Biochemical and Biophysical Research Communications **260,** 251–255 (1999)
Article ID bbrc.1999.0893, available online at http://www.idealibrary.com on IDEAL®

Denervation of Chicken Skeletal Muscle Causes an Increase in Acetylcholinesterase mRNA Synthesis

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Received May 24, 1999

We have examined the changes in enzymatic activity and the levels of transcripts for AChE following denervation of chicken skeletal muscle. Quantitation of RNA blots indicates that AChE transcripts are increased following denervation. AChE transcripts increased ~17-fold in the fast-twitch posterior latissimus dorsi muscle and ~4-fold in the tonic anterior latissimus dorsi muscle 10 days after denervation of adult chickens. Both AChE transcript levels and enzyme activity increased in parallel for the two muscles. AChE transcripts also increased ~4-fold in the shank muscles of 2-day-old chicks following denervation. Transcript synthesis, measured by run-on transcription, increased ~3-fold in these denervated muscles. These results suggest that the increase in AChE transcripts following denervation in the chicken is due, at least in part, to an increase in the rate of its synthesis.

Key Words: **acetylcholinesterase; denervation; run-on transcription; mRNA.**

The expression of genes in muscle encoding synaptic proteins such acetylcholinesterase (AChE, EC 3.1.1.7) are controlled in part by the activity of the innervating motor neuron (1–4). Although innervation is necessary for normal expression of AChE, the response to denervation differs between species. Denervation leads to a decrease in AChE activity in the rat (5), mouse (6) and human (5) but an increase in the chicken (7), rabbit (8), and guinea pig (9). It is not known for any of these species whether transcriptional or posttranscriptional mechanisms underlie the change in activity. In the adult rat, a decrease in mRNA for AChE parallels the change in enzyme activity (4, 10). This response may be due to either a decrease in transcript synthesis or to a decrease in transcript stability. Sketelj *et al.* (4) have suggested that a decrease in transcript synthesis may be involved in the denervation induced response in rat fast twitch muscles, although modulation in transcript stability appears to be the primary mechanism responsible for the increase in AChE activity during differentiation *in vitro* of the mouse myogenic C2C12 cell line (11, 12). In avian muscle, it is unknown whether the increase in AChE activity seen following denervation is accompanied by an increase in its mRNA and, if so, what cellular mechanisms underlie the denervation response. We have isolated and characterized cDNA clones encoding the chicken AChE transcript (13). We examine here the expression of AChE at the mRNA level and show that denervation of adult chicken fast-twitch and tonic skeletal muscle fibers causes an increase in the levels of AChE transcripts that parallel the changes in enzyme activity previously observed (7). The response of AChE to denervation in early developing chick muscle is similar to the adult PLD but attenuated. Transcript levels for AChE increase ~4-fold following denervation of shank muscles of 2-day-old chicks. Further, there is an increase of ~3 fold in the synthesis of nascent transcripts for AChE measured by run-on transcription assays, suggesting that a change in transcript synthesis contributes to the increase in avian AChE mRNA following denervation.

MATERIALS AND METHODS

Denervations. Adult chickens (1.5 year-old, Spafas, PA) were anesthetized intravenously with ketamine (25–50 mg/kg) and thiamytal sodium (50–100 mg/kg). An incision was made under the wing and nerves supplying the anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) were sectioned simultaneously before the bifurcation of the two nerve trunks. The animals were sacrificed 3 to 20 days after surgery and the muscles were removed, frozen in liquid N_2 and stored at −80°C until used. Two-day-old chicks were

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Abbreviations used: AChE, acetylcholinesterase. ALD, anterior latissimus dorsi. PLD, posterior latissimus dorsi.

0006-291X/99 $30.00

anesthetized with ketamine (50–100 mg/kg), an incision made above the lower end of the femur and the peroneal and sciatic nerves were isolated, and sectioned. Chicks were sacrificed two days later.

Muscle preparation. Muscle was ground into a powder under liquid N_2 using a mortar and pestle. An aliquot (15–25 mg) was resuspended in 0.5 ml of extraction buffer (10 mM Hepes pH 7.5, 1 M NaCl, 1% Triton X-100, 0.1 mg/ml apronitin, 1 mg/ml bacitracin, 2 mM benzamidine, 0.03 mg/ml leupeptin and 0.1 mg/ml lima bean inhibitor), incubated at 4°C for 30 min and homogenized with a glass homogenizer. Samples were centrifuged at 4°C in a microcentrifuge at 14,000 rpm for 10 min and the supernatant was assayed for AChE activity in duplicate using a radiometric assay (14, 15).

RNA blotting and quantitation. Total RNA from powdered muscle prepared as above was extracted using an acid phenol method (16). RNA was denatured using 0.5% v/v glyoxal and 50% v/v dimethyl sulphoxide at 50°C (17), separated in a 1% agarose gel, electrotransfered onto nylon membranes (GeneScreen, NEN-DuPont) and crosslinked by UV light. Blots were prehybridized at 42°C for at least 4 hours in a buffer containing 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1.5 mM EDTA, pH 7.4), 50% formamide, 5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 1 mg/ml poly A^+, 100 mg/ml sonicated salmon sperm DNA. Hybridization used the same solution but contained cDNA probes labeled at $\sim 0.5 \times 10^9$ cpm/ug using ^{32}P-dCTP by the random primer method (18). Membranes were hybridized overnight, subsequently washed once in 0.1× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate pH 7.0) for ~30 min at room temperature and twice in 0.1× SSC for 30 min at 65°C. The cDNA encoding AChE (p11.3) was a ~2.2 kb fragment subcloned in the EcoRI site of pGEM7Z+. It contains approximately 70% of the open reading frame, starting at base 767, and includes a poly A^+ tail at the 3′-end (13). RNA blots were quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics). The predominant AChE transcripts (~4.5 and 4.8 kb) were quantified together. Values obtained for each sample were normalized to those of the innervated contralateral control muscle. Ribosomal RNA was used to normalize the sample loading. The blots were stained with methylene blue after crosslinking but prior to pre-hybridization (19), and the 28S rRNA band was quantitated using NIH Image 1.44 software after capturing the image with a CCD camera (Photometrics, CH250). Methylene blue was removed by incubation in 1× SSPE, 1% sodium dodecyl sulfate for 15 min at room temperature before pre-hybridization.

Run-on transcription assay. Shank muscles from 2-day-old chicks were dissected on ice, pooled (6-7 per sample), homogenized for 30 sec at setting 4 using a Polytron homogenizer (Brinkman) and the intact nuclei were separated and concentrated as previously described (20). Nuclear transcription assays (5×10^6–10^7 nuclei per sample) were carried out in the presence of RNasin, ribonucleotides and ^{32}P UTP according to Tsay and Schmidt (20). After digestion with DNase1, nascent RNA transcripts were extracted with phenol–chloroform, precipitated with ethanol, dissolved in 10 mM Tris pH 7.4, 5 mM EDTA, 1% SDS and separated from free label by centrifugation through a Sephadex G25 spun column. The RNA was precipitated with 5% TCA, resuspended in 10 mM Tris pH 7.4, 1 mM EDTA and hybridized to M13 derived single stranded templates immobilized on nitrocellulose membranes (10 μg/slot) using a slot blot (Schleicher and Schuell) under conditions of high stringency as described above for the RNA blots. Single stranded antisense cDNA encoding a 790 bp cDNA fragment of AChE [pCMACE (21)] and chicken myoD, whose rate of transcription increases only slightly following denervation of chicken muscle (22), were used to assay transcription. The sense strand of the AChE probe and M13 alone were used as negative controls. Following hybridization, filters were washed as the RNA blots described above. Hybridizing ^{32}P labeled RNA's were detected by autoradiography using a PhosphorImager.

RESULTS

Adult denervations. Two predominant transcripts were present in the adult fast-twitch fiber containing PLD and the tonic fiber containing ALD muscles when probed with a cDNA fragment (p11.3) that hybridizes specifically to all the known transcripts for AChE in the chicken (21). The apparent sizes were approximately 4.8 kb and 4.5 kb (Fig. 1B). A third minor band at ~6 kb was seen in both the PLD and the ALD after longer exposure times.

The PLD and ALD muscles were denervated simultaneously, removed after periods up to 20 days and analyzed for AChE activity and transcript levels. AChE activity rose in the denervated PLD, reaching a maximum of ~13 times that of the innervated control muscle (zero-day time point) by 10 days following denervation (Fig. 1A). The denervated ALD muscle showed a smaller increase in AChE activity (~4-fold) than the denervated PLD but followed the same time course for the response. Transcript levels in the PLD muscle increased to a maximum of ~12-fold higher than the innervated control muscles (zero-day) by 6 days following denervation (Fig. 2A). Both transcript levels and enzyme activity remained at maximum levels throughout the denervation period. The denervated ALD similarly showed an increase in AChE transcript level (Fig. 2B), but again the change was not as great as for the PLD, reaching maximum levels by day 6 of about 4-fold higher than the innervated control muscles (zero-day). The relative sizes and proportions of the major transcripts for AChE following denervation remained unchanged from the non-denervated muscles (data not shown).

Denervations of newly hatched chicks. AChE activity and mRNA from the shank muscles of non-denervated 2-day-old chicks were measured to provide a baseline for the response to denervation. AChE activity was higher in the two-day-old shank muscles (~2-fold; Fig. 3A) than in the adult PLD (see Fig. 1A) or adult shank muscles (data not shown), indicating that the maturation of the neuromuscular junction and down regulation of extrajunctional AChE within the muscle fibers were still in transition between the embryonic and adult levels. Transcript levels rose ~4-fold compared to contralateral innervated muscles, two-days following denervation of the shank muscles (3.87 ± 0.33, s.d., $n = 3$). This was accompanied by a similar increase in AChE activity in the denervated shank muscles (Fig 3A).

AChE transcriptional activity in newly hatched chicks. Run-on transcription assays were performed to determine if changes in transcript synthesis contributed to the increase in AChE transcripts. We chose to assay for nascent transcript synthesis two-days following denervation because others found in the same aged (2-day-old) chick shank muscles that transcript synthe-

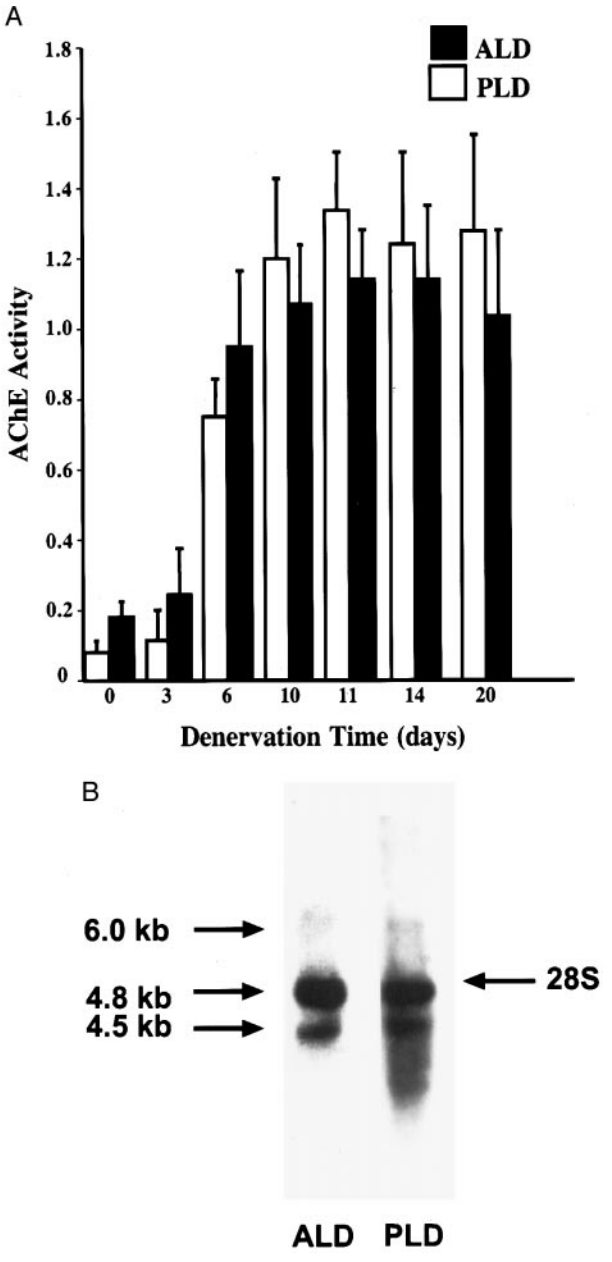

FIG. 1. AChE enzyme levels and transcript sizes in innervated and denervated adult chicken muscles. (A) Time course of AChE activity following denervation of the ALD and PLD muscles. Values expressed in nmoles of acetylcholine hydrolyzed/min × g are the mean ± s.d. of 3-6 animals per time point. (B) RNA blot of adult innervated ALD and PLD muscle probed with the AChE cDNA p11.3. Blots were exposed for 6 hours (ALD), and 3 days (PLD). Arrows indicate apparent sizes (in kb) of the AChE transcripts.

sis for the acetylcholine receptor α-subunit reached a peak at this time after denervation (20). Two-day-old chicks showed significant levels of newly synthesized transcript for AChE, approximately 3-fold above the single stranded M13 phage DNA negative controls, when detected using an antisense probe to AChE [pCMACE; (21)] encompassing 790 bp of coding region common to all transcripts (Fig. 3B). Two-days following denervation of the shank muscles, nascent transcript synthesis increased approximately 3-fold compared to the non-denervated contralateral muscles. A slight increase in transcript synthesis (~1.3-fold) was also observed in run on assays for chicken MyoD as previously reported (22). There was no change above background for either M13 phage DNA or the sense strand of pCMACE when they were used as the negative controls.

DISCUSSION

This report shows that following denervation of chicken adult and newly hatched skeletal muscles AChE transcripts increase sharply and reach levels which parallel the change in AChE enzymatic activity of those muscles. The change in transcript levels of the newly hatched chicks was accompanied by an increase in nascent transcript synthesis indicating that the response to denervation is controlled, at least in part, by transcriptional regulation.

The increase in AChE activity following denervation reported here for the PLD and ALD muscles supports a

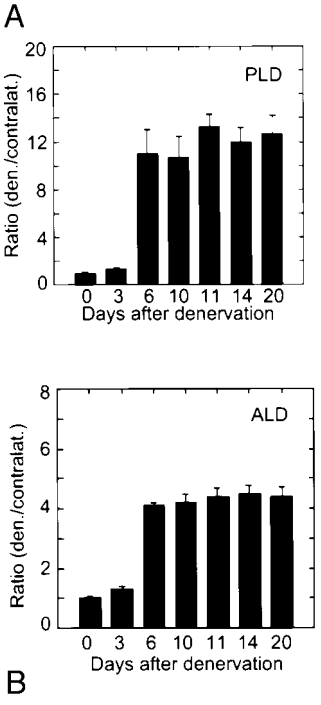

FIG. 2. Denervation time course of the PLD and ALD muscle AChE transcript levels. Ten μg of total RNA from muscles of innervated controls (0-day time point) or denervated for various times (3-, 6-, 10-, 11-, 14- and 20-days) were blotted and probed with a ^{32}P-labeled AChE cDNA fragment (p11.3). Transcript levels are for the PLD (A) and the ALD (B) muscles following denervation. The values (mean ± s.d, n = 5-6 muscles per time point) are the sum of the two major transcripts (4.5, 4.8 kb; see Fig. 1B) and are presented as the ratio of the denervated to its innervated contralateral muscle. RNA levels were normalized to the 18S rRNA detected by methylene blue staining (see Materials and Methods).

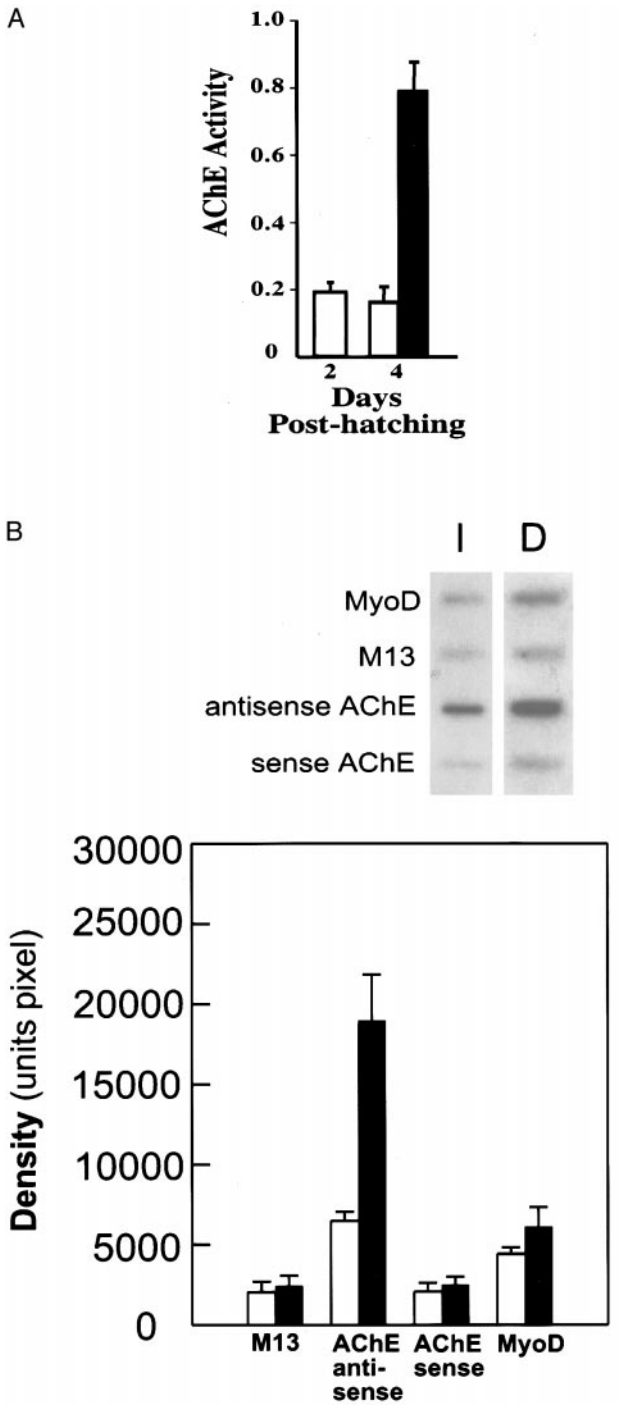

FIG. 3. AChE activity and transcript synthesis following denervation of chick shank muscles. (A) AChE activity of innervated 2-day- and 4-day-old chick shank muscles (open bars) and 4-day-old muscles denervated for two-days (filled bar). (B) Nascent transcript synthesis of 4-day-old shank muscles denervated for two days. Upper panel is a representative slot blot showing hybridization of elongated ^{32}P-labeled transcripts to immobilized single-stranded cDNAs to chicken MyoD, the parental M13 vector, the antisense strand of AChE (pCMACE), and the sense strand of pCMACE. Left column is the innervated contralateral muscle (I), and the right column is denervated muscle (D). Lower panel shows quantitative measurements, using a PhosphorImager, of band densities from the blots (values are means ± s.e.m., n = 3 independent experiments). Open bars are innervated contralateral muscles and black bars are denervated muscles.

previous report (7), and our data on the newly hatched chick shank muscles further extends the observation to muscles from young animals and to muscles also containing slow-twitch fiber types. Although the increase in transcript levels is higher for the PLD (~17-fold) than for the ALD (~4-fold), muscles of both twitch- and tonic-fiber type exhibit the same response following denervation.

The change in AChE activity and transcripts following denervation could be a result of an increase in expression at either or both the synaptic and extrasynaptic regions of the muscle fiber. The observation (23) that AChE transcripts are enriched in the synaptic region of innervated avian muscle fibers *in vivo* supports the notion that the AChE gene is preferentially active in this domain and may respond to denervation by up regulation. However, another study (24) demonstrated that the increase in enzyme activity in denervated adult chicken muscle occurred mainly in the extrasynaptic region of the fibers, suggesting that the primary regulatory response was in areas outside the synapse. Our data would be consistent with the latter observation, although we have not tried to directly measure transcript levels in synaptic and extrasynaptic regions of the fibers. The larger increase in transcript levels in the fast-twitch type PLD muscle (~12-fold) than the tonic ALD muscle (~4-fold) is consistent with this idea, since proportionally more extrasynaptic nuclei would be activated following denervation in the singly-innervated PLD than in the multiply-innervated ALD.

Denervation-induced increase in nascent AChE transcripts. The increase in transcript levels following denervation could arise through transcriptional or post-transcriptional mechanisms. Nascent synthesis of AChE transcripts, measured by run-on transcription assays, increased (~3 fold) to levels nearly equal to the accumulation of transcripts measured by RNA blotting (~4 fold) in denervated chick shank muscles, indicating that transcript synthesis was a primary mechanism contributing to the up regulation. Our data does not exclude the possibility that other posttranscriptional mechanisms, such as the stabilization of transcripts, may participate in the modulation. Transcript stabilization has been shown to be the primary mechanism underlying the increase in AChE activity during early differentiation *in vitro* of the mouse myogenic C2C12 cell line (11, 12). Furthermore, our data does not discriminate between transcriptional regulation of synaptic or extrasynaptic regions of the fibers. Studies directed at the level of the promoter for avian AChE will be required to address these questions.

Young chick muscles were necessarily used for the run-on assays because we were unable to isolate a sufficient quantity of nuclei from adult muscle. The difficulty in extracting nuclei from adult muscle was likely due to the dense meshwork of contractile elements within the fibers. Similar difficulties have been encountered by others (25) applying run-on transcription assays to study the regulation of muscle specific transcripts *in vivo*.

The denervation-induced increase in AChE in chicken muscle is similar to the up regulation of the acetylcholine receptor α- and δ-subunits following denervation or paralysis (20, 26, 27). These studies indicate that the majority of the increase in acetylcholine receptors can be accounted for by an increase in transcript synthesis, primarily in the extrasynaptic region of the muscle fiber. By analogy, similar mechanisms may regulate avian muscle AChE transcript levels. Our data provides the first direct evidence that transcriptional mechanisms play a key role in the modulation of AChE levels in birds.

ACKNOWLEDGMENTS

We thank Dr. B. Patterson for kindly providing the cDNA for chicken MyoD, and Dr. M. M. Salpeter for advice and comments on the manuscript. This work was supported by a grant from the NIH to W.R.R.

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