Acetylcholinesterase mRNA Level and Synaptic Activity in Rat Muscles Depend on Nerve-Induced Pattern of Muscle Activation

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Acetylcholinesterase (AChE) mRNA levels are severalfold higher in fast rat muscles compared with slow. We hypothesized that AChE mRNA levels and AChE activity in the neuromuscular junction depend on a specific nerve-induced pattern of motor unit activation. Chronic low-frequency stimulation, mimicking the activation pattern in slow muscles, was applied to fast muscles in rats. Molecular forms of AChE were analyzed by velocity sedimentation, and AChE mRNA levels were analyzed by Northern blots. AChE mRNA levels in stimulated fast muscles dropped to 10–20% of control after 1 week and became comparable to those in slow soleus muscles. The activity of the junctional A₁₂ AChE form in 35 d stimulated fast muscles decreased to 56% of control value, reaching that in the soleus muscle. Therefore, synaptic AChE itself depends on the muscle activation pattern. Complete inactivity after denervation also

decreased the AChE mRNA level in fast muscles to <10% in 48 hr. In contrast, profuse fibrillations observed in noninnervated immature regenerating muscles maintain AChE mRNA levels at 80% of that in the innervated fast muscles. If protein synthesis was inhibited by cycloheximide, AChE mRNA levels in 3-d-old regenerating muscle, still containing myoblasts, increased approximately twofold. No significant increase after cycloheximide application was observed either in denervated mature fast muscles or in normal slow muscles. Low AChE mRNA levels observed in those muscles are probably not caused by decreased stability of AChE mRNA as demonstrated in myoblasts.

Key words: acetylcholinesterase; denervation; muscle; muscle stimulation; nerve–muscle interaction; nerve impulse pattern; neuromuscular junction; plasticity; regeneration; synapse

Skeletal muscle fibers are able to change their phenotype in response to altered functional demands (Pette and Staron, 1997). Many functional elements in fast muscle fibers can be transformed by chronic low-frequency stimulation, e.g., contractile proteins, the Ca²⁺-handling system, and enzymes of energy metabolism (Pette and Vrbova, 1992). Therefore, fast muscles, stimulated by an impulse pattern similar to that in slow muscles, display several, although not necessarily all, characteristics of a slow soleus muscle (Westgaard and Lomo, 1988; Termin et al., 1989; Schiaffino et al., 1990; Pette and Vrbova, 1994). Acetylcholinesterase (AChE; EC 3.1.1.7) activity in muscle fibers can also be changed by electrostimulation or disuse of muscles, which adds this enzyme to the list of proteins affected by the pattern of muscle activation (Dettbarn et al., 1985; Lomo et al., 1985; Sketelj and Črešnar, 1995; Sketelj et al., 1997).

AChE, which is responsible for fast hydrolysis of acetylcholine in the neuromuscular junction (NMJ) (Marnay and Nachmansohn, 1938; Eccles et al., 1942), is highly concentrated in the junctional basal lamina. It is present in a lower concentration throughout the length of muscle fibers (Sketelj, 1994). AChE displays a rich molecular polymorphism that encompasses globular and asymmetric molecular forms (Massoulié and Bon, 1982).

The asymmetric molecular forms of AChE, bound to the junctional basal lamina, contribute most to the junctional AChE activity in mammalian muscles (Hall, 1973; Fernandez et al., 1984; Sketelj and Brzin, 1985; Inestrosa and Parelman, 1990; Rossi and Rotundo, 1996; Rotundo et al., 1997).

AChE regulation in predominantly fast rat muscles, such as extensor digitorum longus (EDL), is different from that in the slow soleus muscle (Bacou et al., 1982; Groszwald and Dettbarn, 1983; Lai et al., 1986; Sketelj et al., 1992a). Most conspicuously, total AChE activity per unit of muscle weight, as well as activity of the globular molecular forms, is higher in fast than in slow muscles (Groszwald and Dettbarn, 1983; Sketelj et al., 1992b), probably because of the much higher transcript level of the AChE catalytic subunit in fast muscles (Črešnar et al., 1994; Michel et al., 1994).

Some differences in AChE regulation may be attributable to intrinsic properties of the two types of muscles (Sketelj et al., 1991; Dolenc et al., 1994). However, because the patterns of neural activation of motor units in fast and slow rat muscles are very different (Navarrete and Vrbova, 1983; Hennig and Lomo, 1985), we hypothesized that mRNA levels of AChE catalytic subunit and AChE synaptic activity in muscles depend on specific neural activation patterns. Indeed, the AChE mRNA level in fast muscles was found to decrease after 1 week of chronic low-frequency stimulation and became comparable to that in the slow soleus muscle. The activity of the asymmetric AChE forms in the motor endplates of chronically stimulated fast muscles decreased too, revealing a dependence of functional AChE activity in the synapse itself on the muscle activation pattern. Low levels of AChE transcripts found in tonically activated or completely in-

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active muscles are probably not caused by the increased AChE mRNA degradation rate that is characteristic of early stages of muscle development.

MATERIALS AND METHODS

Animals. In accordance with national guidelines, the experiments on animals in Slovenia were approved by the Veterinary Administration of the Ministry for Agriculture, Forestry and Food, permit 326-07-258/95, and in Germany by the Regierungspräsidium Freiburg, authorization Az. 37/9185.815/733.

Experiments were performed on adult male Wistar rats. For surgical procedures, rats were anesthetized by a mixture of Ketalar (Parke-Davis, Vienna, Austria; 60 mg/kg), Rompun (Bayer, Leverkusen, Germany; 8 mg/kg), and atropine (Belupo, Koprivnica, Croatia; 0.6 mg/kg), or by sodium pentobarbital (Werfft-Chemie, Wels, Austria; 55 mg/kg), injected intraperitoneally. The animals were exsanguinated under ether anesthesia.

Low-frequency stimulation of EDL and tibialis anterior (TA) muscles. Stainless steel electrodes were implanted under anesthesia on each side of the peroneal nerve of the left hindlimb (Simoneau and Pette, 1988). Stimulation was performed using a battery-operated, portable stimulator (Tyler and Wright, 1980). Square wave stimuli (10 Hz, single pulse duration 30 msec) were applied continuously for 10 hr per day for either 7, 25, or 35 d. The amplitude of stimulation was adjusted daily so that maximum contraction of the stimulated muscle group could be palpated and stimulation did not cause apparent discomfort to the animals.

Muscle denervation. The sciatic nerve in one leg was exposed and transected in a midhigh region. A piece of the nerve was excised to prevent reinnervation.

Noninnervated regenerating muscle. One leg was denervated as described above, and the EDL muscle was exposed in that leg. The muscle was thereafter injected with the myotoxic local anesthetic Marcaine (0.5% bupivacaine; Astra, Södertälje, Sweden) as described (Hall-Craggs, 1974). The wound was closed, the animals were left to recover, and the muscles were allowed to regenerate for 3 or 8 d.

Cycloheximide treatment. Rats were injected intraperitoneally with 20 mg/kg of cycloheximide in PBS. Four hours later, they were given another 10 mg/kg of cycloheximide. Controls were injected with saline. The animals were killed 8 hr after the first injection, and muscles to be used for further analysis were immediately isolated and frozen.

Velocity sedimentation analysis of AChE. The EDL and soleus muscles of both hindlimbs were isolated (muscles from the nontreated leg served as controls). Regions containing NMJs (junctional regions) were separated from regions without them (extrajunctional regions) (Koenig and Rieger, 1981; Sketelj et al., 1993). The samples were weighed, frozen in liquid nitrogen, and kept at -80°C until they were analyzed. Frozen muscle samples were crushed and pulverized in a mortar chilled in liquid nitrogen and then immediately homogenized. An ice-cold high-salt-detergent medium with a set of protease inhibitors was used to extract AChE, which was then subjected to velocity sedimentation analysis in 5–20% linear sucrose gradients as described (Sketelj et al., 1993). The activity pertaining to an individual molecular form of AChE was calculated by measuring the area under its peak of activity in the velocity sedimentation pattern, using our own computer program (Ribarič et al., 1996).

Northern blot analysis. EDL, TA, diaphragm, or soleus muscles were isolated rapidly under antiseptic conditions and frozen immediately in liquid nitrogen. Frozen muscles were pulverized in a mortar chilled by liquid nitrogen and briefly homogenized in denaturing solution by an Ultra-Turrax homogenizer. Total RNA was isolated by a slightly modified single-step procedure (Chomczynski and Sacchi, 1987), subjected to electrophoresis in 1.2% denaturing formaldehyde–agarose gel (30–50 μg of RNA per lane), and transferred to nylon membranes by capillary blotting (Hybond N, Amersham, Arlington Heights, IL). Specific DNA probe corresponding to a fragment of the cDNA of the rat AChE catalytic subunit (common catalytic domain, nucleotides 920–1683; a gift by Dr. C. Legay and Dr. J. Massoulié, Paris, France) was used for hybridization. Random prime kit components (Boehringer Mannheim, Mannheim, Germany) were used for labeling the probe with $\alpha^{32}\text{P}$ dCTP (DuPont de Nemours, Brussels, Belgium). A specific synthetic oligonucleotide complementary to the 3' end of the probe (Ransom Hill Bioscience, Ramona, CA) was used as the primer instead of random oligonucleotides. The membranes were prehybridized for 1 hr at 42°C in a buffer solution containing $5\times$ SSPE, 50% formamide, $5\times$ Denhardt's

solution, 0.5% SDS, and 20 $\mu\text{g}/\text{ml}$ salmon sperm DNA (Sigma, St. Louis, MO). Hybridization was performed at 42°C overnight in the same solution with the addition of the radioactive probe to $\sim 10^6$ cpm/ml. The blots were washed in $2\times$ SSPE at room temperature, then twice for 15 min in $2\times$ SSPE and 0.1% SDS at 42°C , then for 30 min in $1\times$ SSPE and 0.1% SDS at 42°C , and finally two times for 15 min in $0.1\times$ SSPE and 0.1% SDS at room temperature. The membranes were exposed to x-ray films using intensifying screen for 1–4 d. The AChE probe was then stripped off the membranes, and they were rehybridized by radioactive β -actin probe as described above. The autoradiograms were quantified densitometrically using MCID M4 image analyzer by Imaging Research, and the values for AChE mRNA were normalized in regard to the intensity of the corresponding β -actin signal.

EMG recording. The rats were anesthetized as described and kept on a warm blanket to prevent hypothermia. Skin and muscle fascia on the frontal side of the calf were incised, and the EDL muscle (control, denervated, or regenerating) was carefully exposed. A disposable concentric needle electrode (No. 53155, recording area 0.07 mm^2) was inserted into the muscle at several sites consecutively. Spontaneous electromyographic activity followed and was recorded several times during the next 10–30 min by a Mystro electromyograph (Medelec, Old Woking, UK). The frequency bandwidth used was 0.2–2.0 kHz.

RESULTS

Levels of AChE mRNA and molecular forms of AChE in different rat muscles

Gene transcript levels of AChE catalytic subunit were determined in several rat muscles differing in regard to their speed of contraction, fiber type composition, and expression of myosin heavy chain (MHC) isoforms. AChE mRNA levels were found to be higher in fast muscles such as EDL and TA than in slower ones such as the diaphragm and soleus (Fig. 1B). We used densitometric evaluation and assigned a value of 100 to the mRNA level in the EDL muscle, and the average mRNA levels in the TA were ~ 70 , in the diaphragm ~ 50 , and in the soleus ~ 15 (Fig. 1C). The relative levels of AChE mRNA in the examined muscles were roughly proportional to activities of the monomeric 4 S molecular form of AChE in the extrajunctional regions of these muscles (Fig. 1A,C).

The asymmetric A_{12} molecular forms of AChE are highly concentrated in NMJs of rat muscles and contribute most of the junctional AChE activity (Hall, 1973). We analyzed the patterns of molecular forms of AChE separately in the muscle region containing all of the NMJs (junctional region) and in muscle regions without NMJs (extrajunctional regions). By deducting the specific activity of the A_{12} AChE form in the extrajunctional region from that in the junctional region, the activity of the synaptic A_{12} form proper in the junctional region was calculated, both in the fast EDL muscle and in the slow soleus muscle. The number of muscle fibers (and therefore, of motor endplates) in the two muscles was determined in stained histological cross-sections (EDL, 3120 ± 20 fibers; soleus, 1790 ± 60 fibers; mean $\pm$ SE; $n = 3$), and A_{12} AChE activity per NMJ in the two muscles was calculated. The activity of the A_{12} AChE per endplate in the soleus muscles was $\sim 60\%$ of that in the EDL (4.5 ± 0.5 fmol ACh/min vs 7.3 ± 0.3 fmol ACh/min; mean $\pm$ SD; $n = 4$); the difference was statistically significant ($p < 0.01$).

The effect of chronic low-frequency stimulation on AChE mRNA levels and its synaptic activity in fast muscles

In rats, fast muscles are normally activated by rare, short-lasting high-frequency bursts of nerve impulses (Navarrete and Vrbová, 1983; Hennig and Lomo, 1985). Chronic low-frequency electrical stimulation of fast EDL and TA muscles via their nerves subjected the two muscles to an activation pattern similar to that in

Figure 1. AChE expression in four different rat muscles: soleus (SOL, lane 1), extensor digitorum longus (EDL, lane 2), tibialis anterior (TA, lane 3), and the diaphragm (DIA, lane 4). *A*, Velocity sedimentation patterns of AChE molecular forms, identified by their sedimentation coefficients, in the extrajunctional muscle regions; AChE activity is expressed in arbitrary units per unit of wet weight. *B*, Northern blot analysis of AChE mRNA levels in muscles. β -actin mRNA was used to control uniformity of sample loading. *C*, Relative levels of the 4S AChE form activity (blank bars) and AChE mRNA (hatched bars) in rat muscles (mean $\pm$ SE; the number of determinations is given in parentheses).

the slow soleus muscle. We found that after only 1 week AChE mRNA levels in both stimulated fast muscles fell to low values comparable to those in the soleus muscle (~ 10 – 20% of the AChE mRNA levels in control fast muscles). Low levels of AChE mRNA in chronically stimulated EDL muscle also persisted after 25 d of stimulation (Fig. 2).

In addition, the patterns of AChE molecular forms in the junctional regions of 35 d stimulated and control EDL muscles were analyzed (Fig. 3*A*). Specific activity of the monomeric G_1 form was heavily reduced in the stimulated muscles. Activity of the junctional A_{12} AChE form calculated per NMJ in the stimulated EDL was only $\sim 56\%$ of that in the control EDL muscle ($p < 0.05$) and was about the same as that in the NMJs of normal soleus muscles (Fig. 3*B*).

The effect of muscle inactivity caused by denervation on the levels of AChE mRNA in fast muscles

We focused our attention on an early period after denervation that was characterized by complete muscle inactivity. Northern blot analysis of denervated EDL muscles revealed that mRNA levels of AChE started to decrease during the first 24 hr after denervation (Fig. 4*A,B*). At that time, AChE mRNA levels were already reduced to $\sim 30\%$ of control levels. AChE mRNA levels in the denervated EDL muscles were further reduced to just a small percentage of control levels after 48 hr of inactivity.

AChE mRNA levels also were examined in noninnervated muscles that regenerated after injury. We let muscle regeneration take place in the absence of innervation to be able to compare denervated immature regenerating muscles with denervated ma-

Figure 2. The effect of chronic low-frequency stimulation on AChE mRNA levels in fast muscles tibialis anterior (TA) and extensor digitorum longus (EDL). *A*, Northern blot analysis of control (c) and stimulated (s) muscles, stimulation lasting 7 d (7d) or 25 d (25d). Normal soleus muscle (SOL) is included for comparison; β -actin mRNA was used to control uniformity of sample loading. *B*, Histogram of relative levels of AChE mRNA in control (contr) and stimulated (stim) EDL muscles in comparison to soleus (SOL) muscle (mean $\pm$ SE of several determinations; the number of determinations is given in parentheses).

ture muscles. In contrast to mature 8 d denervated muscles, AChE mRNA levels in noninnervated 8-d-old regenerating fast muscles were fairly high, i.e., $\sim$ 80% of those in control mature innervated muscles (Fig. 4).

Electromyographic recording of spontaneous muscle activity in denervated mature and regenerating muscles was performed to examine the hypothesis that possible differences in spontaneous activity might be responsible for different AChE mRNA levels in mature versus immature denervated muscles (Fig. 5). In denervated mature muscles, we were able to record spontaneous fibrillations at 10 of 18 recording sites in three 8 d denervated EDL muscles, using standard concentric needle electrodes. Action potentials originating from one or a few muscle fibers were observed at recording sites with activity. Individual muscle fibers fibrillated with frequencies ranging from $\sim$ 0.5 to 5 Hz. In the noninnervated

Figure 3. The effect of chronic low-frequency stimulation on the junctional 16 S (A_{12}) AChE form in fast extensor digitorum longus (EDL) muscles. *A*, Velocity sedimentation pattern of AChE molecular forms in the junctional region of control and 35 d stimulated EDL muscles; the peak corresponding to the 16 S form is hatched. AChE activity is expressed in arbitrary units per neuromuscular junction. *B*, Activities of the 16 S AChE form per neuromuscular junction in soleus (SOL) muscles, control EDL muscles (EDL), and 35 d stimulated EDL muscles (EDL stim) (mean $\pm$ SE; the number of determinations is given in parentheses).

regenerating muscles, however, profuse spontaneous fibrillatory activity could always be recorded at each impalement by the same EMG electrode. The number of active fibers within the reach of the electrode was too great to allow more detailed analysis of the firing rate of individual muscle fibers.

The effect of cycloheximide treatment on AChE mRNA levels in very immature regenerating muscle and various mature muscles

Inhibition of protein synthesis in myoblasts *in vitro* by cycloheximide for 6 hr caused a great increase of AChE mRNA levels in these cells, probably because of inhibited synthesis of short-lived proteins responsible for the fast degradation rate of AChE mRNA in myoblasts (Fuentes and Taylor, 1993). We found that the level of AChE mRNA in 3-d-old regenerating EDL muscles was $<50\%$ of that in more mature 8-d-old regenerating muscles (Fig. 6). In accordance with the results obtained *in vitro*, we found an 1.8-fold increase of AChE mRNA levels in early regenerating EDL muscles 8 hr after we treated the rats with a high dose of cycloheximide. It is possible that low AChE mRNA levels observed in either inactive muscles or tonically activated muscles are caused by an accelerated AChE mRNA degradation rate, as

Figure 4. The effect of denervation on AChE mRNA levels in mature and regenerating EDL muscles. *A*, Northern blot analysis of AChE mRNA levels in control EDL (*con*), EDL denervated (*den*) for 24 hr (*1d*), 48 hr (*2d*), or 8 d (*8d*), and in noninnervated 8-d-old regenerating EDL muscle (*reg 8d*). β -Actin mRNA was used to control uniformity of sample loading. *B*, Histogram of relative AChE mRNA levels in control (*contr*) and different kinds of denervated EDL muscles (mean $\pm$ SE; the number of determinations is given in parentheses).

in immature muscle cells. However, in the rats treated with a high dose of cycloheximide, no major increase of AChE mRNA levels could be detected after 8 hr of cycloheximide treatment either in denervated fast muscles or in normal innervated slow soleus muscles (Fig. 6).

DISCUSSION

AChE mRNA levels in rat muscles depend on the muscle activation pattern

AChE expression was examined in four rat muscles displaying different patterns of MHC isoforms: EDL: 60–65% MHCIIb, 25–35% MHCIId/x, 10–12% MHCIIa, 1% MHCI; TA: 60–77% MHCIIb, 19–25% MHCIId/x, 4–14% MHCIIa, 0–4% MHCI; diaphragm: 1% MHCIIb, 53% MHCIId/x, 32% MHCIIa, 14% MHCI; and soleus: 0% MHCIIb, 0% MHCIId/x, 10–20% MHCIIa, 80–90% MHCI (Bär and Pette, 1988; Kirschbaum et al., 1990; Pette and Staron, 1990; Snoj-Cvetko et al., 1996; Sketelj et al., 1997). We found that high cumulative percentages of the fastest MHC isoforms MHCIIb and IId/x and low percentages of the isoform MHCIIa and slow isoform MHCI were associated with high levels of AChE transcripts in a muscle, and vice versa.

Figure 5. Representative examples of electromyographic recordings of spontaneous electrical activity in denervated EDL muscles. *A*, Three muscle fibers firing in the neighborhood of the electrode in an 8 d denervated mature EDL muscle. *B*, Profuse fibrillatory activity in an 8-d-old noninnervated regenerating EDL muscle. *C*, Electrical silence in a normal EDL muscle, showing the level of noise.

Activity of the monomeric 4 S AChE form (G_1), reflecting the concentration of the basic catalytic subunit of AChE, roughly paralleled the levels of AChE mRNA in the examined muscles. Because the most important factor determining the MHC profile of a muscle, although not the only one, is a specific pattern of

Figure 6. The effect of cycloheximide on AChE mRNA levels in different muscles 8 hr after the rats received the drug. *A*, Northern blot analysis of AChE mRNA levels in 3-d-old regenerating EDL muscles (*reg 3d*) in control (*c*) and cycloheximide-treated (*chx*) rats, with an 8-d-old control regenerating muscle (*reg 8d*) shown for comparison. *B*, Northern blot analysis of AChE mRNA levels in normal EDL, 3 d denervated mature EDL (*EDL den*) and normal soleus (*SOL*) muscles from control (*c*) or cycloheximide-treated (*chx*) rats. β -actin mRNA was used for control of uniformity of sample loading. *C*, Relative levels of AChE mRNA in control EDL (*EDL c*), 3-d-old non-innervated regenerating EDL (*EDL reg 3d*), 3 d denervated mature EDL (*EDL den 3d*), and normal soleus (*SOL*) muscles in control (blank bars) or cycloheximide-treated animals (hatched bars) (mean + SE; the number of determinations is given in parentheses).

neural activation of muscle fibers (Pette and Staron, 1997), the above results indirectly support the hypothesis that the AChE mRNA level in muscles also depends on the neural activation pattern. However, although the MHC profiles of EDL and TA muscles are fairly similar, the AChE transcript level and activity of the G_1 AChE form were consistently higher, whereas the activity of the G_4 form was lower in the EDL compared with the TA. The latter observation indicates that this may be attributable to a greater work load imposed on the TA than on the EDL muscle (Fernandez and Hodges-Savola, 1992; Gisiger et al., 1994; Boudreaularviere et al., 1997). Some mechanisms seem to modify AChE expression to a certain extent independently of MHC regulation.

Fast motor units in rat muscles such as EDL are activated phasically, with short high-frequency bursts interspersed with long periods of inactivity (Fischbach and Robbins, 1969; Navarette and Vrbova, 1983; Hennig and Lomo, 1985). We demonstrated that by imposing a chronic low-frequency stimulation, which roughly mimics the tonic activation pattern in slow muscle fibers, on fast muscles such as EDL or TA, high levels of AChE mRNA in these muscles became reduced to a low level characteristic of the soleus muscle after 1 week, and they persisted for at least a few weeks thereafter. Conversely, AChE activity and the

transcript levels in the soleus muscle were reported to increase after its disuse (Dettbarn et al., 1985; Sketelj and Črešnar, 1995), which changed its pattern of activation from tonic to phasic and greatly reduced the aggregate number of nerve impulses per day (Fischbach and Robbins, 1969). Therefore, the AChE mRNA level in a rat muscle depends on specific neural activation patterns: rare phasic bursts of high-frequency activity enhance and continuous tonic low-frequency muscle activation reduces AChE mRNA levels.

Activity of the junctional asymmetric AChE forms is affected by the pattern of muscle activation

We found earlier that activity of the globular molecular forms of AChE in the extrajunctional muscle regions decreased after continuous muscle stimulation (Sketelj et al., 1997). In addition, we show here that activity of the functionally most important part of muscle AChE, i.e., activity of the multimeric asymmetric AChE forms in the neuromuscular junction itself, parallels the available amount of catalytic subunits and the level of AChE mRNA: activity of the synaptic A_{12} AChE form in control EDL was nearly two times higher than in the stimulated EDL or in the normal soleus muscles. Accordingly, transgenically overexpressed AChE in embryonic muscles caused an increase in the size of their

NMJs (Shapira et al., 1994). However, the amount of A₁₂ AChE forms assembled from the catalytic subunits and the noncatalytic tail subunit is also determined by the expression of the collagen-like tail subunit (collagen Q) (Krejci et al., 1991; Duval et al., 1992; Bon and Massoulié, 1997; Krejci et al., 1997). At present, therefore, it is not possible to decide whether changes in expression of either catalytic or tail subunits, or both, are responsible for the observed changes in junctional A₁₂ AChE activity in chronically active muscles. Our data agree well with earlier indirect estimations of junctional AChE activity in slow and fast rat muscles using electrophysiological experiments (Magazanik et al., 1984). Higher junctional AChE activity in fast muscles, as compared with slow ones, may be an advantage when very rapid acetylcholine hydrolysis is required for transmission of nerve impulses at very high frequencies in fast muscles.

The idea that NMJ development is regulated by “trophic” factors released by the nerve ending has gained new support recently (Bowe and Fallon, 1995). Thus, expression of acetylcholine receptor in the subjunctional nuclei might be enhanced by calcitonin gene-related peptide (CGRP) (Fontaine et al., 1986; New and Mudge, 1986) and acetylcholine receptor-inducing activity (ARIA)-neuregulin (Harris et al., 1988; Falls et al., 1993; Chu et al., 1995). AChE transcripts are approximately 10 times more concentrated under the motor endplate than elsewhere in muscle fibers in different vertebrate species (Jasmin et al., 1993; Michel et al., 1994; Legay et al., 1995). Different sensitivity of AChE transcript levels to either denervation or tetrodotoxin paralysis of rat muscles suggested that trophic factors also regulated AChE expression in subjunctional nuclei (Michel et al., 1994). Indeed, CGRP had an innervation-like effect on the junctional G₄ AChE form in rat muscles (Hodges-Savola and Fernandez, 1995). It increased AChE mRNA levels in cultured chick muscles approximately threefold (Choi et al., 1996), but ARIA-neuregulin had no effect (Pun and Tsim, 1995). In addition to this, our results suggest that junctional muscle nuclei, like the extrajunctional ones, still possess the ability to respond to different neural stimulation patterns as far as the regulation of junctional AChE is concerned.

Either muscle inactivity or continuous muscle activation decreases AChE expression in muscles

It has been shown before that the levels of AChE mRNA become very low in 5 to 10 d denervated rat muscles (Črešnar et al., 1994; Michel et al., 1994). In light of the present results, it was not clear whether this was attributable to muscle inactivity or chronic spontaneous low-frequency activity, called fibrillations, which appear in denervated rat muscles ~2–3 d after denervation (Hník and Škorpil, 1962; Salafski et al., 1968). We therefore examined the AChE mRNA level in the denervated EDL muscle before it starts fibrillating. The immediate decrease of AChE mRNA indicates that muscle inactivity itself is primarily responsible. Fibrillatory activity that appears later is insufficient to maintain the AChE mRNA levels, just as it is unable to prevent the extrajunctional appearance of acetylcholine receptors in denervated muscle fibers (Fambrough, 1970; Purves and Sakmann, 1974). Namely, periods of fibrillatory activity in individual denervated rat muscle fibers last on average <1 day and are interspersed with 2- to 3-d-long periods of electromechanical inactivity (Purves and Sakmann, 1974).

However, immature noninnervated rat muscle fibers express fairly high AChE activity either in culture or during regeneration *in vivo* (Sugiyama, 1977; Sketelj et al., 1987). We showed here that

high AChE activity in noninnervated regenerating EDL muscles was caused by fairly high levels of AChE mRNA (~80% of control fast muscles). Because profuse spontaneous fibrillatory activity was recorded by EMG at each impalement of regenerating EDL muscles, it seems that spontaneous fibrillatory activity in noninnervated immature muscle fibers is sufficiently intense to sustain high AChE mRNA levels yet not too persistent to suppress it. Accordingly, a dramatic increase of AChE activity occurs in cultured rat myotubes after the onset of fibrillations but can be prevented if fibrillations are blocked by tetrodotoxin (Brockman et al., 1984; De La Porte et al., 1984; Rubin et al., 1985).

Regarding the mechanisms of AChE mRNA level dependence on muscle activation pattern, it is surprising that both inactivity and continuous muscle activation should have similar suppressive effects on AChE transcript levels. Either a decreased transcription rate or an enhanced mRNA degradation rate might be responsible for these effects. It has been shown that increased AChE expression after fusion of myoblasts into myotubes is attributable to a decrease of the AChE mRNA degradation rate, whereas its transcription rate does not change appreciably (Fuentes and Taylor, 1993). When cultured myoblasts were treated by the protein synthesis inhibitor cycloheximide, a prompt increase of AChE mRNA levels was noted. This “superinduction” was thought to be attributable to the suppressed synthesis of proteins responsible for destabilization of AChE mRNA (Fuentes and Taylor, 1993). We treated the rats with a dose of cycloheximide 10-fold higher than that which produced 80% inhibition of protein synthesis (Dupret et al., 1986). Accordingly, 8 hr later AChE mRNA levels in the early regenerating muscles, still containing myoblasts (Carlson, 1976; Hansen-Smith and Carlson, 1979), became approximately twofold higher than in the untreated controls. However, after we applied the same dose of cycloheximide, we could find no substantial increase of AChE mRNA levels either in normal slow soleus muscles or in denervated fast EDL muscles. This argues against the possibility that either denervated fast muscles or tonically activated muscles display low levels of AChE mRNA because of relapsing to a very immature state in which an increased AChE mRNA degradation rate would reduce its steady-state levels. Accordingly, immature myotubes *in vitro*, although noninnervated, have already achieved AChE mRNA stabilization (Fuentes and Taylor, 1993). These results favor the view that AChE mRNA stabilization is a very early, and possibly irreversible, event during muscle cell differentiation. We propose that at later stages, regulation of AChE mRNA levels by muscle activation pattern is based on regulation of the AChE mRNA transcription rate. Our results support this assumption, although they do not prove it.

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