Neural Regulation of Acetylcholinesterase mRNAs at Mammalian Neuromuscular Synapses

Robin N. Michel, Cathy Q. Vu, Wolfram Tetzlaff, and Bernard J. Jasmin
Department of Physiology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Abstract. We examined the role of innervation on acetylcholinesterase (AChE) gene expression within mammalian skeletal muscle fibers. First, we showed the selective accumulation of AChE mRNAs within the junctional vs extrajunctional sarcoplasm of adult muscle fibers using a quantitative reverse transcription PCR assay and demonstrated by in situ hybridization experiments that AChE transcripts are concentrated immediately beneath the postsynaptic membrane of the neuromuscular junction. Next, we determined the influence of nerve-evoked activity vs putative trophic factors on the synaptic accumulation of AChE mRNA levels in muscle fibers paralyzed by either surgical denervation or selective blockage of nerve action potentials with chronic superfusion of tetrodotoxin. Our results indicated that muscle paralysis leads to a marked decrease in AChE transcripts from the postsynaptic sarcoplasm, yet the extent of this decrease is less pronounced after tetrodotoxin inactivation than after denervation. These results suggest that although nerve-evoked activity per se appears a key regulator of AChE mRNA levels, the integrity of the synaptic structure or the release of putative trophic factors contribute to maintaining the synaptic accumulation of AChE transcripts at adult neuromuscular synapses. Furthermore, the pronounced downregulation of AChE transcripts in paralyzed muscles stands in sharp contrast to the well-documented increase in nicotinic acetylcholine receptor mRNAs under these conditions, and indicates that expression of the genes encoding these two synaptic proteins are subjected to different regulatory mechanisms in adult muscle fibers in vivo.

Expression of several components of the adult neuromuscular synapse is strongly influenced by the presence of the motor nerve, as well as by muscle contractile activity. One dramatic example of such highly refined regulation is that of the nicotinic acetylcholine receptor (AChR). In intact muscle fibers, AChR selectively accumulates at the postsynaptic membrane, where it reaches a density of 10,000–20,000 molecules/μm². Upon surgical denervation, AChRs appear in extrajunctional regions as a result of de novo synthesis (for review see Fambrough, 1979; Salpeter, 1987; Hall and Sanes, 1993). These changes in the spatial distribution of AChR along muscle fibers are accompanied by profound modulations in the expression of genes encoding the various AChR subunits in junctional and extrajunctional regions of skeletal muscle fibers, modulations governed by both nerve-evoked muscle activity and neurochemical factors (Merlie et al., 1984; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Witzemann et al., 1991).

Acetylcholinesterase (AChE) is another major constituent of the neuromuscular synapse responsible for the rapid hydrolysis of acetylcholine released from nerve terminals (Massoulié and Bon, 1982; Brimijoin, 1983; Rotundo, 1987; Taylor, 1991; Massoulié et al., 1993). This enzyme is of particular interest with regard to muscle plasticity since levels of AChE molecular forms are known to be highly sensitive to neural influences. For example, muscle paralysis induced via surgical denervation generally results in the rapid disappearance of the synaptic collagen-like tailed AChE forms (Hall, 1973; Vigny et al., 1976; Fernandez et al., 1979; Bacou et al., 1982; Collins and Younkin, 1982; Lomo et al., 1985). In addition, blockade of action potential propagation with tetrodotoxin (Butler et al., 1978; Cangiano et al., 1980) or of neuromuscular transmission with botulinum toxin A (Stromblad, 1960; Drachman, 1972) lead to pronounced decreases in total AChE activity that include reductions in the amount of the asymmetric form A12 (Skel et al., 1993). Alternatively, enhanced neuromuscular activation causes significant increases in whole muscle AChE activity that are reflected by specific and prominent changes in the levels of the various molecular forms (Fernandez and Donoso, 1988; Jasmin and Gisiger, 1990; Gisiger et al., 1991; Jasmin et al., 1991). Taken together, these studies indicate that regulation of AChE expression in adult skeletal muscle is a multifactorial process that may involve, in addi-

1. Abbreviations used in this paper: AChE, acetylcholinesterase; AChR, acetylcholine receptor; EDL, extensor digitorum longus; PL, plantaris; RT, reverse transcription; SOL, soleus; TTX, tetrodotoxin.

© The Rockefeller University Press, 0021-9525/94/11/1061/9 $2.00
The Journal of Cell Biology, Volume 127, Number 4, November 1994 1061–1069 1061
tion to the pattern of neural activation, nerve-derived trophic substances, as well as passive and active mechanical factors.

In contrast to the recent advances regarding our understanding of the mechanisms involved in AChR expression, knowledge concerning the cellular and molecular basis underlying the neural regulation of AChE molecular forms in muscle is still rudimentary. Within the last few years, however, several laboratories have succeeded in isolating cDNA and genomic clones encoding AChE in a variety of species (Schumacher et al., 1986; Sikorav et al., 1987; Rotundo et al., 1988; Maulet et al., 1990; Rachinsky et al., 1990; Li et al., 1991; Legay et al., 1993a), thus allowing for the study of the cellular and molecular mechanisms involved in the regulation and localization of AChE in muscle fibers.

We have recently initiated a series of experiments to specifically address this issue in vivo (Jasmin et al., 1993). At first, we concentrated on the molecular basis underlying the accumulation of AChE at the avian neuromuscular junction by comparing levels of AChE transcripts in junctional vs extrajunctional regions of skeletal muscle fibers. Using a quantitative reverse transcription PCR (RT-PCR)–based assay, we showed the accumulation of AChE mRNAs within the junctional sarcoplasm. The next step is to investigate the neural factors regulating the selective accumulation of AChE transcripts within the postsynaptic sarcoplasm of the neuromuscular junction.

In the present studies, we have examined this by using two well-characterized models of muscle paralysis, surgical denervation and chronic superfusion of tetrodotoxin (TTX). With denervation the nerve is severed from its target muscle cells, whereas chronic TTX delivery onto the nerve blocks the propagation of action potentials while preserving the integrity of nerve–muscle contacts and maintaining axonal transport (Lavoie et al., 1976). Our results indicate that although nerve-evoked muscle electrical activity per se appears a key regulator of AChE mRNA levels, the integrity of the synaptic structure or the release of putative trophic factors contribute to maintaining the synaptic accumulation of AChE transcripts at adult neuromuscular synapses.

Materials and Methods
Surgery and Muscle Sample Preparation
Female Sprague Dawley rats weighing between 180 and 200 g were randomly assigned to one of three experimental groups: (a) control; (b) denervated (DEN); and (c) TTX-inactivated (TTX). All animal surgery was performed under aseptic conditions with the animals anesthetized with sodium pentobarbital (35 mg/kg i.p.). The left soleus, plantaris, and EDL muscles of control rats were quickly excised and frozen in liquid nitrogen. Left hindlimb muscles of DEN animals were denervated by cutting and removing a 4-mm segment of the sciatic nerve 10 mm distal to the sciatic notch. Using a quanti-

Efficacy of TTX-induced Paralysis
Animals were checked twice daily to ensure the completeness of left hindlimb paralysis using as indicators of recovery: (a) toe spreading in response to hindlimb unweighting; (b) the flexor reflex in response to pinching of the foot pad; and (c) plantar flexion in response to forced dorsiflexion of the ankle. None of the animals included in this study demonstrated any of these responses at any time. In a parallel study (Jasmin et al., 1994b), the efficacy of the TTX block was verified before removal of the hindlimb muscles by stimulating the sciatic nerve proximal to the sciatic cuff with a bipolar platinum electrode at supra-maximal voltages and monitoring the functional response of the exposed triceps surae musculature. This procedure never elicited a contractile response from TTX-inactivated muscles. At the time of muscle excision, the sciatic cuff was removed, and the integrity of the sciatic nerve was assessed. Signs of swelling and connective tissue proliferation near the sciatic nerve were observed. Firstly, the average body weight loss caused by TTX inactivation was identical to that induced by cutting the sciatic nerve in all muscles studied indicating that the entire muscle fibre population had been successfully inactivated.

In separate experiments, the possibility that partial or total denervation may have occurred as a result of the sciatic cuff was also examined. This was done using both histological and functional criteria. First, by combining acetylcholinesterase histochemical stain with silver staining of nerve terminals (Alderson et al., 1989), we observed that the number of postsynaptic membranes contacted by a single mature myelinated nerve terminal in TTX-inactivated muscles was identical to unoperated control muscles (Boudreau, C., and R. N. Michel, manuscript in preparation). Second, comparison of muscle maximal force production between stimulation of the sciatic nerve distal to the sciatic cuff with direct stimulation of the muscle never differed by >5% (see also Michel and Gardiner, 1990; and Michel et al., 1994).

Microdissection of Small Bundles of EDL Muscle Fibers
In another series of experiments, microdissected muscle samples consisting of junctional and extrajunctional regions were examined (see Jasmin et al., 1993). For these studies, the EDL muscle was excised and pinned at resting length on a paraffin bed. To visualize neuromuscular junctions, muscles were then injected with and immersed in an AChE histochemical buffer solution (Karnowsky and Roots, 1964). After 1 h of staining, small bundles containing 10–15 muscle fibers were microdissected under a stereomicroscope. This involved separating, but not detaching, the bundle from the muscle by gently teasing off the connective tissue. The region containing the neuromuscular junctions was then cut from the bundle along with an adjacent extrajunctional segment of similar size. Total RNA from each sample was then immediately extracted (see below). Time for dissection was limited to 1 h to minimize possible RNA degradation. We determined that RNA degradation was negligible under these time and dissection conditions (see also Jasmin et al., 1993). In our experiments, this was done by comparing levels of AChE and dystrophin transcripts in EDL muscles homogenized immediately after excision to those observed in EDL muscles incubated for 2 h in the Karnowsky and Roots buffer-substrate solution. Under these conditions, there was no detectable decline in the mRNA levels of AChE and dystrophin.

Extraction of RNA and Quantitative RT-PCR
Total RNA from whole muscles was extracted using the acid guanidinium phenol chloroform procedure previously described by Chomczynski and Sacchi (1987). Whole muscles were placed in an appropriate volume (10 /g tissue) of denaturing solution D and homogenized using a Polytron (Kinematica, Littau, Switzerland) set at maximum speed for 2 x 15 s. Total RNA from each sample was then immediately extracted (see below). The reverse transcription mixture contained 5 mM MgCl2, 1x PCR buffer II (50 mM KCl, 10 mM Tris- HCl [pH 8.3], 1 mM dNTPs, 20 U RNase inhibitor, 50 U Moloney murine leukemia virus reverse transcriptase, and 2.5 mM random hexamer primers (GeneAmp RNA PCR kit; Perkin-Elmer Cetus Instruments, Norwalk, CT). For microdis-

The Journal of Cell Biology, Volume 127, 1994 1062
negative control reactions were used in which RNA was replaced by RNase-free water. Reverse transcription was performed for 45 min at 42°C, and the reaction was terminated by heating 99°C for 5 min.

To amplify AChE cDNAs, specific synthetic primers based on the rat AChE cDNA sequence were used (Legay et al., 1993b). These 5' (CTGGGTCGCAGGATCCTG) and 3' (TCAAGGCTGACGGCCG) primers were located in exons 2 and 6 of the AChE consensus gene map, respectively. The expected PCR product using 5' (TAAATTAGGAGGAACGGACCTCG) and 3' (GCCGCTACCTCTCTTGGAGAAA) primers for dystrophin was amplified by adding 5 µl of reverse transcription mixture to 20 µl of a solution containing 0.625 U AmpliTaq DNA polymerase, 0.25 µg each of the appropriate 5' and 3' primers, and MgCl2 and PCR buffer II (final concentrations of 2 mM and 1x, respectively). Each PCR reaction mixture was overlaid with mineral oil before the tubes were placed in a DNA thermal cycler (Perkin-Elmer Corp.). For both AChE and dystrophin, each cycle consisted of denaturation at 94°C for 1 rain, and primer annealing and extension at 70°C for 3 min. A final 10-min elongation step at 72°C was added after the last cycle. PCR products were kept at 4°C until gel electrophoresis. Typically, 34 and 42 cycles of amplification were performed for whole muscle and microdissected samples, respectively, since preliminary data showed that these cycle numbers were within the linear range of amplification. Ten µl of the PCR products were visualized on either a 1 or 1.5% agarose gel containing ethidium bromide. The 100-bp ladder (Gibco BRL, Gaithersburg, MD) DNA molecular weight marker was used to estimate the size of the PCR products. Quantitative PCR experiments under noncompetitive conditions were performed since, in the present studies, we were primarily interested in comparing the relative abundance of AChE mRNAs in different muscles subjected to various experimental conditions. These experiments were performed as described in detail in Jasmin et al. (1993). Counts per minute obtained for AChE PCR products from junctional and extrajunctional regions were adjusted according to the levels of dystrophin present in the same RT samples. Thus, for comparison of the relative abundance of AChE transcripts in junctional vs extrajunctional regions of EDL and soleus muscles, microdissected bundles of fibers containing 10-15 neuromuscular junctions were carefully teased from the muscle, and cut into junctional and extrajunctional regions of similar sizes. Total RNA was then extracted from each sample, reverse transcribed using random hexamers, and the resulting AChE and dystrophin cDNAs were amplified. An example of an ethidium bromide-stained gel showing AChE and dystrophin PCR products in junctional vs extrajunctional regions of small bundles of microdissected fibers is shown in Fig. 1. As illustrated, AChE mRNA levels were considerably higher in junctional regions as compared to extrajunctional regions, while dystrophin transcripts were relatively equal in both samples (n = 9 different EDL muscles representing a total of 53 junctional vs extrajunctional comparisons). Quantitative analysis revealed that, on average, AChE mRNA levels were ~5- to 14-fold higher in junctional vs extrajunctional regions of EDL muscle fibers (n = 6 EDL muscles), which values correspond closely to our earlier findings in avian muscle (Jasmin et al., 1993).

To complement our quantitative RT-PCR experiments and to ascertain that the accumulation of AChE mRNAs was located immediately beneath the postsynaptic membrane of rat neuromuscular synapses, we performed a series of in situ hybridization experiments on EDL and soleus muscles. Longitudinal cryostat sections obtained from these two muscles were incubated with a synthetic 35S-labeled oligonucleotide (50 mer) complementary to the common coding region of AChE mRNAs. The localization of AChE transcripts in these tissue sections was compared to either its respective serial cryostat section histochemically stained to visualize neu-
Figure 1. Comparison of AChE mRNA levels between junctional and extrajunctional regions of skeletal muscle fibers. Small muscle fiber bundles each containing between 10-15 individual fibers were isolated from the EDL muscle. From each fiber bundle, junctional segments and their respective adjacent extrajunctional regions were microdissected. RT-PCR products corresponding to AChE and dystrophin (DYS) mRNAs within these specific muscle compartments were visualized on an ethidium bromide-stained agarose gel. Junctional and extrajunctional levels of AChE (lanes 1 and 2, respectively) and dystrophin (lanes 3 and 4, respectively) transcripts are shown. Note the greater accumulation of AChE mRNAs in junctional vs extrajunctional regions compared to dystrophin mRNAs, which were similar in these two compartments.

Figure 2. Subcellular localization of AChE mRNA in individual EDL (A and B) and soleus (C and D) muscle fibers. (A) A representative bright-field photomicrograph of a longitudinal cryostat section of EDL muscle fibers stained for the histochemical demonstration of AChE to localize neuromuscular junctions (Karnovsky and Roots, 1964). (B) A dark-field photomicrograph of its corresponding serial section processed for in situ hybridization using a synthetic probe complementary to AChE transcripts. Comparison of these two panels highlights the codistribution of neuromuscular junctions and silver grains corresponding to the expression of AChE mRNAs (curved arrows point to some examples). This indicates that the selective accumulation of AChE transcripts within the junctional region of individual skeletal muscle fibers is confined to the area of the sarcoplasm immediately beneath the postsynaptic membrane. Also, note the presence of a neuromuscular junction without a corresponding accumulation of AChE mRNAs (A and B), open arrowhead. Bar, 60 μm. (C and D) Representative bright-field and dark-field photomicrographs of a single longitudinal cryostat section from a soleus muscle processed first for AChE histochemistry and subsequently for in situ hybridization to localize AChE transcripts. Note the colocalization of neuromuscular junctions and AChE mRNAs although the accumulation of AChE transcripts appears less striking than for EDL muscle. Bar, 120 μm.
onto the sciatic nerve. This approach allowed us to examine the roles of nerve-induced electrical activity vs putative nerve-derived trophic factors in the regulation and accumulation of AChE transcripts within the postsynaptic membrane domain. The efficacy of the TTX treatment was verified using several functional and morphological criteria (see Materials and Methods). The fact that the mass of EDL, plantaris, and soleus muscles that were inactivated by either denervation or TTX paralysis was reduced to a similar extent after 10 d of inactivity indicates that all fibers of TTX paralyzed muscles had been completely inactivated (Fig. 4).

Although the loss in mass was similar after paralysis, denervation and chronic TTX superfusion affected total AChE enzyme activity to different extents. 10 d after denervation, AChE activity was downregulated considerably in all three muscles studied with the effect being more pronounced in EDL muscles (Fig. 5). The effect of denervation was indeed severe in this latter muscle since denervated EDL muscles had only ~9% of AChE activity remaining as compared to control muscles. Chronic superfusion of TTX onto the sciatic nerve also resulted in decreases in total AChE enzyme activity (Fig. 5). However, in contrast to what was observed with muscle mass, the loss in AChE activity was attenuated after TTX inactivation. For instance, in EDL muscles inactivated for 10 d by TTX, AChE activity was reduced by 75% compared to the 90% seen with denervation. The differential impact of denervation and TTX inactivation on AChE activity was also observed for plantaris and soleus muscles.

Similar to the effects of paralysis on AChE enzyme activity, transcript levels were markedly affected by the period of inactivity. Interestingly, the effects of both inactivity models paralleled the downregulation of enzyme levels since AChE transcript levels in all three muscles studied decreased to a greater extent after denervation than after TTX treatment. For instance, AChE transcripts levels in EDL muscles were decreased by more than 10-fold after denervation, but were much less reduced in TTX-paralyzed muscles (Fig. 6). This was also the case for plantaris and soleus muscles. The pronounced decrease in AChE transcript levels after either denervation or TTX treatment was confirmed with a series
denervated counterparts. Similar results were obtained for SOL and linear over two orders of magnitude of RNA input, the highest RNA primers incorporated. Since AChE PCR product amplification was of AChE PCR products for control

Figure 6. Effect of denervation and TTX-induced paralysis on AChE transcript levels. (A) Ethidium bromide–stained agarose gels of AChE PCR products for control (CON), denervated (D), and TTX-treated (T) EDL muscles. The negative control lane is marked with a −. Serial dilutions of RNA extracts from CON muscles are shown (from left to right: 1/10, 1/100, 1/1000, and 1/10000), and the linear phase plotted in B is based on the amount of 32P-labeled primers incorporated. Since AChE PCR product amplification was linear over two orders of magnitude of RNA input, the highest RNA concentration (1/10 dilution) was used to compare PCR products from D and T muscles. Note attenuated loss in AChE transcripts from control values in TTX-inactivated muscles compared to denervated counterparts. Similar results were obtained for SOL and PL muscles (not shown).

of in situ hybridization experiments and, accordingly, accumulations of AChE transcripts within the postsynaptic sarcoplasm of inactivated EDL and soleus muscle fibers could no longer be observed after the period of inactivity. Yet, neuromuscular junctions were still easily detected by AChE histochemical staining (Fig. 7).

Discussion

In the present studies, we have examined the effects of two well-characterized models of hindlimb muscle disuse, surgical denervation, and chronic superfusion of TTX onto the sciatic nerve, on the selective accumulation of AChE transcripts within the postsynaptic sarcoplasm of rat neuromuscular synapses. With denervation, action potential propagation and axonal transport are eliminated, whereas chronic TTX delivery onto the nerve blocks the propagation of action potentials while preserving the integrity of nerve–muscle contacts and maintaining axonal transport. This approach, therefore, allowed us to examine the influence of nerve-induced electrical activity vs putative trophic factors constitutively expressed (activity-independent) in the regulation and selective accumulation of AChE transcripts within the postsynaptic membrane domain.

Neural Regulation of AChE mRNA at the Mammalian Neuromuscular Synapse

Our recent studies have indicated that AChE transcripts are accumulated within the junctional region of avian neuromuscular synapses (Jasmin et al., 1993). Our current results confirm and extend these findings. Quantitative RT-PCR results obtained from microdissected bundles showed an accumulation of AChE transcripts within the junctional region of mammalian skeletal muscle fibers. In addition, in situ hybridization experiments revealed that this accumulation of AChE mRNA was confined to the area of the sarcoplasm that lies immediately beneath the postsynaptic membrane at the level of the "fundamental" myonuclei. The mechanisms responsible for the preferential accumulation of AChE mRNAs have not yet been elucidated, but selective expression of the AChE gene by the junctional myonuclei may itself explain the observed compartmentalization. This proposed mechanism fits well with a model where genes encoding postsynaptic proteins are transcribed strictly by the end plate myonuclei and locally translated (Rotundo, 1990) such that the nascent peptide chains are processed within the specialized postsynaptic Golgi apparatus (Jasmin et al., 1989, 1994a) and are focally transported to the postsynaptic membrane by way of a microtubule-based transport mechanism (Jasmin et al., 1990; Rossi and Rotundo, 1992). However, other mechanisms may also account for the selective accumulation of AChE mRNA within the postsynaptic sarcoplasm. In particular, transcription of the AChE gene may occur in all nuclei throughout the muscle fiber with transport of the AChE mRNA to the postsynaptic membrane domain or, alternatively, transcription of the AChE gene may involve all nuclei of the muscle fiber with enhanced stabilization of the mRNA within the postsynaptic sarcoplasm.

The marked decrease in AChE enzyme activity that we observed after denervation corresponds well with other published reports (Hall, 1973; Vigny et al., 1976; Fernandez et al., 1979; Bacou et al., 1982; Collins and Younkin, 1982; Lomo et al., 1985), as does the attenuated loss in AChE enzyme activity seen after blockade of action potential propagation with TTX (Butler et al., 1978; Cangiano et al., 1980). Interestingly, these changes in enzyme activity were paralleled by similar decreases in AChE transcript levels that were clearly more evident in denervated muscles in comparison to TTX-inactivated counterparts, regardless of the hindlimb musculature involved. These decreases in AChE transcript levels led to an essentially complete disappearance of the selective accumulation of AChE mRNAs within the postsynaptic sarcoplasm, as disclosed by our in situ hybridization experiments. Taken together, these findings indicate that although nerve-evoked activity per se appears a key regulator of both AChE enzyme and mRNA levels in vivo, other factors such as the integrity of the synaptic structure and the transport of molecules down the axons are capable of maintaining AChE enzyme and transcript levels close to normal. One implication is that release of nerve-derived trophic factors may exert a significant influence on expression of AChE in skeletal muscle fibers. This proposal has received par-
Regulation of AChE Expression with Respect to AChR

During the early stages of myogenesis, expression of several muscle-specific proteins is greatly enhanced. In particular, levels of both AChE and AChR increase sharply during the transitional phase from myoblasts to myotubes. As a result of exploratory motor axons reaching the surface of the muscle fibers, these two proteins eventually become concentrated within the synaptic region of muscle fibers. The apparent coregulation of AChE and AChR expression is not surprising since the assembly and maintenance of the postsynaptic membrane domain of the neuromuscular synapse require that expression of all molecular components be temporally and spatially coordinated. This concept of coregulation of synaptic proteins has, in fact, been put forward previously (Merlie and Sanes, 1986; Klarsfeld, 1987). One prediction based on this model is that expression of genes encoding synaptic proteins is coregulated. Results obtained in several laboratories have revealed a differential accumulation of mRNAs encoding the various AChR subunits in the junctional sarcoplasm (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990) resulting from compartmentalized gene transcription at the level of junctional myonuclei (Klarsfeld et al., 1991; Sanes et al., 1992; Simon et al., 1992; Ducler et al., 1993). Taken together with our findings that AChE transcripts are also found accumulated within the postsynaptic sarcoplasm (Jasmin et al., 1993; this work), these results suggest that expression of AChE and AChR genes is spatially coregulated along the length of muscle fibers. Our results from paralyzed muscles allowed us to address the issue of whether expression of these genes is also temporally coregulated.

It is well established that upon denervation and TTX-induced paralysis of adult skeletal muscle, transcripts encoding the subunits of AChR increase significantly beyond levels normally observed in innervated muscles (Merlie et al., 1984; Fontaine et al., 1988; Goldman and Staple, 1989; Witzemann et al., 1991). This elevation of mRNA levels possibly reflects transcriptional activation of AChR genes in nuclei located in the extrasynaptic sarcoplasm (Fontaine and Changeux, 1989; Tsay and Schmidt, 1989). Our observation that the levels of AChE mRNA are markedly reduced in paralyzed muscles stands in sharp contrast to the findings on the expression of AChR. It strongly indicates that under these conditions, expression of these transcripts encoding synaptic proteins is independently controlled. Since we did not measure transcriptional rates of the AChE gene after paralysis, our result may be explained in two ways. First, transcription of the AChE gene may be severely depressed in paralyzed muscles and, therefore, decreased AChE mRNA levels may result from a mechanism involving transcriptional control. Alternatively, the possibility that transcription of the AChE gene is either unaffected or even enhanced with a concomitant increase in the rate of mRNA degradation also exists. Although we cannot distinguished between these two possibilities at the present time, it is clear that either transcriptional or posttranscriptional differences exist between the regulatory mechanisms involved in the expression of AChE and AChR mRNAs in adult skeletal muscle fibers.

During differentiation and fusion of C2-C12 cells in culture, levels of both AChE and AChR increase along with their respective mRNAs (Fuentes and Taylor, 1993). In contrast to the transcriptional activation of the genes encoding the AChR subunits, the increase in the abundance of AChE
mRNA appears to involve stabilization of the transcripts, suggesting that expression of these genes is independently regulated during myogenesis (Fuentes and Taylor, 1993). However, it still remains to be determined whether expression of these genes is also subjected to distinct regulatory mechanisms in vivo. This appears particularly essential in light of the recent evidence indicating that the mechanisms regulating gene expression in vivo vs in vitro may differ (see for example Duclert et al., 1993). Thus, monitoring transcript levels during embryogenesis may provide another opportunity to test whether expression of the AChE gene is coregulated with that of the AChR subunit genes. For example, if the downregulation of one or more of the AChR subunit genes (Witzemann et al., 1989) is paralleled by a decline in AChE transcript levels, it would provide a first indication that expression of some of these genes may be coregulated. A further indication of coregulation would be provided by a similar and concomitant pattern of progressive resection of these mRNAs as the neuromuscular junction develops and is stabilized on the muscle surface. The implication in this case is that during myogenesis and synaptogenesis, expression of these genes is under the control of common signalling systems. If this hypothesis is confirmed, then a picture could emerge where expression of these genes is coregulated during development of muscle fibers and assembly of postsynaptic membrane domains, but is independently regulated in the adult state as demonstrated in the present work.

This work was supported by grants from the Medical Research Council (MRC) and Muscular Dystrophy Association of Canada to B. J. Jasmin. These experiments were performed in the laboratory of B. J. Jasmin while R. N. Michel was on sabatical leave from Laurentian University (Sudbury, Ontario, Canada). B. J. Jasmin is a Scholar from the MRC.

Received for publication 20 June 1994 and in revised form 5 August 1994.

References

Ahn, A. H., and L. M. Kunkel. 1993. The structural and functional diversity of dystrophin. Nature Genet. 3:283–291.

Alderson, K., A. Peastronk, W. Yee, and D. Drachman. 1989. Silver cholinesterase immunoprecipitation: a new neuromuscular junction stain. Muscle & Nerve. 12:9–14.

Bacu, F., P. Vigeron, and J. Massoulie. 1982. Acetylcholinesterase forms in fast and slow rabbit muscle. Nature (Lond.). 296:661–664.

Bendayan, B. R., V. Witzemann, and B. Sakmann. 1990. Imprinting of acetylcholine receptor α-subunit transcripts during myogenesis and motor endplate development in chick. J. Cell Biol. 108:1025–1037.

Fontaine, B., and J.-P. Changeux. 1989. Localization of nicotinic acetylcholine receptor α-subunit transcripts during myogenesis and motor endplate development in chick. J. Cell Biol. 108:1025–1037.

Gisiger, V., and H. R. Stephens. 1988. Localization of the pool of G4 acetylcholinesterase characterized fast muscles and its alteration in murine muscular dystrophy. J. Neurosci. Res. 19:62–78.

Gisiger, V., B. J. Jasmin, S. Sherker, and P. F. Gardiner. 1991. The pool of G4 acetylcholinesterase characterizing rodent fast muscles is differentially regulated by the predominant type, dynamic or tonic, of natural activity. In Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology. P. Massoulié, F. Bacou, E. A. Barnard, A. Chatenet, B. P. Doctor, and D. M. Quinn, editors. American Chemical Society, Washington, DC. pp. 81–85.

Goldman, D., and J. Staple. 1989. Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. Neurotron. 3:219–228.

Hill, Z. W. 1973. Multiple forms of acetylcholinesterase and their distribution in endplate and nonendplate regions of rat diaphragm muscle. J. Neurobiol. 4:343–361.

Jasmin, B. J., J. Cartaud, M. Bornens, and J.-P. Changeux. 1989. Golgi apparatus in chick skeletal muscle: changes in its distribution during endplate development after denervation. Proc. Nat. Acad. Sci. USA. 87:7218–7222.

Jasmin, B. J., J.-P. Changeux, and J. Cartaud. 1990. Compartmentalization of cold-stable and acetylated microtubules in the subsynaptic domain of chick skeletal muscle fibers. Nature (Lond.). 344:673–675.

Jasmin, B. J., and V. Gisiger. 1990. Regulation by exercise of the pool of G4 acetylcholinesterase characterizing fast muscles: opposite effect of running training in antagonist muscles. Neurosci. 10:1444–1454.

Jasmin, B. J., P. P. Gardner, and V. Gisiger. 1991. Muscle acetylcholinesterase adapts to compensatory overload by a general increase in its molecular forms. J. Appl. Physiol. 70:2485–2489.

Jasmin, B. J., R. K. Lee, and J.-P. Changeux. 1993. Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neurotron. 11:467–477.

Jasmin, B. J., C. Antony, J.-P. Changeux, and J. Cartaud. 1994a. Plasticity of the Golgi complex in skeletal muscle fiber: compartmentalization within the subsarcolemmal sarcoplasm. Eur. J. Neurosci. In press.

Jasmin, B. J., R. J. Campbell, and R. N. Michel. 1994b. Nerve-dependent regulation of succinate dehydrogenase in transgenic mice. Mol. Gen. Genet. 245:499–511.

Karnovsky, M. J., and L. Roots. 1964. A "direct coloring" thiocicholine method for cholinesterase. J. Histochem. Cytochem. 12:219–221.

Katz, B., and R. Millet. 1969. Tetrodotoxin and neuromuscular transmission. Proc. Royal Soc. Lond. 167:9–21.

Klarsfeld, A. 1987. Coordinate control of synaptic protein expression at the neuromuscular junction. Biochimie. 69:433–437.

Klarsfeld, A., J. L. Besseur, A. M. Salmon, A. Triller, C. Babinet, and J.-P. Changeux. 1991. An acetylcholine receptor α-subunit promoter conferring preferential synaptic expression in muscle of transgenic mice. EMBO (Eur. Mol. Biol. Organ.) J. 10:625–632.

Legay, C., S. Bon, P. Vernier, F. Coussen, and J. Massoulié. 1993a. Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a Torpedo collagenic subunit. J. Neurobiol. 26:349–350.

Legay, C., S. Bon, and J. Massoulié. 1993b. Expression of a cDNA encoding...
the glycolipid-anchored form of rat acetylcholinesterase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 315:163-166.

Li, Y., S. Camp, T. L. Rachinsky, D. Getman, and P. Taylor. 1991. Gene structure of mammalian acetylcholinesterase: alternative exons dictate tissue-specific expression. *J. Biol. Chem.* 266:23083-23090.

Lomo, T., J. Massoulie, and M. Vigony. 1985. Stimulation of denervated rat soleus muscle with fast and slow activity patterns induces different expression of acetylcholinesterase molecular forms. *J. Neurosci.* 5:1180-1187.

Massoulie, J., and S. Bon. 1982. The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5:57-106.

Massoulie, J., L. Pezzennenti, S. Bon, E. Krejci, and F. M. Vallette. 1993. Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* 13:31-91.

Matsumura, K., and K. P. Campbell. 1994. Dystrophin-glycoprotein complex: its role in the molecular pathogenesis of muscular dystrophies. *Muscle & Nerve.* 17:2-15.

Maulet, Y., S. Camp, G. Gibney, T. Rachinsky, T. J. Ekstrom, and P. Taylor. 1990. Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells. *J. Cell Biol.* 99:332-335.

Merlie, J. P. 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. *Nature (Lond.)*. 317:66-68.

Merlie, J. P., and J. R. Sanes. 1986. Regulation of synapse-specific genes. *In Molecular Aspects of Neurobiology.* R. Levi Montalcini, editor. Springer-Verlag, Heidelberg. pp. 75-80.

Michel, R. N., and P. F. Gardiner. 1990. To what extent is hindlimb suspension a model of disuse? *Muscle & Nerve* 13:646-653.

Michel, R. N., G. Cowper, M. M.-Y. Chi, J. K. Manchester, H. Falter, and R. N. Michel et al. 1990. The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5:57-106.

Michel, R. N., and P. F. Gardiner. 1990. To what extent is hindlimb suspension a model of disuse? *Muscle & Nerve* 13:646-653.

Michel, R. N., G. Cowper, M. M.-Y. Chi, J. K. Manchester, H. Falter, and R. O. Lowry. 1994. Effects of tetrodotoxin-induced neural inactivation on single muscle fiber metabolic enzymes. *Am. J. Physiol.* (Cell Physiol.) 267:C55-C66.

Pestronk, A., D. B. Drachman, and J. W. Griffin. 1976. Effect of muscle disuse on acetylcholinesterase: appearance during embryogenesis and reinnervation of rat muscle. *J. Neurochem.* 26:154-160.

Rotundo, R. L. 1987. Biogenesis and regulation of acetylcholinesterase in multinucleated muscle cells. *J. Cell Biol.* 110:715-719.

Salpeter, M. M. 1987. Development and neural control of neuromuscular junctions and of junctional acetylcholine receptors. *In The Vertebrate Neuromuscular Junction.* M. M. Salpeter, editor. Alan R. Liss Inc., New York. pp. 1-54.

Sanes, J. R., Y. R. Johnson, P. T. Kotzbauer, J. Mudd, T. Hanley, J. C. Martino, and J. P. Merlie. 1992. Selective expression of an ACh receptor-LacZ transgene in synaptic nuclei of adult mouse muscle fibers. *Development (Cambr.)*. 113:1181-1191.

Schalling, M., A. Dagerlin, S. Brené, H. Hallman, M. Djurfeldt, H. Persson, L. Terenius, M. Goldstein, D. Schesinger, and T. Hokfelt. 1988. Coexistence and gene expression of phenyl ethanolamine N-methyltransferase, tyrosine hydroxylase, and neuropeptide tyrosine in the rat and bovine adrenal gland: effects of reserpine. *Proc. Natl. Acad. Sci. USA.* 85:8306-8310.

Schumacher, M. S., S. Camp, Y. Maulet, M. Newton, K. Macphee-Quigley, S. S. Taylor, T. Friedman, and P. Taylor. 1986. Primary structure of *Torpedo californica* acetylcholinesterase deduced from a cDNA sequence. *Nature (Lond.)*. 319:407-409.

Sikorav, J. L., E. Krejci, and J. Massoulie. 1987. cDNA sequences of Torpedo marmorata acetylcholinesterase: primary structure of the precursor of a catalytic subunit: existence of multiple 5' untranslated regions. *EMBO (Eur. Mol. Biol. Organ.)* 6:1865-1873.

Skeijel, J., N. Cre-Finderle, D. Sket, W.-D. Dettbarn, and M. Brzin. 1993. Comparison between the effects of botulinum toxin-induced paralysis and denervation on molecular forms of acetylcholinesterase in muscles. *J. Neurochem.* 61:501-508.

Simon, A. M., P. Hoppe, and S. J. Burden. 1992. Spatial restriction of AChR gene expression to subsynaptic nuclei. *Development (Cambr.)*. 114:545-553.

Stromblad, B. C. R. 1960. Cholinesterase activity in skeletal muscle after botulinum toxin. *Experientia (Basel).* 16:458-459.

Tanaka, H. and E. Ozawa. 1990. Expression of dystrophin mRNA and the protein in the developing rat heart. *Biochem. Biophys. Res. Commun.* 172:825-829.

Taylor, P. 1991. The cholinesterases. *J. Biol. Chem.* 266:4025-4028.

Tsay, H. J., and J. Schmidt. 1989. Skeletal muscle denervation activates acetylcholine receptor genes. *J. Cell Biol.* 108:1523-1526.

Vigny, M., J. Koenig, and F. Rieger. 1976. The motor endplate specific form of acetylcholinesterase: appearance during embryogenesis and reinnervation of rat muscle. *J. Neurochem.* 27:1347-1353.

Witzemann, V., B. Barg, M. Criado, E. Stein, and B. Sakmann. 1989. Developmental regulation of five subunit mRNAs encoding acetylcholine receptor subtypes in rat muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 242:419-424.

Witzemann, V., H. R. Brenner, and B. Sakmann. 1991. Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.* 114:125-141.

Younkin, S. G., L. H. Younkin. 1988. Trophic regulation of skeletal muscle acetylcholinesterase. *In Nerve-Muscle Cell Communication.* H. L. Fernandez, editor. CRC Press, Boca Raton, FL. pp. 41-59.