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Acetylcholine Receptor α-, β-, γ-, and δ-Subunit mRNA Levels Are Regulated by Muscle Activity

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Summary

Denervation of adult skeletal muscle results in increased sensitivity to acetylcholine in extrajunctional regions of the muscle fiber. This increase in acetylcholine sensitivity is accompanied by a large increase in the level of mRNAs coding for the α-, β-, γ-, and δ-subunits of the acetylcholine receptor. To determine whether muscle activity is sufficient to regulate expression of extrajunctional acetylcholine receptor mRNA levels, denervated muscles were stimulated with extracellular electrodes. Direct stimulation of denervated muscle suppresses both the increase in extrajunctional acetylcholine sensitivity and the expression of mRNA encoding the α-, β-, γ-, and δ-subunits of the acetylcholine receptor. These results show that muscle activity regulates the level of extrajunctional acetylcholine receptors by regulating the expression of their mRNAs.

Introduction

Of central importance to understanding how the nervous system functions is to determine how synapses form during development and how they can be modified in the adult. The neuromuscular junction is one of the best studied synapses to date. The acetylcholine receptor (AChR) of the neuromuscular junction is the best studied neuroreceptor of any synapse. It is a pentameric protein composed of five subunits with the stoichiometry $\alpha_2\beta\gamma\delta$. The neuromuscular junction and the AChR provide one with a model synapse and a model synaptic protein for which one can investigate synapse development and modulation.

In adult skeletal muscle, AChRs are concentrated at the neuromuscular junction, where their density is over 1000 times that in extrajunctional regions. However, prior to muscle innervation, AChRs are found throughout the muscle fiber's surface (Schuetze and Role, 1987). The ion channels of receptors concentrated at the neuromuscular junction differ in their gating and conduction characteristics from those found throughout the muscle prior to innervation. This difference may result in part from expression of different gene products (Mishina et al., 1986). That nerve muscle interactions influence extrajunctional AChR levels can be shown by denervating adult muscle, which results in synthesis of new extrajunctional AChRs (Linden and Fambrough, 1979). In addition, direct stimulation of muscle with extracellular electrodes can suppress the denervation-induced synthesis of extrajunctional AChRs (Linden and Fambrough, 1979; Lomo and Westgaard, 1975; Reiness and Hall, 1977). Therefore, it appears that it is the activity induced in muscle by the innervating axon that results in suppression of extrajunctional AChRs during development of the neuromuscular junction.

Using cDNA clones encoding the α-, β-, γ-, δ-subunits of the AChR as probes for gene expression, we and others have found muscle denervation to result in an increase in the levels of mRNAs encoding these proteins (Evans et al., 1987; Goldman et al., 1985; Merlie et al., 1984; Shieh et al., 1987). A recent study on cultured avian myotubes has shown an increase of α-subunit mRNA content following pharmacological blockade of their spontaneous activity, suggesting that activity may control extrajunctional AChRs by lowering AChR mRNA levels (Klarsfeld and Changeux, 1985). However, AChR levels only increased about 2-fold in the blocked myotubes, whereas blockage by denervation in vivo caused a much larger increase in AChR levels (Shieh et al., 1987). Thus, muscle fibers in vitro may not regulate AChR expression as observed in vivo. Furthermore, since all four subunits are required for expression of functional AChRs, it is important to characterize the effect activity has on all these subunits. In this paper we examined, in vivo, the effect muscle activity has on the level of mRNAs encoding all four subunits of the extrajunctional AChR. We found that activity elicited in vivo by a controlled stimulation pattern suppresses the mRNA levels of the four subunits down to or below the levels in innervated muscle.

Results

Time Course of Induction of AChR α-,β-,γ-, and δ-Subunit mRNAs

Extrajunctional AChR mRNA levels increase after muscle denervation. To determine whether this increase occurs with a similar time course for all four subunit mRNAs, their levels were determined at various times after muscle denervation. Rat soleus muscles were denervated by sectioning the sciatic nerve. At various times after denervation, soleus muscles were dissected out of the animal and RNA was isolated. In innervated control muscles, the levels of the four subunit mRNAs were low. Chronic denervation produced a marked increase in the mRNA content of all subunits in nonstimulated muscles within 48–72 hr (Figure 1). The final increase was larger for the α-, γ-, and δ-subunit mRNAs, whereas the β-subunit mRNA, which was highest in innervated

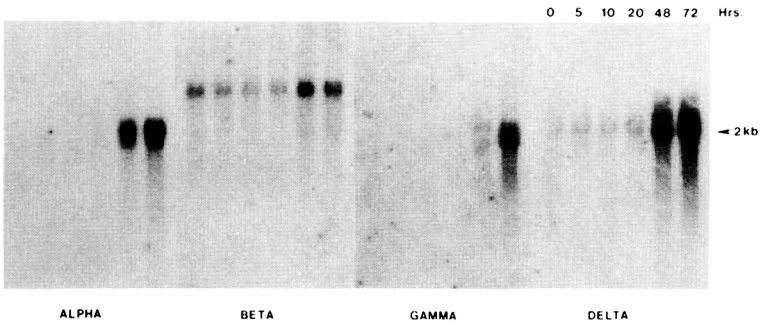

Figure 1. Time Course of AChR Gene Expression after Muscle Denervation

Experiments were carried out on soleus muscles of male Sprague Dawley rats. Muscles were denervated under Nembutal anesthesia by bilateral removal of a 5 mm segment of the sciatic nerve. At 0, 5, 10, 20, 48, and 72 hr after denervation, poly(A)$^+$ RNA was isolated and size-fractionated on denaturing agarose gels. RNA blots were probed with mouse muscle AChR α-, β-, γ-, and δ-subunit cDNA probes. Each lane contains approximately 5 μg of poly(A)$^+$ RNA.

controls, increased to a lesser extent after denervation. Quantitation of the autoradiograms by densitometry shows the α-, γ-, and δ-subunit RNA levels to increase at least 50-fold, whereas the β-subunit RNA level increased approximately 5-fold, after 72 hr of muscle denervation. The α-, γ-, and δ-subunit RNAs are approximately 2 kb, and the β-subunit RNA is 2.4 kb.

Muscle Activity Regulates the Levels of Extrajunctional AChRs and Their mRNAs

To test the idea that muscle activity suppresses expression of extrajunctional AChRs by decreasing the level of mRNA encoding these proteins, we determined acetylcholine (ACh) sensitivity and compared the levels of α-, β-, γ-, and δ-subunit mRNAs in denervated and denervated but electrically stimulated muscle in vivo.

Rat soleus muscles were denervated bilaterally. One muscle in each animal was stimulated chronically via implanted electrodes for 6 days, beginning at the time of denervation. In 26 muscles, the median extrajunctional ACh sensitivity of the denervated/stimulated muscles was <0.1 mV/nC (range 0–7.8 mV) and was thus somewhat lower than the extrajunctional sensitivity in innervated control soleus muscle (Lomo and Westgaard, 1975). In contrast, extrajunctional ACh sensitivity in the denervated/nonstimulated contralateral muscles averaged about 380 mV/nC and was thus about 4000 times higher than in nonstimulated muscle. Sensitivity ratios between nonstimulated and stimulated muscles of each animal averaged 2700. Taking into account that the input resistance of the denervated/nonstimulated fibers increases about 2-fold over the period of the experiment (Westgaard, 1975), this sensitivity ratio corresponds to a ratio of AChR densities of about 1300.

To determine whether muscle activity suppresses extrajunctional AChR levels by decreasing the level of mRNA encoding the AChR subunits, the muscles were divided into three sets of 6 to 14 denervated and denervated/stimulated muscles, respectively, and RNA was isolated from each set individually as well as from innervated control muscles derived from muscles of untreated animals. Poly(A)$^+$ RNA was purified and size-fractionated on denaturing agarose gels. RNA was transferred to GeneScreen Plus and hybridized with radiolabeled cDNA clones coding for the α-, β-, γ-, and δ-subunits of the mouse muscle AChR. Consistent with the above electrophysiological data, little or no increase in these subunit mRNA levels was observed in those muscles stimulated after denervation (Figure 2). In addition, the β- and δ-subunit mRNA levels were reduced, in stimulated

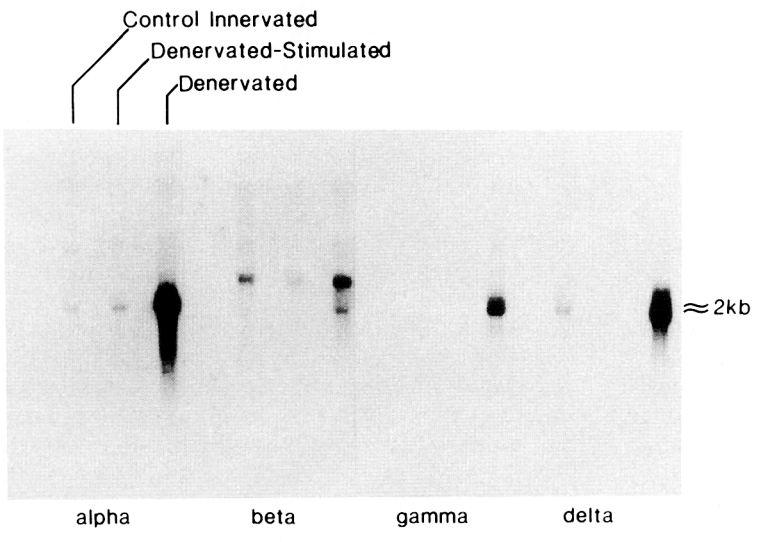

Figure 2. Effect of Muscle Activity on AChR Gene Expression in Denervated Rat Soleus Muscle

At the time of bilateral soleus denervation, stimulating electrodes were implanted into one hindlimb and the soleus muscle was stimulated chronically for 6 days. At the end of the stimulation period, the stimulated and the contralateral nonstimulated muscles were excised and their ACh sensitivities were determined electrophysiologically. Muscles were then pooled in denervated/stimulated, denervated/nonstimulated, and innervated control groups, and AChR subunit mRNA levels were estimated by hybridization of RNA blots with cDNA probes coding for the α-, β-, γ-, and δ-subunits. Each lane contains approximately 5 μg of poly(A)$^+$ RNA. The hybridization observed at 2 kb in the blot probed with the β-subunit clone represents residual signal from a previous hybridization with an α-subunit probe.

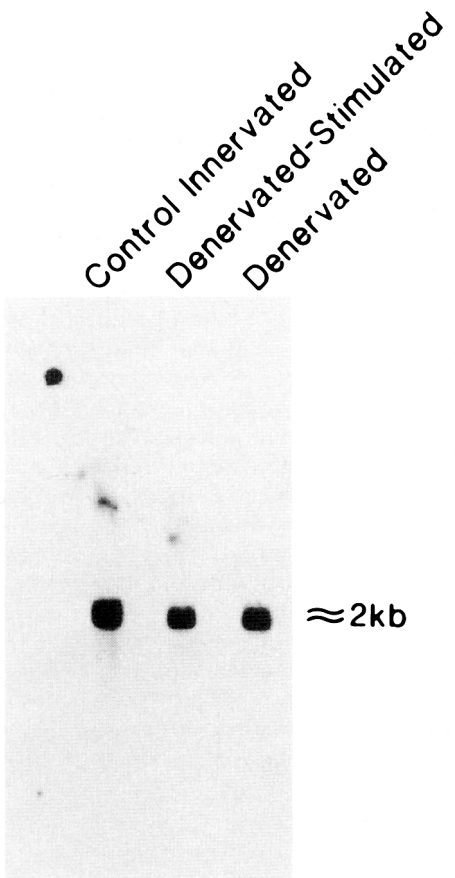

Figure 3. Effect of Muscle Activity on Actin Gene Expression

An RNA blot probed with the α-subunit AChR cDNA clone presented in Figure 2 was stripped of radiolabel by boiling in 10% SDS. The blot was then probed with radiolabeled plasmid containing an insert specific for mouse skeletal muscle α-actin.

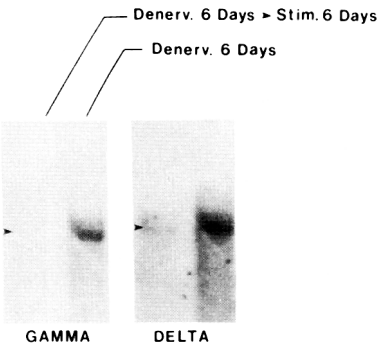

Figure 4. Effect of Muscle Activity on AChR Gene Expression in Denervated Rat Soleus Muscle

In 6 animals, muscles were denervated bilaterally; unilateral stimulation was begun 6 days after denervation and maintained for 5–7 days. AChR subunit mRNA levels in denervated and denervated/stimulated muscles were estimated by hybridization of RNA blots with the appropriate AChR subunit cDNA clone.

muscle, to below control innervated levels. Quantitation of these blots by densitometry showed that RNA levels encoding the α-, γ-, and δ-subunits increased at least 50- to 100-fold, while the β-subunit increased only 3-fold after 6 days of muscle denervation. In contrast, in those muscles denervated and stimulated for 6 days, the α-subunit RNA level increased 2-fold, the β-, and δ-subunit RNA levels decreased about 2-fold, and no detectable change was observed for the γ-subunit RNA level. Similar results were obtained in two more sets of muscles.

To determine whether activity had a specific effect on AChR mRNA, we stripped one of the RNA blots shown in Figure 2 and hybridized it with an α-actin cDNA probe. This cDNA contained only 3′-untranslated sequences and was specific for adult skeletal muscle α-actin (Minty et al., 1982). Hybridization of the RNA blots with this probe showed that α-actin RNA levels are unaffected by muscle activity (Figure 3). These results indicate that the effect of muscle activity on AChR mRNA is specific. The above experiments show that muscle activity can prevent the development of extrajunctional ACh supersensitivity, as noted by others (Lomo and Westgaard, 1975), and the increased levels of AChR mRNA observed in denervated muscle.

Muscle activity also reduces the level of extrajunctional ACh sensitivity in muscle that had been left unstimulated after denervation (Lomo and Westgaard, 1975). To test whether this is also reflected in AChR mRNA content, stimulation of one set of 6 animals was started 6 days after denervation, when supersensitivity had fully developed, and was then maintained for 6 days. The sensitivity ratios between nonstimulated and stimulated sides of each animal then averaged about 2000. An RNA blot containing mRNA isolated from these muscles was hybridized with cDNA probes corresponding to γ- and δ-subunits of the AChR (Figure 4). It is clear that activity reduced the γ- and δ-subunit mRNA levels in 6 day denervated muscle (Figure 4). Densitometric quantitation of these autoradiograms showed muscle activity reduce the γ-subunit mRNA level by 20-fold and the δ-subunit mRNA level by 8-fold. Similar results were obtained when either an α- or a β-subunit cDNA probe was used (data not shown).

Discussion

AChR levels are high in extrajunctional regions of the muscle fiber prior to innervation or after denervation of adult muscle. Innervation of muscle results in a dramatic decrease in these extrajunctional receptors (for review see Schuetze and Role, 1987). Activity, induced in the muscle by the innervating axon, can suppress the denervation-induced increase in extrajunctional AChRs. This has been shown to be a result of suppressing synthesis of new AChRs (Linden and Fambrough, 1979; Reiness and Hall, 1977). To test whether activity suppresses extrajunctional AChR synthesis by regulating the levels of mRNAs encoding these proteins, we investigated the influence activity has on extrajunctional ACh sensitivity and mRNA in vivo.

Adult skeletal muscle contains low levels of extrajunc-

tional AChRs and their corresponding RNAs; however, inactivity induced by muscle denervation results in a large increase in both extrajunctional receptor protein and mRNA levels (Evans et al., 1987; Goldman et al., 1987; Merlie et al., 1984; Shieh et al., 1987). The time course for this induction of AChR mRNAs shows a lag of approximately 2 days before the levels begin to increase (Figure 1). This is consistent with the findings of other investigators studying α-subunit mRNA levels following muscle denervation (Merlie et al., 1984; Shieh et al., 1987). We find that the α-, β-, γ-, and δ-subunit mRNAs are coordinately regulated by muscle denervation. The reason for a 2 day lag before large changes in AChR mRNA levels are observed is not clear; however, it is possible that the synthesis or degradation of an inducer or repressor of AChR gene expression is necessary (Shieh et al., 1987).

To determine whether activity alone is sufficient to suppress denervation-induced AChR mRNA levels, we compared ACh sensitivity and AChR mRNA levels in denervated and denervated/stimulated soleus muscles in vivo. The extrajunctional sensitivity to ACh in 6 day denervated/stimulated muscle was found to be comparable to that found in innervated muscle. Muscle denervation for 6 days resulted in an increased extrajunctional ACh sensitivity of about 2700-fold compared with stimulated muscle. This difference in AChR levels, as measured electrophysiologically, was paralleled by the levels of AChR mRNA (Figure 2). Muscles stimulated for 6 days, starting at the time of denervation, showed little or no denervation-induced increase in α-, β-, γ-, and δ-subunit mRNA levels. In fact the β- and δ-subunit mRNA levels were reduced to below innervated control levels in the denervated/stimulated muscles. This reduction is AChR mRNA by stimulation to below levels in innervated control muscles would be consistent with the lower extrajunctional sensitivity in stimulated versus control innervated muscle found by Lomo and Westgaard (1975).

The specificity of the response of AChR mRNAs to activity was shown by hybridizing RNA blots with an α-actin-specific cDNA probe. The α-actin probe contains only 3'-untranslated sequences and does not cross-hybridize with the other actin isoforms (Minty et al., 1982). This probe detected no change in α-actin mRNA in denervated or denervated/stimulated muscle. It is clear that the effect of muscle denervation and stimulation is a specific one and not a general effect on muscle mRNAs.

During development of the neuromuscular junction, the innervating axon is proposed to result in a decrease in extrajunctional AChRs as a result of the activity it evokes in muscle. This is supported by the observation that the high levels of AChRs found in extrajunctional regions of denervated fibers can be reduced to innervated levels after stimulating these fibers with extracellular electrodes (Lomo and Westgaard, 1975). Using a similar paradigm of in vivo stimulation, we show here that this reduction in AChRs is also accompanied by a decrease in the mRNA levels encoding these proteins. Thus muscle activity appears to regulate extrajunctional AChR expression largely by influencing the level of mRNA encoding the receptor. In light of experiments employing actinomycin D (Fambrough, 1970), these changes in mRNA levels are probably a result of affecting transcription of the AChR genes.

In addition to the four different subunits constituting the extrajunctional receptor studied above, a fifth subunit (ε), which is proposed to replace the γ-subunit in adult junctional AChRs, has been identified (Mishina et al., 1986). The innervating axon is thought to play a role in stabilizing junctional AChRs. Thus, one might speculate that the ε-subunit mRNA would not be regulated by muscle activity in the same way as the other subunits. Although we did not have a cDNA probe to study this subunit, it will be important to determine the influence muscle activity has on regulating expression of the RNA and protein encoding this subunit.

The regulation of important synaptic proteins by synaptic activity, as demonstrated here for the AChR, may represent a general mechanism by which synapses can be modified. Many long-term changes at the synapse will likely result from different patterns of gene expression. It will be important to determine whether synaptic activity in the central nervous system also influences expression of synaptic proteins, as found for the AChR in the periphery.

Experimental Procedures

Muscle Denervation and Stimulation

Experiments were carried out on soleus muscles of male Sprague Dawley rats of about 350 g in weight. Muscles were denervated, while animals were under Nembutal anesthesia, by bilateral removal of a 5 mm segment of the sciatic nerve. For those muscles to be stimulated extracellularly, stimulating electrodes were implanted into one hindlimb (Lomo et al., 1985) and the soleus muscle was stimulated chronically for 6 days in 100 Hz trains, 1 s duration, applied once every 100 s (Lomo and Westgaard, 1975). Stimulus pulses within trains were of alternating polarity, their duration was 0.5 ms, and their strength was 10–15 mA. At the end of the stimulation period, the stimulated and the contralateral nonstimulated muscles were excised and their ACh sensitivities were determined electrophysiologically (Lomo and Westgaard, 1975). ACh sensitivity, expressed as the amount of depolarization (ΔVm) per amount of charge ejected iontophoretically from a 150 MΩ ACh pipette by means of a current pump, was corrected for nonlinear summation and standardized to −60 mV (Martin, 1955) according to $\Delta Vcorr = 60 \times \Delta Vm / (Vm - \Delta Vm)$, where ΔVcorr is the corrected, ΔVm is the measured amplitude of the response, and Vm is the membrane potential of the muscle fiber. The reversal potential of the endplate current was assumed at 0 mV (Sakmann, 1978). In each muscle the sensitivities of 10 or more fibers were averaged, and each pair of muscles was examined with the same ACh pipette using constant ACh pulse and backing current strengths. The sensitivity ratio of the nonstimulated to the stimulated muscle was taken as an estimate of the ratio in AChR density.

RNA Isolation

Soleus muscles isolated from 4 to 15 animals were pooled in denervated/stimulated, denervated/nonstimulated, and innervated control groups, frozen in liquid nitrogen, and stored at -70°C until needed. RNA was isolated from 0.5–2 g of muscle tissue as described previously (Goldman et al., 1985). Briefly, muscle tissue was homogenized in buffered guanidine thiocyanate. After clarification, the homogenate was layered over a cushion of CsCl and centrifuged for 15 hr at 35,000 rpm in a Beckman SW41 rotor. The RNA

pellet was suspended in water, guanidine hydrochloride was added, and the pellet was ethanol-precipitated. The RNA precipitate was resuspended in water and ethanol-precipitated again. Poly(A)$^+$ RNA was selected by chromatography over an oligo(dT)-cellulose column (Aviv and Leder, 1972).

RNA Blots

RNA was denatured in formaldehyde at 65°C and then electrophoresed in 2.2 M formaldehyde, 1.4% agarose gels. The RNA was transferred to a GeneScreen Plus membrane. Prehybridization and hybridization conditions were 5X SSPE (0.75 M NaCl, 57 mM Na_2HPO, 5 mM EDTA [pH 7.4]), 1% SDS, 10% dextran sulfate, 50% formamide, at 42°C. After hybridization, the blot was washed in 0.2X SSPE, 1% SDS, at 65°C and exposed to X-ray film with an intensifying screen at −80°C. In some cases a single blot was hybridized multiple times with different probes. In these cases, after each hybridization and washing, blots were boiled in 10% SDS for 10–30 min and exposed to X-ray film before hybridization with a subsequent probe.

cDNA Probes

RNA blots were hybridized with mouse muscle AChR α-, β-, γ-, and δ-subunit cDNA probes (Boulter et al., 1985, 1986; Evans et al., 1987; LaPolla et al., 1984). In addition, some RNA blots were hybridized with a mouse skeletal muscle α-actin-specific cDNA clone, p91-1 (Minty et al., 1982). cDNAs were radiolabeled by nick translation (Rigby et al., 1977) to specific activities of 2×10^8 to 4×10^8 cpm/μg of DNA. cDNA probes were denatured by placing them in boiling water for 10 min, then on ice. Approximately 10^6 cpm/ml hybridization solution was used for probing RNA blots.

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