis. In case of reinnervation and hindlimb suspension the firing patterns are reduced and therefore AChE synthesis is increased. The re-establishment of muscle function appears to down regulate the intrinsic potential to synthesize AChE in slow SOL, while the EDL appears to be less dependent on functional activity.

The muscles in our hypokinesia experiments were no-loadbearing but free to contract, thus there is a need for a loadbearing function to prevent atrophy. The lesser degree of atrophy in the EDL indicates that this muscle is normally not used as an antigravity muscle during locomotion, while the SOL is normally an antigravity muscle.

These data also indicate that AChE of skeletal muscle is strongly dependent on level of loadbearing and patterns of motor unit activity. Since the control enzyme activity of the slow SOL is about half that of the fast EDL, the increase in the SOL AChE with disuse is to a level somewhat greater than the activity of the control EDL. Disuse produced little change in AChE activity in EDL.

The reasons for the SOL showing this more pronounced sensitivity to inactivity are not clear, however its greater dependency on nerve regulated influences has been observed previously. The data suggest that in the absence of normal patterns of weightbearing activity, i.e. no loadbearing and without trauma to the lower motor neuron slow SOL may revert to a faster and perhaps more immature, i.e. neonatal character. The slow SOL muscle is more dependent on activity related mechanisms than the EDL. Further studies involving axoplasmic transport, release of ACh from nerve terminals, changes in contractile characteristics and careful monitoring

of electrical activity during disuse will help to more clearly elucidate the role of the nerve and nerve induced activity in the maintenance of muscle.

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