Skeletal Muscle Denervation Activates Acetylcholine Receptor Genes

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Abstract. Transcriptional activity of acetylcholine receptor subunit genes was investigated in innervated and denervated chick skeletal muscle. The sciatic nerve of 3-d-old White Leghorn chicks was sectioned unilaterally; after various intervals, nuclei were isolated from operated and sham-operated animals, and run-on assays performed. Nuclei were incubated with ^{32}P-UTP, and total RNA was extracted and hybridized onto filters containing an excess of subunit-specific DNA. Specific transcripts were detected by autoradiography and quantitated densitometrically. A sharp increase in transcriptional activity was observed to begin ∼1/2 d after the operation and peak 1 d later when transcriptional rates reached approximately seven-, six-, and fivefold control levels for the α-, δ-, and γ-subunit genes, respectively. The specificity of the effect was ascertained by normalization to total RNA synthesis and by the demonstration that several non-receptor genes respond differently to denervation.

These results suggest that a denervation signal reaches the genome to induce receptor expression. In addition, since the increase in mRNA levels significantly exceeds what can be accounted for by increased gene activity, posttranscriptional effects are suggested.

WHEN a skeletal muscle is denervated, chemosensitivity—i.e., responsiveness to the neurotransmitter acetylcholine—spreads from the neuromuscular endplate over the entire plasma membrane (Axelsson and Thesleff, 1959; Miledi, 1960). This is the result of an increase in the number of acetylcholine receptors (AChRs)[1] displayed on the fiber surface (Miledi and Potter, 1971; Berg et al., 1972). The rise in receptor levels is accounted for by an increase in de novo synthesis (Brockes and Hall, 1975; Devreotes and Fambrough, 1976). Recent investigations have shown that the increase in receptor synthesis rate is probably a consequence of increased levels of mRNAs coding for receptor subunits (Merlie et al., 1984; Klarsfeld and Changeux, 1985; Goldman et al., 1985; Shieh et al., 1987, 1988; Evans et al., 1987; Moss et al., 1987). That these increases are likely to result from transcriptional activation has been shown by Shieh et al. (1987) who used genomic probes and found that the appearance of a putative α-subunit mRNA precursor precedes the increase in the level of mature message.

To establish directly that AChR genes are activated as a result of denervation, we have carried out run-on experiments with nuclei isolated from innervated and denervated chick muscle. Here we report findings that confirm the validity of Fambrough's suggestion, inspired almost two decades ago by experiments with metabolic inhibitors, that "the neuronal regulation of acetylcholine sensitivity probably involves regulation of gene activity in muscle fibers" (Fambrough, 1970).

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1. *Abbreviation used in this paper:* AChR, acetylcholine receptor.

Materials and Methods

Chicken Operation

Unilateral section of the sciatic nerve was performed as described previously (Shieh et al., 1988); in all instances, both proximal and distal nerve stumps were ligated to prevent reinnervation.

Probe Preparation

For the nuclear run-on experiments, the PstI fragment of pC25.1Bgl (Wang et al., 1988), which contains 1.4 kb of α-subunit genomic sequence including exons I and II, was inserted into the polylinker of M13mpl0 in both orientations. The HindIII-EcoRI fragment of pL3, a plasmid containing 4.8 kb of the 5' portion of the δ-subunit gene including exons I through IV, and the HindIII-PstI fragment of pB5, a plasmid containing 0.5 kb of the 5' region of the γ-subunit gene including exon I, each were cloned into M13mpl0 and M13mpl1. Single-stranded DNA was prepared as described in the M13mp7 Cloning/Dideoxy Sequencing Manual (Bethesda Research Laboratories, Bethesda, MD). Plasmids containing full-length cDNAs of chicken β-actin and chicken β-tubulin in the pBR322 vector (Cleveland et al., 1980) were linearized with HindIII and BglII, respectively.

For nuclease protection assays, pα7, which comprises exon VII of the α-subunit (224 nt with 75-nt and 100-nt flanking sequences attached; referred to as "pexon2" in Shieh et al., 1987); p2, which contains portions of the 5' untranslated sequence and exon I of the δ-subunit; and pB5, which comprises γ-subunit exon I and flanking sequences, were cloned into Bluescript SK+ (Stratagene, La Jolla, CA; a plasmid vector which contains transcriptional promoters for T3 and T7). The recombinant plasmids were linearized with appropriate restriction enzymes, and riboprobes synthesized by in vitro transcription.
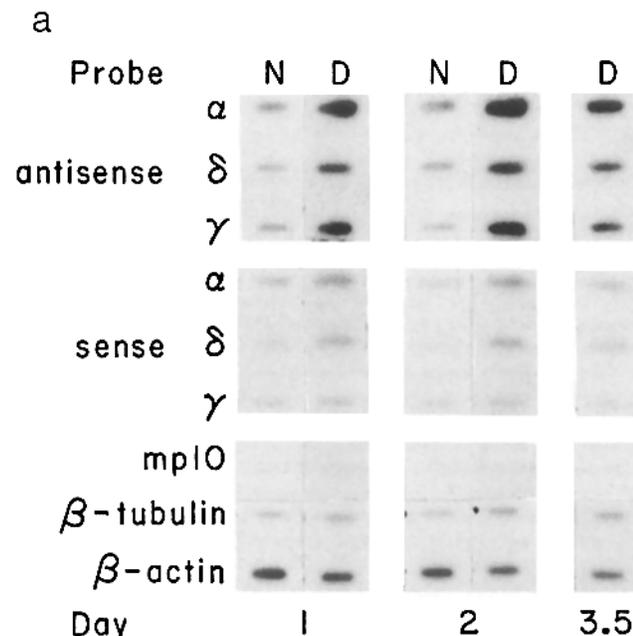
Isolation of Nuclei and Elongation of Nascent Transcripts

Nuclei were purified and assayed for transcriptional activity by adapting the methods of Schibler et al. (1983). Briefly, calf muscles were dissected free

of bone and connective tissue and homogenized with a motor-driven tissue grinder (B pestle; Thomas Scientific, Philadelphia, PA) in 0.3 M sucrose in buffer A with 1 mM PMSF. The homogenate was filtered twice through a double layer of cheese-cloth to remove residual connective tissue, layered over a cushion of 30% sucrose in buffer A, and spun for 10 min at 2,500 rpm in a centrifuge (RT 6000B; Du Pont Co. Diagnostic & BioResearch Systems, Wilmington, DE). The crude nuclei were resuspended in 0.1% NP-40 in buffer A, left for 5 min on ice, and recentrifuged. The resulting pellets were resuspended in 5 ml nuclei storage buffer, centrifuged (Eppendorf centrifuges made by Brinkmann Instruments, Inc., Westbury, NY) for 30 s, and resuspended in storage buffer containing 100 U/ml RNasin (Promega Biotec, Madison, WI). Nuclei were either used immediately or aliquotted, frozen in liquid nitrogen, and stored for up to 6 mo without loss of activity. For run-on assays, ^{32}P-UTP at 600 Ci/mmol was used. In a modification of the protocol of Schibler et al. (1983), RNAse treatment was omitted, and incubations were carried out in the absence of heparin sulfate at 26°C for 30 min, with 4 mM MgCl added. Nascent transcripts were purified as described by Nepveu and Marcu (1986) using centrifugation through a G-50 spun column followed by TCA precipitation. Samples were then exposed to 0.2 M NaOH (10 min; ice bath), followed by quenching with Hepes and ethanol precipitation. For hybridization, radioactively elongated transcripts were dissolved in small volumes of hybridization buffer, and aliquots containing 2 × 10^6 cpm were incubated in small culture dishes with sections of nitrocellulose filters containing probe DNA samples at 10 μg/slot.

Isolation of RNA and Nuclease Protection Analysis

Total RNA was isolated from tissue frozen in liquid nitrogen by extraction with guanidinium isothiocyanate/phenol (Protter et al., 1982) and subjected to solution hybridization and nuclease protection analysis. Total transcript was measured by TCA precipitation followed by liquid scintillation counting. The proportion of individual components (primary transcript; splicing intermediate; mature mRNA) was deduced using electrophoresis, autoradiography, and densitometric quantitation as described previously (Shieh et al., 1988).
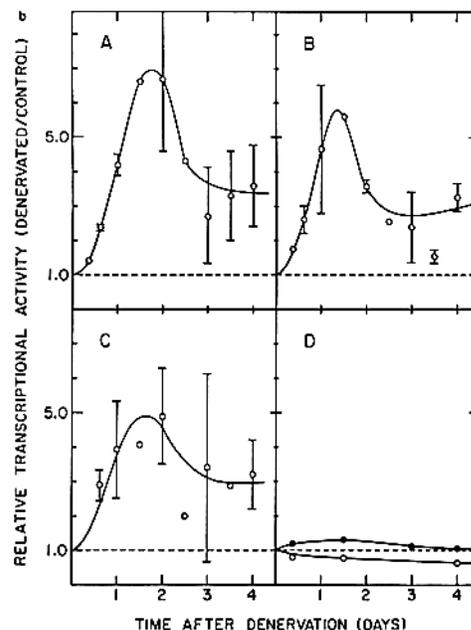
Receptor Assay

Total AChR content in skeletal muscle was quantitated by ^{125}I-α-bungarotoxin as described (Shieh et al., 1988), except that filtration over glass fiber disks (GF/C; Whatman Inc., Clifton, NJ) was substituted for adsorption to DEAE-cellulose.

Results and Discussion

Young White Leghorn chicks (2–3 d after hatching) were subjected to unilateral section of the sciatic nerve. After varying intervals, animals were killed and muscle nuclei analyzed for receptor gene transcriptional activity. Examples of results of such run-on assays are shown in Fig. 1 a. Elongation of sense transcripts (i.e., subunit mRNA precursors) is significantly enhanced after the operation. This activity was monitored for the α-, δ-, and γ-subunit genes for several days after denervation. Timecourses are displayed in Fig. 1 b. A sharp increase in transcriptional activity is observed to begin ∼1/2 d after denervation and peak during the second postoperative day when transcriptional rates reach approximately seven-, six-, and fivefold control levels for the α-, δ-, and γ-subunit genes, respectively. Relative rates drop to about half-maximal levels by the fourth day after the operation. In contrast, transcription of the β-tubulin gene is not significantly affected, while that of β-actin is actually reduced. It is noteworthy that antisense transcription of receptor genes, which proceeds at 25–35% of sense transcription in innervated muscle, is also increased upon denervation although only about twofold for α and δ, and less than 1.5-fold for γ.

Figure 1. Nuclear run-on analysis. (a) Transcript elongation was assayed in nuclei isolated from operated (D) and control (N) muscle at several timepoints after denervation. 10^7 nuclei were used in each run-on incubation, and 2 × 10^6 cpm of ^{32}P-labeled RNA for each hybridization. Results shown are those obtained with α-, δ-, and γ-subunit-specific sequences derived from both coding and noncoding strands, with the vector alone (mp10), and with probes specific for β-tubulin and β-actin. Numbers refer to days after denervation, except in the case of β-tubulin and β-actin, which were assayed after 9 h, 4 d, and 1 wk (first, second, and third column, respectively). (b) Timecourse of transcriptional activity of the coding strands of the α- (A), δ- (B), and γ- (C) subunit gene and of β-tubulin (D, ●) and β-actin (D, ○). Each receptor subunit panel contains between 1 and 5 independent measurements per timepoint that are presented as mean and SEM of relative activity (experimental divided by control); in the case of the α-subunit the transcriptional activity of the control declined by approximately a factor of 2 over a 1-wk period after the operation. Control data in D represent averages of two experiments.
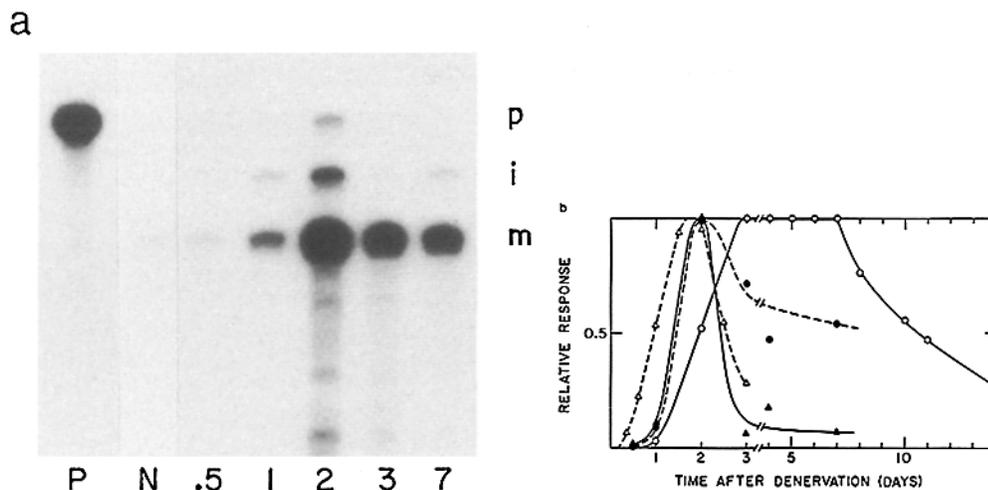
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Figure 2. Timecourse of α-subunit gene expression. (*a*) Electrophoretic analysis of RNase digests. Nuclease protection analysis was performed as described in Materials and Methods, with 30 μg of total RNA and an excess of 10-fold or greater of radiolabeled riboprobe. Transcription was monitored over a 1-wk period after denervation (*P*, probe; *N*, nondenervated control; the numbers under the remaining lanes indicate days after denervation). Three types of transcript can be discerned: *p*, primary; *i*, splicing intermediate; *m*, mature mRNA. (*b*) Denervation responses (*D/N* − 1; where *D* and *N* refer to values obtained in denervated and control muscles, respectively) are normalized to the maximal value attained and plotted as a function of time after denervation for transcript elongation activity ($\triangle$, from Fig. 1 *a*), levels of splicing intermediate ($\blacktriangle$) and mature mRNA ($\bullet$), and AChR density ($\bigcirc$). Data points represent averages of 2–5 experiments.

To correlate transcriptional activity with message levels, we determined α-subunit mRNA concentration using nuclease protection analysis (Fig. 2 *a*). Within 48 h, message levels rose about 400-fold, from 0.4 fmol to 0.17 pmol/g tissue. The rise in the level of a putative splicing intermediate (*i* in Fig. 2 *a*; see also Shieh et al., 1987) from undetectable to >10 fmol/g tissue follows transcriptional activation and narrowly precedes the increase in mature mRNA (Fig. 2 *b*). On the third day, transcriptional activity and precursor levels drop sharply, and the concentration of mature α-subunit level begins to decline. Upon denervation, mRNA levels for the δ- and γ-subunits also become elevated by about two orders of magnitude to reach 0.06 pmol/g tissue each; they begin to drop after 3 d and reach ~25–40% of maximum 1 wk after the operation (data not shown). Changes in receptor density (from 5 to 125 pmol/g tissue) follow changes in mRNA concentration.

It has long been suspected that the pronounced induction of AChR after denervation of skeletal muscle is controlled at the genome level (Fambrough, 1979). Our observations now indicate that receptor upregulation after denervation of skeletal muscle is, indeed, at least partly caused by enhanced transcriptional activity of receptor subunit genes. The increase in receptor gene activity is a specific effect, since it is evident after normalization to total RNA synthesis. In addition, none of several control genes (β-actin, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase) is similarly affected by denervation. In fact, β-actin transcription is suppressed in agreement with the recent finding that denervation diminishes total skeletal actin mRNA in chick muscle (Shimizu et al., 1988).

Since, for the three AChR subunit genes investigated, transcriptional activation is only on the order of five-to sevenfold while mRNA levels increase to a much larger extent, a post-transcriptional contribution is suggested. One such mechanism would be decelerated message turnover. Variations in mRNA half-life by more than an order of magnitude are not uncommon in eucaryotic gene expression control (see chapter 12 in Darnell et al., 1986); mRNA stabilization could

therefore easily provide for the share of receptor message increase (10- to 50-fold) that is not accounted for by transcriptional activation. Yet other mechanisms are conceivable: a block to elongation (either due to premature chain termination or pausing, at sites downstream of the gene regions covered by the run-on probes), which in control tissue results in low transcript levels, might be released after denervation and thereby raise mRNA concentrations. This mode of regulation is well documented for the protooncogenes c-*myc* (Bentley and Groudine, 1986; Nepveu and Marcu, 1986) and c-*myb* (Bender et al., 1987).

Contrary to the expectation that denervation may bring about a specific "denervated" steady state maintained by a characteristic set of receptor gene transcription rates, a significant fraction of the transcriptional activity is transient. This is not only seen in the reduction of specific transcript elongation on day 3 after denervation, but, more strikingly, in the rapid loss of the putative α-subunit splicing intermediate (Fig. 2, *a* and *b*). The fall in transcriptional activity is unlikely to be a result of reinnervation, which was mechanically prevented by nerve stump ligation. Perhaps transient stimulation reflects a denervation-triggered wave of general RNA polymerase II activity (as described for the rat extensor digitorum longus and soleus muscles by Held, 1978) superimposed on the more permanent receptor-specific elevation in transcription rates.

To our knowledge, the increase in transcriptional activity of the α- and δ-subunit genes observed 9 h after the operation is the earliest receptor-related response to denervation yet recorded. Unfortunately, this period is still too long to permit conclusions regarding the underlying mechanisms. In particular the distinction between de novo synthesis and activation/inhibition of a regulatory factor is not yet possible. More work will be necessary to delineate all events that lead to the increase of receptor messages after denervation. Nevertheless we can now state with confidence that a denervation signal reaches the genome and that it is appropriate to study its targets and mode of action.

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