Neural Factors Regulate AChR Subunit mRNAs at Rat Neuromuscular Synapses

V. Witzemann,* H.-R. Brenner,‡ and B. Sakmann*

*Max-Planck-Institut für Medizinische Forschung, Abteilung Zellphysiologie, D-6900 Heidelberg, Germany;
‡Physiologisches Institut der Universität Basel, CH-4051 Basel, Switzerland

Abstract. To elucidate the nature of signals that control the level and spatial distribution of mRNAs encoding acetylcholine receptor (AChR), α-, β-, γ-, δ- and ε-subunits in muscle fibers chronic paralysis was induced in rat leg muscles either by surgical denervation or by different neurotoxins that cause disuse of the muscle or selectively block neuromuscular transmission pre- or postsynaptically and cause an increase of AChRs in muscle membrane. After paralysis, the levels and the spatial distributions of the different subunit-specific mRNAs change discoordinately and seem to follow one of three different patterns depending on the subunit mRNA examined. The level of ε-subunit mRNA and its accumulation at the end-plate are largely independent on the presence of the nerve or electrical muscle activity. In contrast, the γ-subunit mRNA level is tightly coupled to innervation. It is undetectable or low in innervated normally active muscle and in innervated but disused muscle, whereas it is abundant along the whole fiber length in denervated muscle or in muscle in which the neuromuscular contact is intact but the release of transmitter is blocked. The α-, β-, and δ-subunit mRNA levels show a different pattern. Highest amounts are always found at end-plate nuclei irrespective of whether the muscle is innervated, denervated, active, or inactive, whereas in extrasynaptic regions they are tightly controlled by innervation partially through electrical muscle activity. The changes in the levels and distribution of γ- and ε-subunit-specific mRNAs in toxin-paralyzed muscle correlate well with the spatial appearance of functional fetal and adult AChR channel subtypes along the muscle fiber. The results suggest that the focal accumulation at the synaptic region of mRNAs encoding the α-, β-, δ-, and ε-subunits, which constitute the adult type end-plate channel, is largely determined by at least two different neural factors that act on AChR subunit gene expression of subsynaptic nuclei.

THE nicotinic acetylcholine receptor (AChR)[1] of mammalian skeletal muscle is a heterooligomeric membrane protein composed of α-, β-, δ-, and either γ- or ε-subunits (Mishina et al., 1986; Witzemann et al., 1990). Functionally, the two AChR subtypes (termed AChRγ and AChRε, respectively) differ in their gating and ion conductance properties and their density in the sarcolemmal membrane changes during development. Before innervation, the fetal AChR subtype containing the γ-subunit is distributed over the entire surface of the muscle fiber and accumulates at the end-plate as the nerve contacts the muscle fiber. The adult AChR subtype containing the ε-subunit appears only postnatally and is predominantly localized at the end-plate. Concomitantly with the switch of the AChR subtypes in the end-plate, the fetal AChRs disappear from the extrajunctional membrane. When the muscle is surgically denervated the fetal AChRs are expressed again along the whole fiber length and disappear upon reinnervation. Thus the abundance, subunit composition, and localization of AChRs are under control of the motor nerve (see Schuetze and Role, 1987 for review).

The synaptic accumulation, the "subunit switch" underlying the conversion from fetal to adult AChR subtype, and the maintenance of the synaptic accumulation of adult type AChRs in innervated fibers may be caused by neurogenic factors that are released from the nerve ending as well as by signals linked to the electrical activity of the muscle induced by the nerve. At the level of AChR subunit-specific mRNAs, the involvement of specific neurotrophic signals is based on the observation that in innervated adult muscle the mRNAs are locally accumulated at the end-plate (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990). This focal accumulation of subunit mRNAs may be the result of several regulatory mechanisms since both the abundance and the cellular location of the various AChR subunit-specific mRNAs are regulated differentially during postnatal development (Witzemann et al., 1989; Brenner et al., 1990). For example, whereas α-subunit mRNA is expressed along the entire muscle fiber before innervation and becomes restricted to the

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; α-BuTX, α-bungarotoxin; BuTX, botulinum toxin; m.e.p.c., miniature end-plate current; m.e.p.p., miniature end-plate potential; TTX, tetrodotoxin.

end-plate only postnatally (Fontaine and Changeux, 1989; Brenner et al., 1990), the increase in ϵ-subunit mRNA level is induced locally at the end-plate after birth and is not prevented by denervation immediately after birth, suggesting a locally restricted mechanism of gene activation (Brenner et al., 1990). Activity linked signals may also be important for the focal accumulation of mRNAs at the end-plate. This view is based on experiments in which muscles were surgically denervated and the effects of muscle inactivity and of exogenous electrical stimulation on the levels of subunit specific mRNAs were measured (Goldman et al., 1988). Surgical denervation of the muscle, however, may affect AChR mRNA levels by interrupting the action of putative neurotrophic factors as well as of nerve-induced electrical activity of the muscle. Here we describe experiments that differentiate between the role of neural factors that depend on an intact nerve-muscle contact, and signals linked to electrical muscle activity. We have compared the changes in the levels of AChR-subunit mRNAs in rat muscles that were paralyzed chronically either by cutting the motor nerve, which destroys the presynaptic nerve terminal, or by different neurotoxins which leave the nerve terminal structurally intact but interrupt functional neuromuscular transmission. The results revealed that the AChR subunit mRNA accumulation and maintenance is mediated by neural factors.

Materials and Methods

Surgery and Toxin Paralysis of Lower Leg Muscle

Experiments were performed on soleus or entire triceps muscles or diaphragm of male Sprague-Dawley rats weighing 200–300 g. Muscles were paralyzed surgically by denervation or pharmacologically either by chronic application of tetrodotoxin (TTX) to the sciatic nerve, by injection of Botulinum toxin (BoTX) type A into the triceps muscle or by chronic application of α-bungarotoxin (α-BuTX) to the soleus muscle. Reinnervation experiments were made as detailed in Witzemann et al. (1987). In all blocking experiments with neurotoxins precautions were taken to avoid undetected muscle activity to affect AChR mRNA levels. They are listed below for the different types of blockades.

Tetrodotoxin Blockade

Chronic blockade of impulse conduction in the sciatic nerve was carried out as described by Brenner et al. (1987). Osmotic minipumps (Alzet 2002; Alza, Palo Alto, CA) were filled with Hanks' solution containing 370 or 1,000 μg TTX/ml (Sigma Chemical Co., St. Louis, MO or Sankyo, Tokyo), 200 IU of penicillin, and 200 μg streptomycin/ml (Amimed, Basel, Switzerland). At a nominal pumping rate of these pumps of 0.5 μl/h, this corresponds to a delivery of 4.5 and 12 μg TTX per d, respectively. The minipump was implanted into the peritoneum. It was connected by sterile silicone rubber tubing threaded under the skin to a silastic cuff (inside diameter 1.8 mm, length 8 mm), which was placed around the sciatic nerve in the upper thigh. Paralysis of the triceps muscles as assessed by the missing toe spreading reflex was observed within 3 h of pump implantation. Impulse conduction block was maintained for 3–19 d in different experiments.

The following tests suggested that the conduction block was complete during the entire duration of the experiments: (*a*) the absence of the toe spreading reflex was checked at least once daily. Conduction block was also checked by nerve stimulation when the muscles were excised for an acute experiment. In none of the rats produced a supramaximal single or a 30-Hz tetanic stimulus applied to the sciatic nerve >10 mm proximally from the cuff a visible contraction of the lower leg muscles. In five animals, this was ascertained quantitatively by isometric contraction measurements, using a strain gauge attached to the Achilles tendon in situ. In contrast, distal stimulation always elicited vigorous contractions. Comparison of measurements with proximal and distal stimulation indicated that with distal stimulation the effect was below the detection limit (<1% of the contraction measured with distal stimulation). (*b*) In four animals, TTX-treated sciatic nerves were excised and placed in saline (see below) at room temperature. Washout of the conduction block as assessed by compound action potential recordings from the sciatic nerve then required >3 h or was not achieved at all within 5 h when the experiment was discontinued. (*c*) In five experiments, total TTX delivery during the blocking period was checked at the end of an experiment by recovering the TTX solution remaining in the pump and dividing its volume by the duration of the block; the nominal pumping rate was always maintained and, thus, no indication of a long-lasting interruption of TTX delivery was observed. (*d*) No difference in the effects of the two TTX dosages (4.5 and 12 μg/d) on AChR subunit mRNA levels were detected. These findings combined show that impulse conduction block was complete during the entire period during which TTX was applied.

Botulinum Toxin Block

Botulinum toxin (BoTX) type A, which induces muscle paralysis by blockade of quantal release of transmitter, was kindly tested for biological activity before use and supplied by Drs. E. Habermann and F. Dreyer (Universität Giessen). 0.2 ng of toxin (for 250-g rats) was injected into the triceps surae muscle, and paralysis was observed within 12 h of the injection. Completeness of the block of neuromuscular transmission was assessed by the missing toe spreading reflex and by direct stimulation of the sciatic nerve before an acute experiment.

α-Bungarotoxin Block

Chronic blockade of neuromuscular transmission was induced by blocking endplate AChRs chronically with α-bungarotoxin (α-BuTX); the blockade was performed as described by Drachmann et al. (1982). Alzet 2002 pumps were filled with 1 mg α-BuTX (Boehringer, Mannheim, FRG) per ml Hanks' solution, containing penicillin and streptomycin. Paralysis was produced by a priming injection of 25–30 μg α-BuTX dissolved in 90 μl of Hanks solution into the soleus muscle followed by chronic application of α-BuTX from the minipump onto the surface of the soleus muscle. Delivery was via finely tapered polyethylene tubing placed on the endplate region of the muscle. In one experiment it was found that complete block of neuromuscular transmission as assessed by the absence of endplate potentials upon sciatic nerve stimulation was achieved within 2 h after the intramuscular α-BuTX injection. Completeness of the block was ascertained daily by the missing toe spreading reflex and by the paralysis observed upon nerve stimulation before an acute experiment. In four α-BuTX blocked muscles, intracellular recordings of membrane potentials were made from the synaptic fiber regions (see below). In none of the experiments were miniature endplate potentials detected. In two muscles that were examined for the occurrence of stimulus-evoked endplate potentials (e.p.p.s) it was found that in <10% of the examined fibers (50–70 fibers were examined in each muscle) e.p.p.s were detectable. When present, however, e.p.p. amplitudes were <1 mV, which is much smaller than those seen in normal muscles (>10 mV at resting potentials of −60–−70 mV). Thus even in these fibers where e.p.p.s could be detected the block of end-plate channels would be >95% complete (Pennefather and Quastel, 1981). Four different animals were used for the Northern blot hybridization analysis. The RNA was isolated from muscles ranging between 0.4 and 0.5 g and yielded 50–125 μg of total RNA.

RNA Isolation and RNA Blot Hybridization Analysis

For analysis of mRNA levels, animals were killed with CO_2 and the excised muscles were frozen in liquid nitrogen until further use. Total RNA was extracted and analyzed as described (Methfessel et al., 1986). RNA hybridization analysis was performed using 10 μg of total RNA from each muscle. After electrophoresis on 1.5% agarose gels RNA was transferred to Biodyne nylon membranes (Pall; Ultrafine Filtration Corporation, Glen Cove, NY) and UV-cross-linked using the UV Stratalinker 1800 (Stratagene Corp., La Jolla, CA). Equal loading was controlled by ethidium bromide staining of the RNA-agarose gels and by using the actin-mRNA levels as internal standards. The mRNAs were completely transferred by diffusion (Thomas, 1980) as verified by the analysis of increasing amounts of total RNA. AChR- and actin-specific mRNAs increased in a linear fashion (Witzemann et al., 1987). Hybridization was carried out after the procedure recommended by Pall. Hybridization probes were prepared as described (Witzemann et al., 1989). AChR-subunit specific DNA probes were also derived from full-length cDNA clones (Witzemann et al., 1990). All hybridization probes were labeled with [α-^{32}P]dCTP using the random primer labeling kit from Boehringer following procedures based on the method of Feinberg and Vogelstein (1983). The specific activities of the

probes ranged from 1-2 × 10^9 cpm/μg DNA. Autoradiograms obtained upon blot hybridization were scanned densitometrically (Witzemann et al., 1987).

RNase Protection Assay

RNase protection assays were performed according to Melton et al. (1984). The α-subunit-specific probe was synthesized from rACRα DNA (Witzemann et al., 1990) cloned into pSP65 in 3'-5' orientation. The 3' end was deleted by digestion with XbaI/BstxI and the blunt ended BstxI and XbaI (on pSP65) sites were ligated. Linearization with EcoRV yielded a cRNA probe of 211 nucleotides protecting 176 nucleotides of the α-subunit mRNA. The γ-subunit specific probe was synthesized from the 370-bp BamHI-SmaI fragment from rACRγ10 DNA (Witzemann et al., 1990) that had been cloned into HincII/BamHI-digested pSP64. The probe obtained from the BamHI-linearized vector is 391 nucleotides long and protects 370 nucleotides of the γ-subunit mRNA. 10 μg of total RNA was hybridized at 60°C with both α- and γ-subunit-specific ^{32}P-labeled cRNA probes. The specificity of the protection assay was established using synthetic sense and antisense cRNA together with poly(A)$^+$ RNA or poly(A)$^-$ RNA from denervated rat muscle. Only the sense cRNA hybridized with poly(A)$^+$ RNA yielded protected fragments. These fragments were visualized upon polyacrylamide gel electrophoresis by autoradiography. Densitometric evaluation of the autoradiograms was performed to estimate the amounts of α- and γ-subunit-specific mRNA. Calibration was done by subjecting α- and ε-subunit sense cRNA of known concentration (ranging from 100 to 1,000 pg) to the RNase protection assay using the same hybridization probes and conditions as for the total muscle RNA. In this concentration range the relation between optical density and cRNA concentration is linear.

In Situ Hybridization Experiments

Soleus muscles were fixed and stained for acetylcholinesterase (AChE). Sections of 8-10 μm thickness were mounted on aminoalkylsilane-treated glass slides. After rehydration, the sections were scanned for end-plate AChE stain deposits and their locations with respect to the scales of the microscope stage were recorded. Sections were then pretreated and hybridized essentially as described (Fontaine and Changeux, 1989; Brenner et al., 1990).

α-Bungarotoxin Binding

^{125}I-α-Bungarotoxin (α-BuTX) was purchased from New England Nuclear (Boston, MA). Native α-BuTX was from Boehringer. Rat muscle frozen in liquid nitrogen was homogenized in 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 10 mM EDTA, 0.1 μM PMSF, and 0.5% Triton X-100 at a ratio 10 ml buffer/g wet tissue. Toxin binding was assayed using the DEAE-filter disc method described by Schmidt and Raftery (1973).

Electrophysiology

In TTX-paralyzed muscles to be used for electrophysiological experiments, preexisting end-plate AChRs were blocked with α-BuTX at the beginning of the nerve blockade; the soleus muscle was exposed and the AChRs were blocked by superfusion of the exposed muscle in situ with 2.5 μM α-BuTX in physiological solution for 30 min. Labeling of such muscles with ^{125}I-α-BuTX and subsequent γ-counting of the radioactivity bound to end-plate containing segments of superficial muscle fibers showed that the end-plate AChRs were 90-95% blocked by this preblock treatment (n = 6). BoTX-blocked soleus muscles were injected, on the fourth day of the block, with 10 μg α-BuTX, resulting in 70-80% block of preexisting AChRs (n = 2). None of these treatments severely impaired the behavior of the animals.

Electrophysiological experiments were carried out as described by Brenner et al. (1987). The bath solution consisted of: 40% Leibovitz L-15 medium added to a solution consisting of (in millimolar): NaCl, 140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 2.5; glucose, 5; Hepes, 5 (pH 7.2). This solution was used for recording of end-plate currents and for single channel current measurements. Fluctuation analysis experiments were performed in a bath solution containing (in millimolar): NaCl, 155; KCl, 4; EGTA, 2; Hepes, 5 (pH 7.2). End-plates in TTX-blocked muscle were localized by moving a blunt pipette filled with 1 M sucrose solution slowly along an individual muscle fibre. A marked increase in the frequency of miniature end-plate potentials (m.e.p.ps) indicated the presence of an end-plate underneath the pipette. In BoTX-blocked muscles, where not all end-plates responded to hypertonic solution, end-plates were localized by impaling the fibres near the finest nerve branches visible at a magnification of 200. End-plates were voltage clamped to −70−−80 mV membrane potential at such sites and impalements revealing miniature end-plate currents (m.e.p.c.s) with rise times <2 ms were accepted for analysis. The subtypes of AChRs expressed in the junctional and extrajunctional membranes were assessed from the gating properties of their ion channels by analysis of the decays of m.e.p.c.s or of ACh-induced membrane current fluctuations as described in Brenner et al. (1987). Properties of extrajunctional AChR channels were also examined on isolated muscle fibers by recording ACh-activated single channel current using patch-clamp techniques. In these experiments, fetal and adult AChR subtypes were distinguished by their different slope conductances as determined in the cell-attached configuration (Hamill et al., 1981).

To assess the block of neuromuscular transmission after chronic α-BuTX application soleus muscles were dissected with their nerves attached and placed in a recording chamber that allowed electrical stimulation of the attached motor nerve via bipolar electrodes. Intracellular recordings were made with KCl filled microelectrodes in the end-plate region to measure the size of stimulus evoked end-plate potentials. To ensure action potential generation in the motor nerve by the electrical stimulus (200 μs, 4-6 V) evoked extracellular field potentials were recorded.

Results

Changes in mRNA Levels after Denervation of Adult Muscle

Fig. 1 *a* compares autoradiograms obtained from Northern blots of total RNA from innervated and 7-d denervated rat triceps muscle hybridized with α-, β-, γ-, δ-, and ε-subunit specific cDNA probes. Quantitative changes in the levels of individual subunit mRNAs were estimated densitometrically from the autoradiograms. In innervated muscle, all subunit mRNAs except that coding for the γ-subunit are clearly resolved. Upon chronic denervation the levels of α- and δ-subunit-specific mRNAs increase between 30- and 50-fold. The relative increase of the γ-subunit specific mRNA is even stronger. The relative increase in β- and in particular the ε-subunit mRNAs is about one order of magnitude less (Fig. 1 *b*).

Changes in mRNA Levels upon Reinnervation of Denervated Muscle

To analyze changes of mRNA levels as a consequence of reinnervation of chronically denervated muscle the left phrenic nerve was crushed to allow development of denervation changes and subsequent reinnervation of the denervated hemidiaphragm ~7 d after the crush (Witzemann et al. 1987). The mRNA amounts measured 7 d after nerve crush were similar to the amounts present in muscle denervated by cutting the nerve (Fig. 2 *a*). Reinnervation of diaphragm begins 7-9 d after crushing the nerve and is complete after 15-19 d following denervation by crush. As shown in Fig. 2 *b*, the increase in subunit specific mRNAs is reversed upon muscle reinnervation since the levels of α-, β-, δ-, and ε-subunit mRNA are reduced to amounts characteristic of normal, innervated muscle and γ-subunit mRNAs become undetectable.

Thus the Northern blot hybridization experiments shown in Figs. 1 and 2 demonstrate that in adult normal muscle the total amounts of α-, γ-, and δ-subunit mRNA and, to a much smaller extent, that of the β- and ε-subunit mRNA are reduced by signals that depend on innervation of the muscle. These signals could be factors released from the nerve terminal or from cells associated with it or could depend on nerve evoked electrical activity of the muscle.

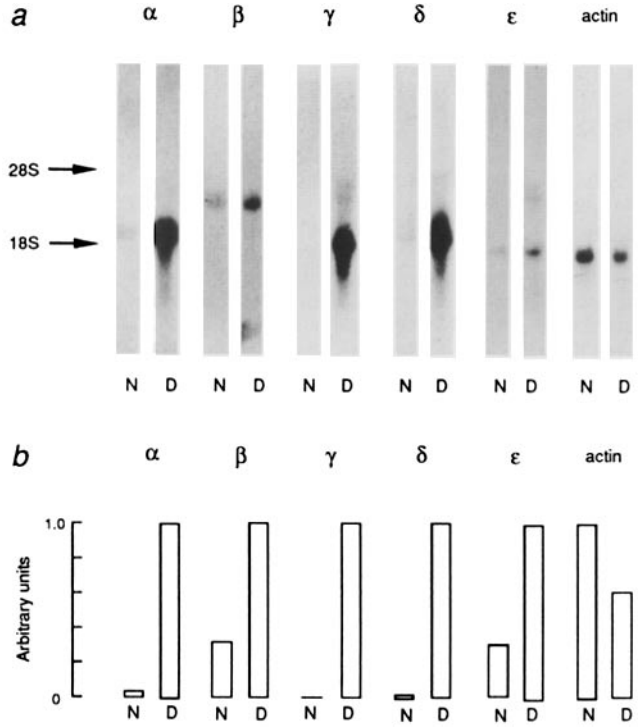

Figure 1. Effect of denervation on AChR subunit-specific muscular mRNA levels. (*a*) Autoradiograms of Northern blot hybridization analysis of RNA from normal (*N*) and 7 d denervated muscle (*D*). Total RNA was analyzed using AChR α-, β-, γ-, δ-, and ε-subunit specific cDNA probes and a β-actin cDNA probe. Autoradiography was performed at −80°C with an intensifying screen. Since actin mRNA levels are much higher than AChR mRNA levels, actin-hybridized blots were exposed only for a few hours instead of one to five days as for the AChR transcripts. The positions of rat ribosomal RNA are indicated by arrows. (*b*) Bar histogram showing the relative contents of AChR-subunit specific mRNAs and actin mRNA of normal (*N*) and 7-d denervated muscle (*D*). The autoradiograms obtained upon blot hybridization analysis, as shown in *a*, were scanned densitometrically. The relative contents of the respective mRNAs evaluated from a single experiment are normalized with respect to the values obtained for the mRNA levels of 7-d denervated muscle and which were set arbitrarily to 1. In case of actin mRNA values were normalized with respect to values of normal, innervated muscle.

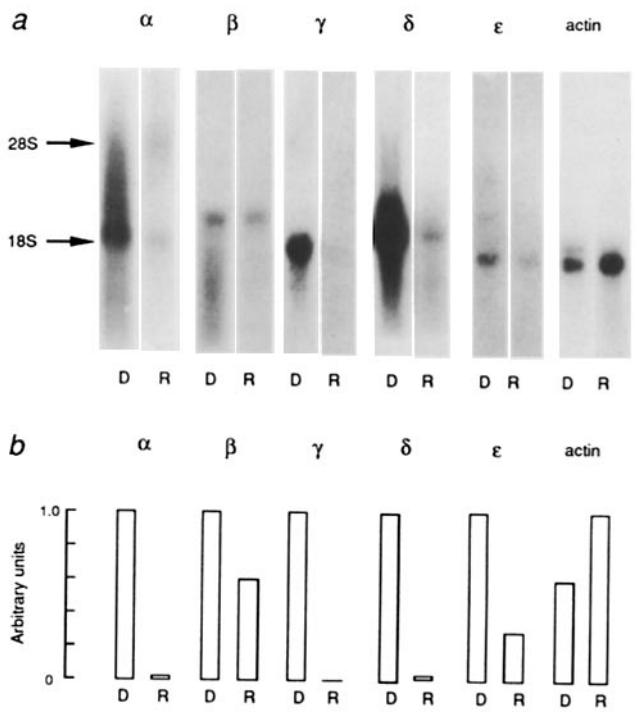

Figure 2. Effect of reinnervation of chronically denervated hemidiaphragm on AChR subunit-specific mRNA levels. (*a*) Autoradiograms of Northern blot hybridization of total RNA from 7-d denervated (*D*) and from denervated and subsequently reinnervated (*R*) muscles. Denervation was performed by crushing the left phrenic nerve. Reinnervation of hemidiaphragm begins ~7–9 d after nerve crush and is complete by 15–19 d. RNA was prepared from hemidiaphragm 30 d after nerve crush. Analysis was performed using AChR α-, β-, γ-, δ-, and ε-subunit-specific cDNA probes and a β-actin cDNA probe. Arrows indicate the positions of rat ribosomal RNA. (*b*) Bar histogram showing the relative contents of AChR-subunit-specific mRNAs and actin mRNA of denervated (*D*) and reinnervated (*R*) muscles as described in *a*. The autoradiograms shown in *a* were evaluated densitometrically and the relative contents of the respective mRNAs are normalized with respect to the values obtained for the mRNA levels of 7-d denervated muscles (arbitrarily set to 1). In case of actin the value of the reinnervated muscle was arbitrarily set to 1.

Changes in mRNA Levels in Denervated Muscle after Exogenous Stimulation

Previous results (Goldman et al., 1988) showed that electrical muscle stimulation, beginning at the time of denervation, prevented the increase in α-, β-, γ-, and δ-subunit specific mRNAs observed in denervated electrically inactive muscles. In the present experiments the effect of electrical activity alone on the AChR subunit-specific mRNA levels was examined by exogenous stimulation of chronically denervated muscles via implanted electrodes (Lømo et al., 1985). Stimulation was begun 7 d after the muscle had been denervated and stimulation was then maintained for a further 7 d. Muscles were stimulated chronically in 100-Hz trains of 1 s duration, applied once every 100 s. This high frequency stimulation pattern has been found to be highly effective at suppressing extrajunctional denervation supersensitivity (Lømo and Westgaard, 1975).

Northern analysis showed that the mRNAs encoding the α-, γ-, and δ-subunit were strongly reduced by electrical stimulation (Fig. 3 *a*), whereas β-subunit mRNAs displayed a much smaller decrease and ε-subunit mRNA remained unchanged. Densitometric analysis of the autoradiograms suggested that electrical activity reduced mRNA levels in denervated muscles to the levels observed in innervated controls, i.e., the γ-subunit mRNA became undetectable, whereas the α- and δ-subunit mRNAs were strongly reduced but not abolished. Similar data were found in three experiments (Fig. 3 *b*). Fig. 4 shows that the AChR subunit mRNA in chronically denervated muscle that is resistant to electrical stimulation is located in the former end-plate region. Autoradiograms of Northern blots of total RNA from denervated and denervated/stimulated muscles hybridized with α-, β-, δ-, and ε-subunit-specific probes reveal a characteristic difference between end-plate-rich muscle strips and strips lacking end-plates. The effect of denervation and of electrical stimulation on α-subunit-specific mRNA level (Fig. 4 *a*) reveals a dramatic

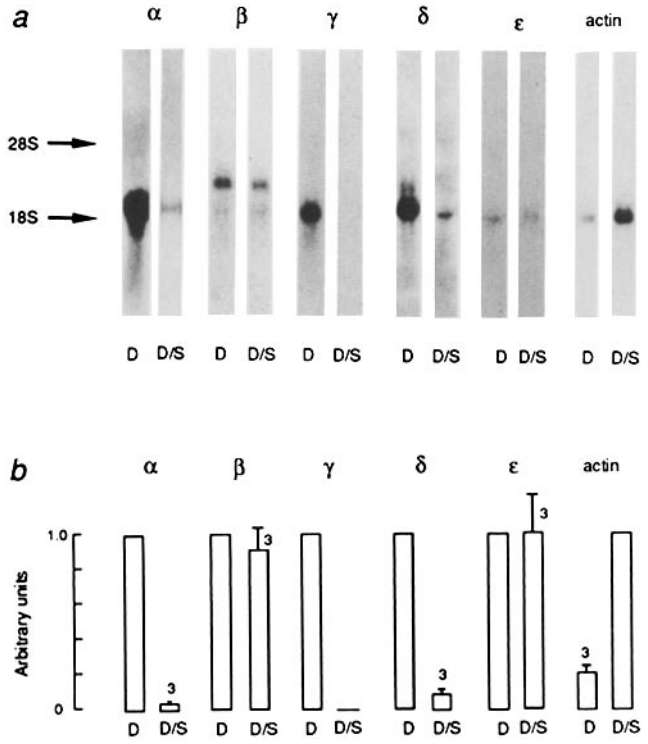

Figure 3. Effect of electrical stimulation on AChR subunit-specific mRNA levels in chronically denervated muscle. (*a*) Autoradiograms of Northern blot hybridization analysis of total RNA from 14-d denervated (*D*) and from denervated (14 d) and stimulated (last 7 d; *DS*) muscle. Analysis was performed using AChR α-, β-, γ-, δ-, and ε-subunit specific cDNA probes and a β-actin cDNA probe. Arrows indicate the positions of rat ribosomal RNA. (*b*) Bar histogram showing the relative contents of AChR-subunit-specific mRNAs and actin mRNA of denervated (*D*) and denervated/stimulated (*D/S*) muscles as described in *a*. Autoradiograms obtained from three separate experiments (number above bars) were evaluated densitometrically and the relative contents of the respective mRNAs are normalized with respect to the values obtained for the mRNA levels of 14-d denervated muscles (arbitrarily set to 1). The standard deviation of the mean (SD) is shown by the error bars.

change on extrasynaptic muscle segments. In stimulated muscles, α-subunit mRNA was no longer detectable. In synaptic regions, a comparable strong downregulation was observed, with the difference that a low amount of α-subunit mRNA was resistent to muscle activity. The decrease in mRNA may reflect the fact that end-plate-rich muscle strips contain extrasynaptic fiber segments. In contrast, the ε-subunit mRNA is barely affected by electrical stimulation (Fig. 4 *b*). The β- and δ-subunit mRNAs showed a behavior comparable to that of the α-subunit mRNA (Fig. 4 *c*), except that for the β-subunit mRNA almost no changes occurred in synaptic regions, regardless of whether the muscle was denervated or dener-

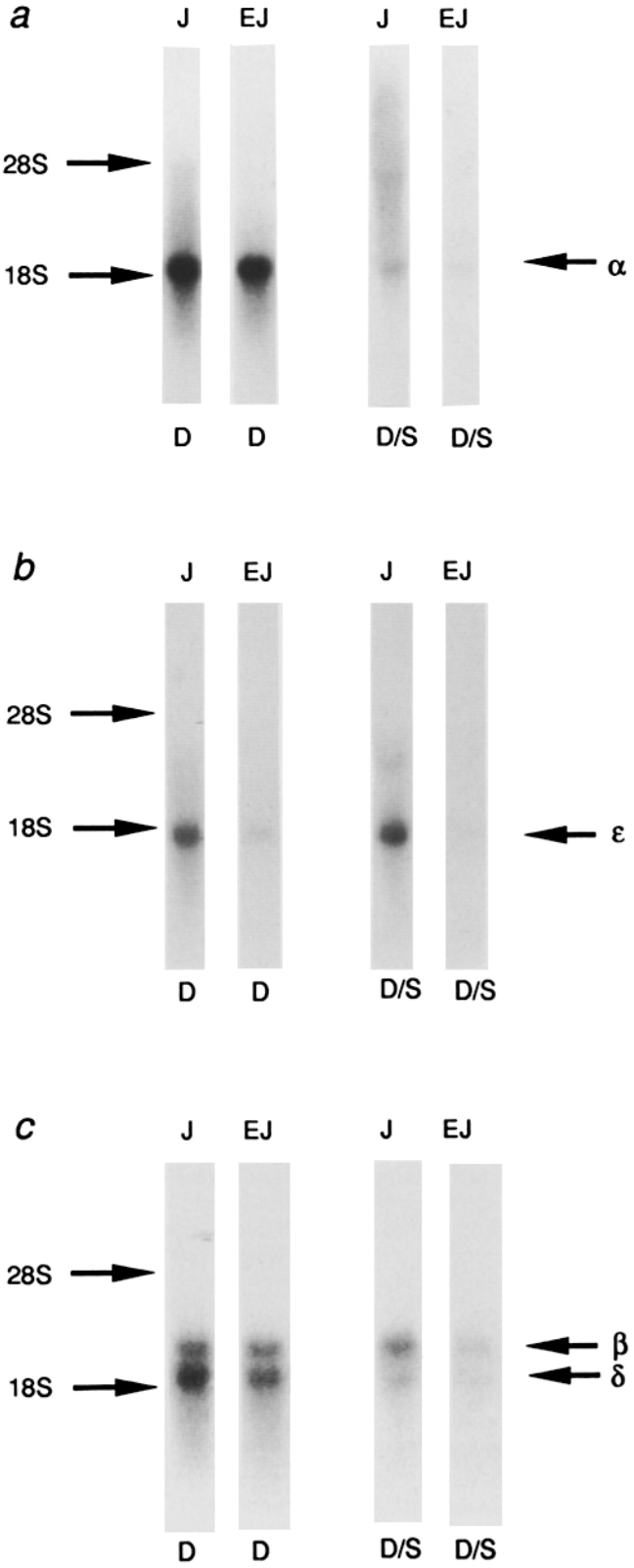

Figure 4. Differential effect of denervation and exogenous electrical stimulation on α-, β-, δ-, and ε-subunit mRNA levels in synapse rich and extrasynaptic fiber segments. Denervation and stimulation protocols as in Fig. 3. (*a* and *b*) Autoradiograms of Northern blot hybridization of total RNA from junctional (*J*) and extrajunctional (*EJ*) strips of denervated muscle (*D*) and of denervated and subsequently stimulated (*D/S*) muscle using α- and ε-subunit-specific cDNA probes. Arrows on the left indicate the positions of rat ribosomal RNA. (*c*) Autoradiograms of Northern blot hybridization of total RNA from junctional and extrajunctional muscle segments in denervated and denervated/stimulated muscle using β- and δ-subunit specific cDNA probes. The same blot was used for hybridization with the two probes. Note differences in the response of mRNAs in end-plate containing muscle segments where β-subunit mRNA level is barely affected by electrical stimulation.

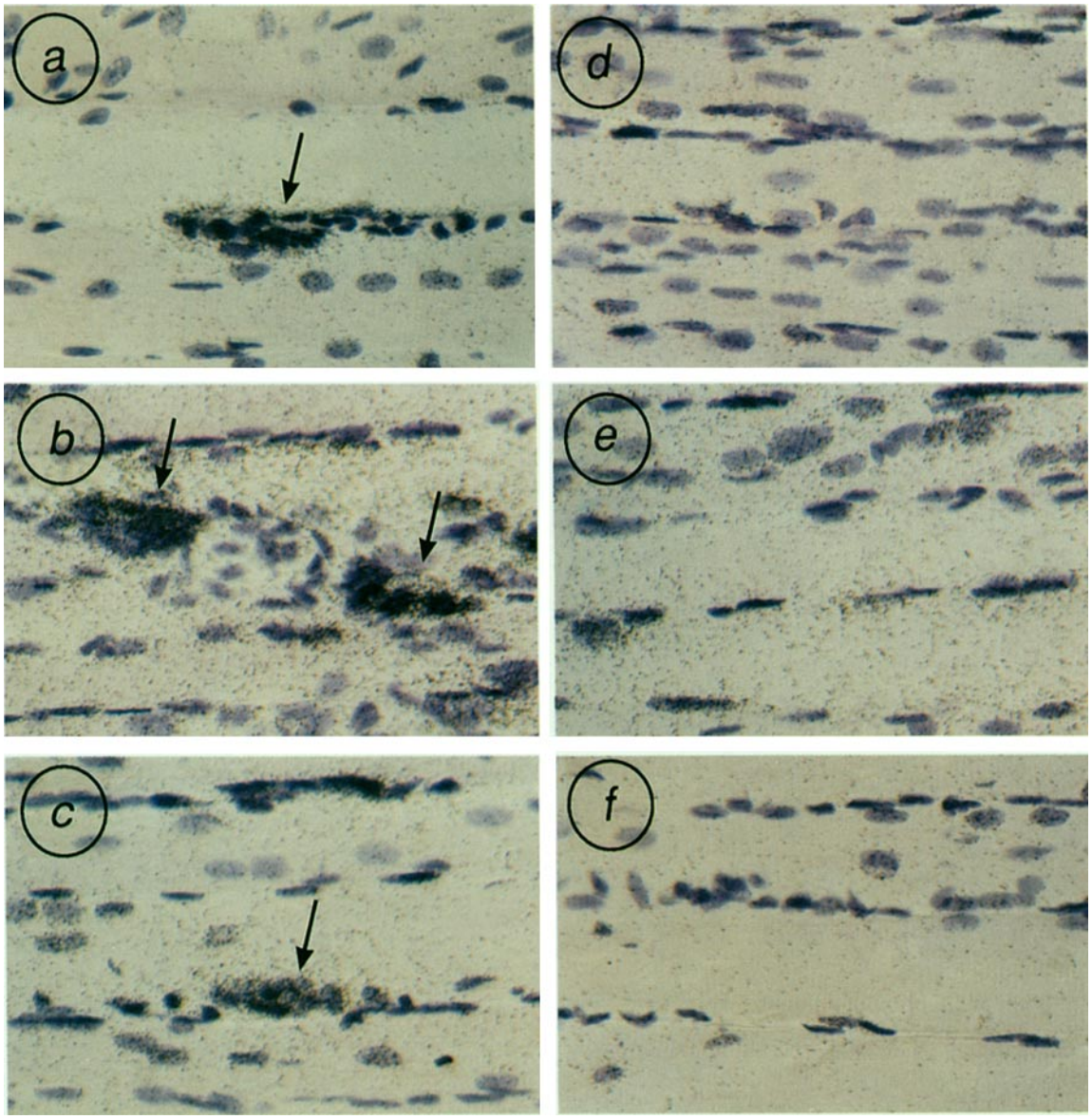

Figure 5. In situ hybridization of normal, denervated and denervated/stimulated muscle with α-subunit-specific RNA probes. Distribution of α-subunit-specific mRNA in synaptic (*left column*) and extrasynaptic (*right column*) fiber segments in control (*a* and *d*), denervated (*b* and *e*) and denervated/stimulated muscle (*c* and *f*). The areas of high grain density in *a–f* correspond to the end-plate region of the fiber as determined by staining for ACh esterase activity as described in Brenner et al. (1990). All sections were hybridized with the same batch of probes and simultaneously processed for in situ hybridization.

vated/stimulated. In the extrasynaptic region there was a significant increase in β- and δ-subunit mRNA upon denervation, which could be largely repressed upon stimulation.

This result was confirmed by in situ hybridization experiments. In denervated fibers α-subunit mRNA was produced by nuclei both in the synaptic and extrasynaptic fiber segments (Fig. 5, *b* and *e*), whereas in denervated/stimulated fibers, grains were restricted to end-plate nuclei (Fig. 5, *c* and *f*). On the other hand, ε-subunit mRNA remained restricted to the end-plate nuclei in denervated (Brenner et al., 1990) and in denervated/stimulated muscle (Fig. 6 *a*). The β- and δ-subunit mRNAs showed a behavior comparable to that of the α-subunit and the remaining mRNA in denervated/stimulated muscle was restricted to end-plate nuclei as shown by in situ hybridization experiments (Fig. 6, *b* and *c*). These data suggest that in adult muscle fibers the α-, β-, δ-, and ε-genes are expressed permanently and independently of electrical activity in synaptic nuclei even in the absence of the nerve terminal.

Changes in mRNA Levels in Innervated But Disused Muscle

To examine the regulation of AChR subunit mRNA levels by putative neurotrophic factors in the absence of electrical muscle activity muscles were paralyzed while the nerve-muscle connection was left intact. This can be achieved by chronic application of highly specific neurotoxins that cause muscle

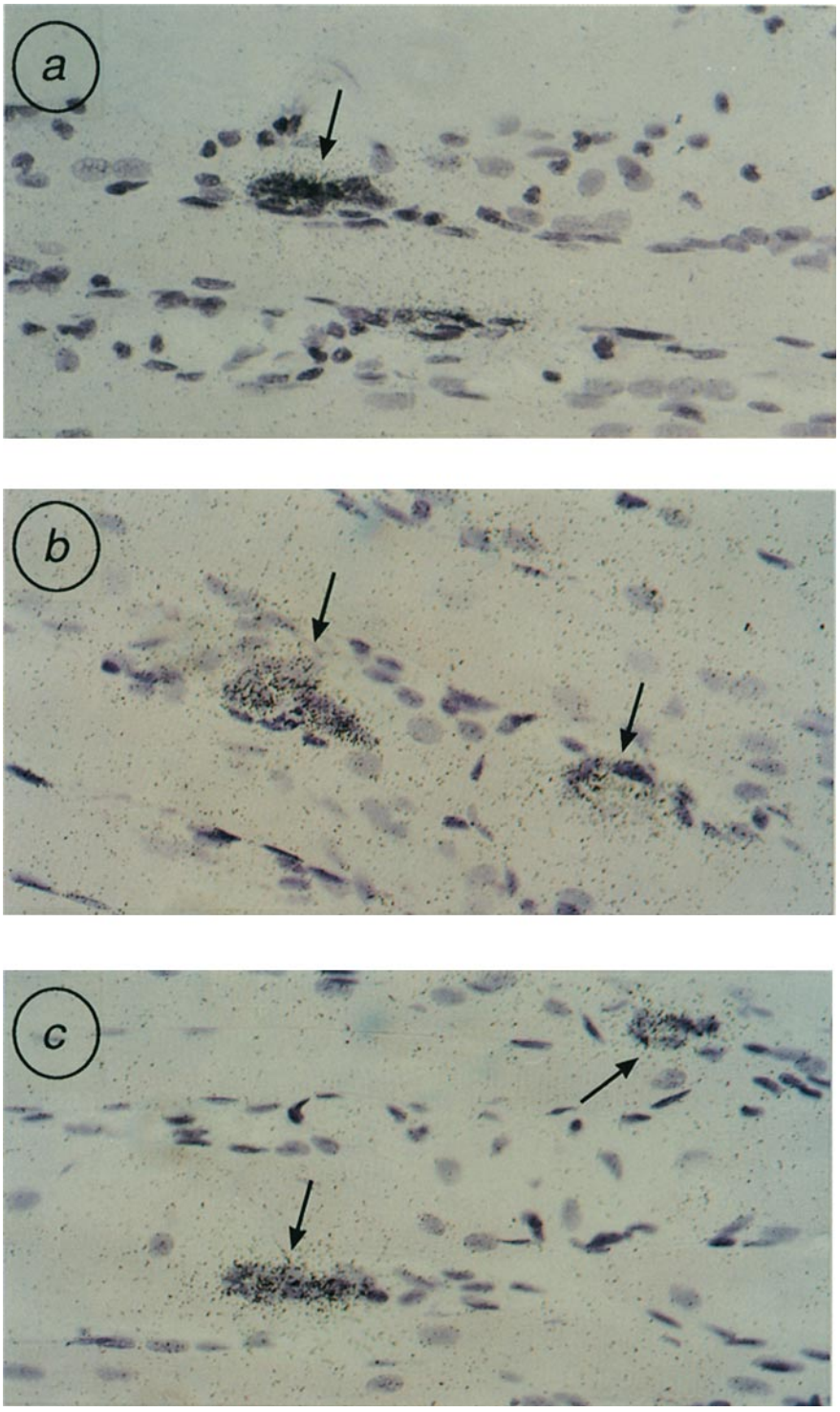

Figure 6. In situ hybridization of denervated and subsequently stimulated muscle with β-, δ-, and ε-subunit-specific mRNA probes in synaptic fiber segments. Areas of high-grain density correspond to the former end-plate region as determined by staining for ACh esterase activity and are indicated by arrows. (*a*) ε-subunit mRNA (muscles were denervated for 8 d followed by 6 d of stimulation), (*b*) β-subunit mRNA (denervation 5 d, stimulation 8 d), *c*: δ-subunit mRNA (denervation 4 d, stimulation 10 d).

paralysis by blocking impulse conduction in the motor nerve or by blocking different steps of neuromuscular transmission. The two types of muscle paralysis, denervation or toxin blockade, affected subunit-specific mRNA levels in a different fashion.

In Fig. 7 *a*, autoradiograms of blot hybridization analysis of total RNA from rat triceps muscle after 10 d of nerve conduction block with TTX and after 10 d of denervation are compared. In TTX-paralyzed muscle α- and δ-subunit transcripts increased strongly and reached 60–70% of the amounts found after denervation. The small increase in β- and ε-subunit mRNA was comparable to that after denervation. In contrast, the γ-subunit mRNA increased only marginally in TTX paralyzed muscle compared to what is seen in surgically denervated muscle. Densitometric estimates of changes in AChR subunit mRNAs are given in Fig. 7 *b*. Similar results were obtained when only soleus muscles were analyzed. In four muscles paralyzed for 8–15 d with a very high daily dose of

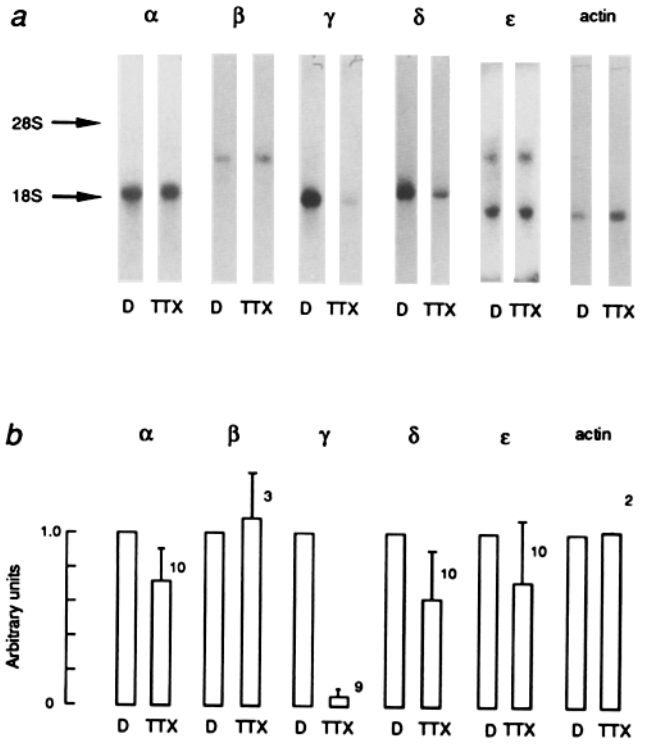

Figure 7. Differential effect of TTX-induced paralysis and of denervation on AChR subunit-specific mRNA levels. (*a*) Autoradiograms of Northern blot hybridization of total RNA from 9–10 d denervated (*D*) and 9–10-d TTX-paralyzed (TTX) muscles. Analysis was performed using α-, β-, γ-, δ-, and ε-subunit-specific cDNA probes and a β-actin cDNA probe. Arrows indicate the positions of rat ribosomal RNA. (*b*) Bar histogram showing the relative contents of AChR subunit-specific mRNAs and actin mRNA of denervated (*D*) and TTX-paralyzed (*TTX*) muscles as described in *a*. Autoradiograms obtained from separate experiments were evaluated densitometrically and the relative contents of the respective mRNAs were normalized with respect to the values obtained for the mRNA levels of 9–10 d denervated muscles (arbitrarily set to 1). The larger RNA species hybridizable with the ε-subunit specific probe which may represent incompletely spliced RNA (Mishina et al., 1986) was not included for densitometric evaluation. Height of each bar represents mean value from separate experiments. SD of the mean is indicated by error bars. The numbers given with bar represent number of separate experiments.

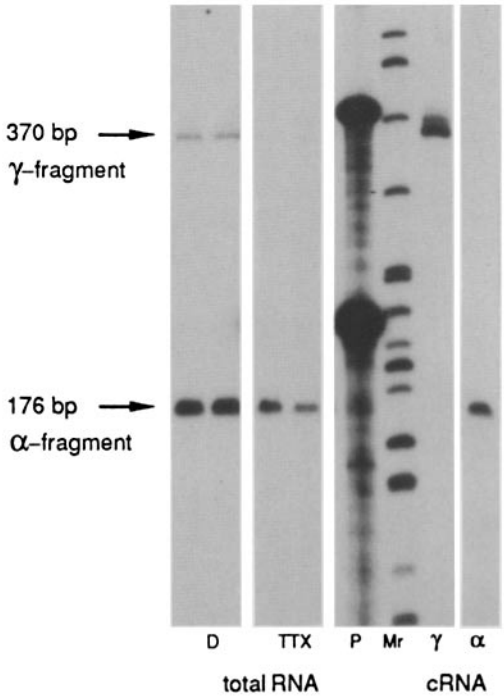

Figure 8. Liquid hybridization analysis of α- and γ-subunit-specific RNA content in denervated and TTX paralyzed muscles. 10 μg total RNA from muscle treated as described in the legend of Fig. 6 *a* was hybridized with α- and γ-subunit-specific ^{32}P-labeled antisense cRNA probes. The figure shows, from left to right, autoradiograms of two separate RNA preparations of denervated (*D*) and TTX-paralyzed (*TTX*) muscles subjected to RNase protection analysis. The lane marked *P* shows the sizes of the ^{32}P-labeled antisense cRNA probes, the α-subunit-specific probe containing 211 and the γ-subunit-specific probe 391 nucleotides. Lane M_r, ^{32}P-labeled HpaII-DNA fragments of pBR322 used as size markers. 1,000 pg of γ- and α-subunit-specific sense cRNAs were subjected to RNase protection assays under identical conditions as the total RNAs of muscle. Additional assays using 500, 200, and 100 pg of α- and γ-subunit-specific cRNA were performed (not shown) to obtain a calibration curve for the quantitation of α- and γ-subunit mRNA content in muscle. The RNase protected fragment of the α-subunit mRNA corresponds to 176 bp as indicated on the left side. The content of α-subunit mRNA in denervated muscle is 168 ± 28 amol (number of RNase protection assays, n = 8) in TTX paralyzed muscle and 126 ± 42 amol (n = 9). The RNase protected fragment of the γ-subunit mRNA corresponds to 370 bp as indicated on the left side. The content of γ-subunit mRNA in denervated muscle is 32 ± 18 amol (n = 8) and in TTX paralyzed muscle 5.8 ± 1.2 amol (n = 9).

12 μg of TTX (see Materials and Methods) the increase in γ-subunit mRNA was only 12% (±0.04 SEM) of that seen in surgically denervated muscle.

Absolute Levels of Subunit-specific mRNAs in Normal, Denervated, and Disused Muscle

To confirm the Northern blot hybridization analysis of TTX-blocked muscle mRNA we used liquid RNA hybridization analysis, which allows quantitation of the absolute contents in subunit specific mRNA. Fig. 8 shows α- and γ-subunit-specific fragments obtained upon RNase protection assays. Denervated muscle contained 168 ± 28 amol (n = 8) of α-subunit mRNA and 32 ± 18 amol (n = 8) of γ-subunit mRNA per μg of total RNA. TTX-treated muscle contained only a slightly reduced amount of α-subunit mRNA (126 ± 42 amol/μg of total RNA; n = 9) but much lower amounts of γ-subunit mRNA (5.8 ± 1.2 amol/μg of total RNA; n = 9). Thus, both the Northern and liquid hybridization experiments show that in disused muscle the intact nerve–muscle contact suffices to inhibit drastically γ-subunit mRNA levels even in electrically silent muscle which is very different from the strong increase seen in surgically denervated muscle.

To examine the possibility that in these experiments, the increase in γ-subunit-specific mRNA may have been selectively suppressed by undetected electrical muscle activity, for example due to incomplete nerve conduction block by the TTX cuffs, we tested for a differential effect of impulse activity on α- and γ-subunit mRNA levels. The levels of α- and γ-subunit-specific mRNAs were compared in soleus muscles that were stimulated for 4 d by 10 or 0.5 Hz trains of 10 s

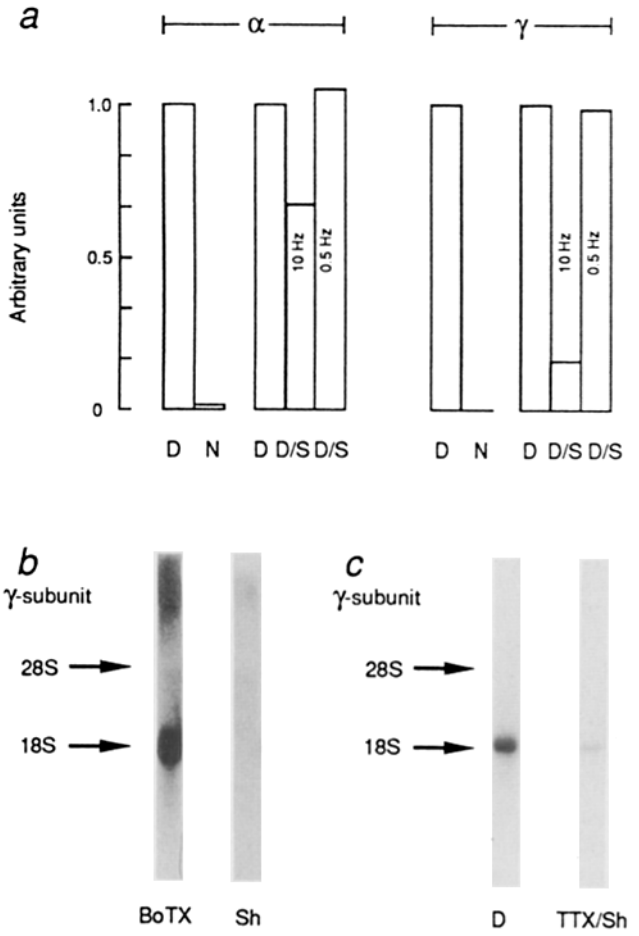

Figure 9. Control experiments to demonstrate the specificity of the difference between γ-subunit mRNA level in muscles paralyzed by surgical denervation and BuTX paralysis or TTX application. (*a*) Bar histogram of densitometric evaluation of autoradiograms obtained upon Northern blot hybridization of total RNA from denervated (*D*) and denervated/stimulated (*D/S*) muscle. Muscles were denervated for 4 d and subsequently stimulated for 7 d. Hybridization was performed using α- and γ-subunit-specific cDNA probes. Stimulus patterns of 0.5 and 10 Hz were applied to show that high α- and γ-subunit mRNA levels persist in muscles stimulated by low frequency. (*b*) Autoradiograms upon Northern blot hybridization of RNA from BoTX injected (*BoTX*) and sham injected muscles (*Sh*). Hybridization was performed using γ-subunit-specific cDNA probe to show that the increase in γ-subunit mRNA in BoTX treated muscle is not a consequence of injection procedure. (*c*) Autoradiogram upon Northern blot hybridization of total RNAs from muscle where the muscle was denervated (*D*) and from muscle in which the motor nerve was TTX cuffed and which, in addition, was sham injected (*TTX/Sh*) with saline. Hybridization was performed with γ-subunit-specific cDNA probe.

duration applied once every 100 s, beginning with the time of denervation (Fig. 9 *a*). In the muscles stimulated with the 10-Hz protocol the levels of both the α- and the γ-subunit specific mRNAs were significantly reduced with respect to the unstimulated control, the effect on the γ-subunit mRNA being somewhat stronger than on the α-subunit mRNA level. With the 0.5-Hz stimulation protocol, however, their levels were similar to that in denervated muscles (Fig. 9 *a*) suggesting that in rat muscle these two subunit mRNAs are affected by low levels of muscle activity in a comparable manner. Furthermore, previous experiments in cultured chick muscle cells suggested that γ-subunit mRNA expression is less affected by electrical activity than α-subunit mRNA (Shieh et al., 1988; Harris et al., 1988). Together, these data indicate that the differential effect of TTX paralysis on α- and γ-subunit mRNA levels could not have been caused by incomplete blockade of motor nerve induced muscle activity.

Degenerating nerve terminals in unstimulated muscles contribute significantly to the development of extrajunctional ACh sensitivity for up to ~15 d after denervation (Cangiano, 1985). To assess a possible role of degenerating nerve in our experiments, we also examined triceps muscles after 14 and 19 d of chronic TTX blockade. Whereas the levels of α-, δ-, and ε-subunit mRNAs were comparable in denervated and in paralyzed muscle, that of γ-subunit mRNA always remained significantly lower in TTX paralyzed muscles. In one experiment with 19 d of TTX block, the γ-subunit mRNA was only 15% of that seen in surgically denervated muscle.

In summary, the data suggest that the level of γ-subunit mRNA is much less affected than that of the α- and δ-subunit mRNAs by mere disuse of muscles in which the neuromuscular junction is left intact. In innervated muscle γ-subunit mRNA is, therefore, downregulated by an activity independent inhibitory neural signal.

Changes in α-BuTX Binding after Denervation and after Disuse by Impulse Conduction Block

AChR expression in TTX paralyzed and in denervated muscles was compared by measuring the amount of ^{125}I-α-bungarotoxin bound to respective muscle homogenates. Specific toxin binding to AChR was estimated from the difference in radioactivity bound in the presence or absence of a hundredfold excess of unlabeled native α-BuTX to triton X-100 solubilized muscle homogenates. The TTX-blocked muscles contained 69 ± 13 fmol (n = 9) of α-BuTX binding sites/mg of protein. This is only ~42% of the number of toxin binding sites found in denervated muscle which contained 163 ± 30 fmol (n = 7)/mg of protein, in accordance with previous measurements (Bray et al., 1979; Drachman et al., 1982).

Changes in mRNA Levels in Muscle after Block of Neuromuscular Transmission

Presynaptic Block. Muscle fibers can be paralyzed chronically by blocking functional neuromuscular transmission by botulinum toxin (BoTX) (Habermann and Dreyer, 1986; Thesleff, 1989*a*,*b* for reviews) causing muscle supersensitivity to ACh (Thesleff, 1960). With this toxin the muscle paralysis is caused by the block of evoked and spontaneous Ca^{2+}-dependent quantal release of acetylcholine from the motor nerve terminals. The functional block is caused by a reduction of the size of evoked end-plate potentials and is accompanied by a reduced frequency of spontaneous miniature end-plate potentials (m.e.p.p.s). Specifically, the latter is reduced to a few percent of the control and nerve impulses rarely release more than a single quantum (Tsujimoto and Kuno, 1988). The invasion of the nerve terminal by action potentials, the nonquantal release of ACh (Stanley and Drachman, 1983) and axonal transport (Pestronk et al., 1976) remain unaffected, however. To examine how this type of muscle paralysis affected the increase in the AChR subunit-specific mRNAs we measured their abundance in BoTX-paralyzed

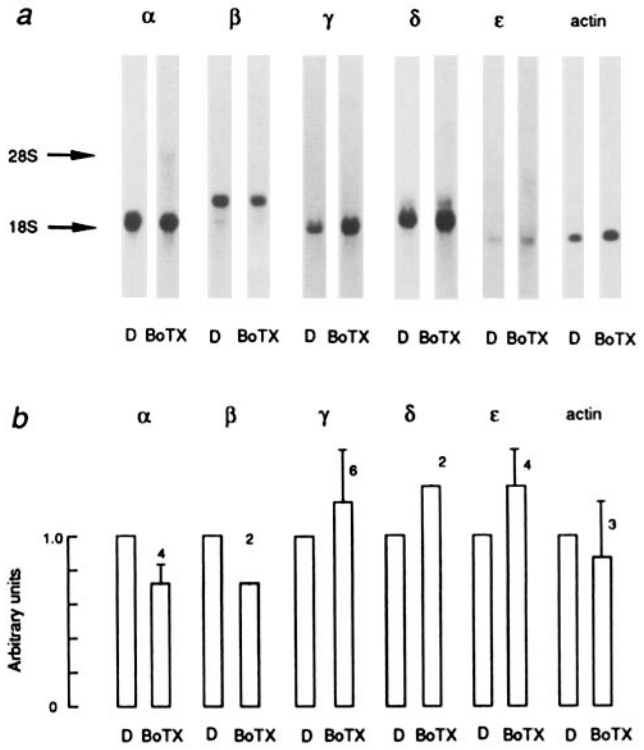

Figure 10. Effect of BoTX-induced paralysis on AChR subunit-specific mRNA levels. (*a*) Autoradiograms of Northern blot hybridization analysis of RNA from 7-d denervated (*D*) and 7-d BoTX paralyzed (*BoTX*) muscle. Total RNA was analyzed using AChR α-, β-, γ, δ-, and ε-subunit specific cDNA probes and a β-actin cDNA probe. Arrows indicate the positions of ribosomal RNA. (*b*) Bar histogram showing the relative contents of AChR-subunit specific mRNAs and actin mRNA of denervated (*D*) and BoTX paralyzed (*BoTX*) muscles (as in *a*). Autoradiograms obtained from separate experiments were evaluated densitometrically and the relative contents of the respective mRNAs were normalized with respect to the values obtained for the mRNA levels of 7-d denervated muscles (arbitrarily set to 1). SD is indicated by error bars. The numbers given in the graph represent number of separate experiments.

muscle. Fig. 10 *a* shows autoradiograms of Northern blot hybridization experiments with total RNA obtained 5–7 d after the injection of BoTX. They revealed an effect on AChR mRNAs similar to muscle denervation, i.e., there is a strong increase in α- (Lipsky et al., 1989), as well as in γ- and δ-subunit-specific mRNAs and a much smaller increase in ε-subunit mRNA whereas the β-subunit-specific mRNA remained almost unchanged (Fig. 10 *b*). The difference in γ-subunit mRNA levels in TTX and BoTX paralyzed muscle suggests that BoTX interrupts a neural signal, which in the innervated muscle reduces γ-subunit mRNA levels and which is not affected by block of nerve impulse conduction.

It might be argued that the stronger effect of BoTX blockade versus that of TTX-induced muscle disuse on AChR-specific mRNAs could be due to the muscle lesion inflicted by the toxin injection (Cangiano, 1985). To test this possibility we injected normal muscle with saline, which, however, did not cause an increase in γ-subunit mRNA (Fig. 9 *b*). We also found that in two rats, where the effect of BoTX was more generalized and led to the partial paralysis of the uninjected contralateral leg muscle, a marked increase in all subunit-specific mRNAs was also seen in the uninjected muscle which was similar to that observed in injected muscle. Finally, sham injections into TTX paralyzed muscle did not lead to an additional increase in γ-subunit mRNA levels (Fig. 9 *c*). Thus, the possible muscle damage due to the injection procedure in BoTX experiments did not cause the difference in the increase in γ-subunit mRNA levels in muscles paralyzed by BoTX and TTX, respectively.

Postsynaptic Block. Another way to paralyze the leg muscle chronically is to interrupt neuromuscular transmission by blocking end-plate AChRs by α-bungarotoxin (α-BuTX). After 3–4 d of α-BuTX paralysis, muscles have become supersensitive to ACh (Drachman et al., 1982). Comparing the rise in α-, γ-, and δ-subunit mRNA in α-BuTX paralyzed muscle with that observed in surgically denervated muscle shows that the muscular content in all three subunit mRNA rises significantly less than in denervated muscle (Fig. 11). RNase protection experiments were performed using total RNA from 3-d denervated and 3–4-d α-BuTX paralyzed muscles. Using the α-subunit-specific RNA probe (see Materials and Methods) the content of α-subunit mRNA was 145 amol/μg of total RNA (one experiment) but only 15 amol/μg RNA in the α-BuTX-treated muscle. With the γ-subunit-specific RNA probe the amount of γ-subunit mRNA was 22.5 amol/μg RNA in the denervated muscle but only 4.4 amol/μg RNA in the α-BuTX-paralyzed muscle. The considerably lower levels of α- and γ-subunit-specific mRNAs in toxin blocked muscles suggest that either activation of a remaining small percentage of unblocked AChR channels could be sufficient to inhibit the rise in α- and γ-subunit mRNA levels or that the level of both mRNAs is reduced by a neural signal that bypasses activation of the AChR channel. We therefore measured the occurrence and size of miniature end-plate potentials (m.e.p.p.s) and stimulus evoked e.p.p.s after 3–4 d of α-BuTX paralysis before Northern analysis of the muscle. In none of the α-BuTX paralyzed muscles (n = 4) m.e.p.p.s were detected. In two muscles that were examined for the occurrence of stimulus evoked e.p.p.s it was found that in <10% of the examined fibres (50–70 examined per muscle) e.p.p.s were detectable. E.p.p. amplitudes were <1 mV in all cases which is much smaller than seen in normal muscles (>10 mV at resting potentials of −60–−70 mV). Thus even in these fibers in which e.p.p.s could be detected the block of end-plate channels would be >95% complete.

To compare the effects of α-BuTX-blockade with that of TTX-induced blockade under similar conditions we measured the α- and γ-subunit mRNA levels also in 3–4 d TTX paralyzed muscles. Both subunit mRNAs are present in significantly lower amounts than in 3–4-d denervated muscle (Fig. 11, *c* and *d*).

Comparison of Muscle Disuse and Pre- and Postsynaptic Block of Neuromuscular Transmission

The difference in increase in α- and γ-subunit mRNAs in TTX-, BoTX-, and α-BuTX-paralyzed muscles as determined from Northern blot hybridization analysis is summarized in Fig. 12. To illustrate the difference in the effect of different toxins the mRNA levels were normalized with respect to those seen in surgically denervated muscle. It is obvious that denervation and BoTX blockade have similar effects on α- and γ-subunit specific mRNA levels when compared after 5–7 d

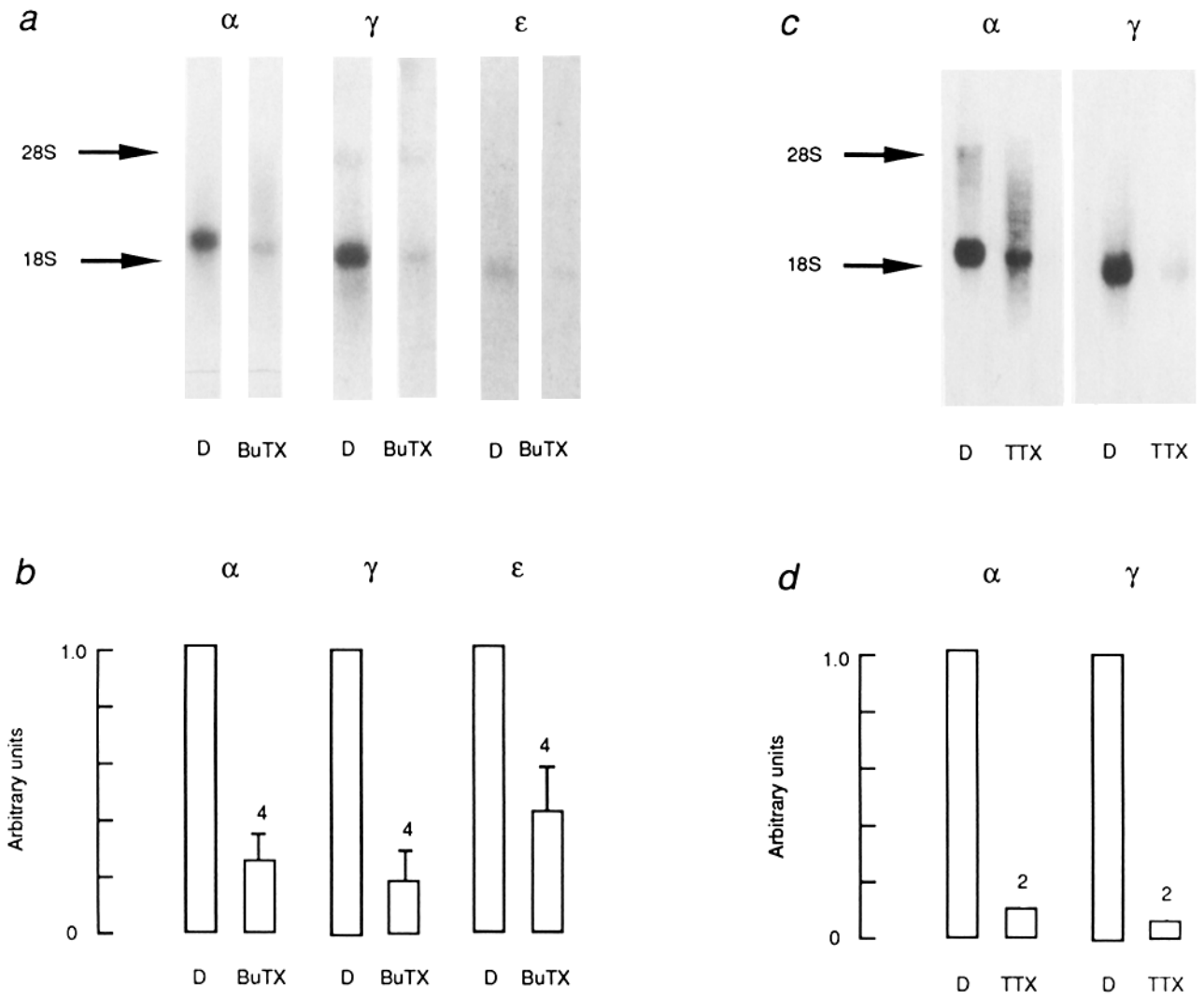

Figure 11. Effect of α-BuTX induced paralysis on the level of α-, γ-, and ε-subunit-specific mRNA. For comparison the effects induced by surgical denervation and by TTX paralysis are also shown. (*a*) Autoradiograms of Northern blot hybridization analysis of RNA from 3–4 d denervated (*D*) and 3–4 d α-BuTX paralyzed (BuTX) soleus muscle. Total RNA was analyzed using AChR α-, γ-, and ε-subunit specific cDNA probes. Arrows indicate the positions of ribosomal RNA. (*b*) Bar histogram showing the relative contents of α-, γ-, and ε-subunit specific mRNAs of denervated (*D*) and α-BuTX paralyzed (*BuTX*) muscles (as in *a*). Autoradiograms obtained from separate experiments were evaluated densitometrically and the relative contents of the respective mRNAs were normalized with respect to the values obtained for the mRNA levels of 3–4 d denervated muscles (arbitrarily set to 1). SD is represented by error bars. The numbers given with error bars represent numbers of separate experiments. (*c*) Autoradiograms of Northern blot hybridization analysis of RNA from 3 d denervated (*D*) and 3–4-d TTX-paralyzed (*TTX*) muscle. Total RNA was analyzed using AChR α- and γ-subunit specific cDNA probes. Arrows indicate the positions of ribosomal RNA. (*d*) Bar histogram showing the relative contents of α- and γ-subunit-specific mRNAs of denervated (*D*) and TTX paralyzed (*TTX*) muscles (as in *c*). Autoradiograms obtained from two independent experiments were evaluated densitometrically and the values were normalized with respect to the values obtained for the mRNA levels of 3-d denervated muscles (arbitrarily set to 1). Bar height represents mean of the two experiments.

of paralysis. In contrast, the paralysis by TTX or by α-BuTX causes a very different pattern of mRNA increase. In TTX-blocked muscle the α-subunit mRNA rises to a slightly smaller degree than seen with the BoTX or denervation paralysis and significantly less in α-BuTX blocked muscle. The γ-subunit mRNA level increases much less both with TTX and α-BuTX induced paralysis. This suggests that a presynaptic factor that is dependent on Ca^{2+}-mediated release suppresses predominantly the level of γ-subunit mRNA in electrically silent muscle and to a lesser degree also the α-subunit mRNA level. Secondly, the data show that activation of end-plate channels and ion flow across postsynaptic membrane might not be required for α- and γ-subunit mRNA suppression by the putative inhibitory neural factor.

AChR Channel Subtypes in Innervated Disused and Blocked Muscle

The differences in γ-subunit-specific mRNA levels seen in muscles paralyzed by different toxins suggested that the subunit composition of the AChRs in these muscles might be different, i.e., different ratios of $AChR_\epsilon$ and $AChR_\gamma$ channel subtypes might be observed. Therefore, we compared the proportions of the two AChR subtypes present in synaptic

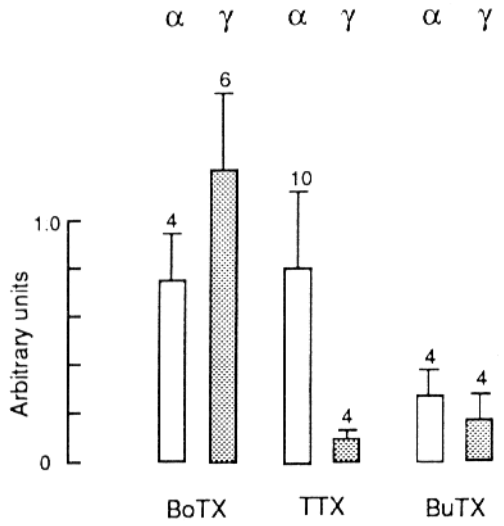

Figure 12. Comparison of the differential effect of various types of muscle paralysis on α- (*light bars*) and γ-subunit (*stippled bars*) specific mRNA levels. Bar histograms of densitometric evaluation of autoradiograms of total muscle RNA hybridized with subunit specific cDNA probes. All densitometric readings of toxin paralyzed muscle (*BoTX*, *TTX*, and *BuTX*) were normalized with respect to the values obtained in denervated muscle (set arbitrarily to 1). Error bars indicate standard deviation. The numbers above each bar refer to the number of independent experiments. In each experiment mRNAs of a toxin paralyzed and a surgically denervated muscle were prepared in the same way to allow pairwise comparison of mRNA levels. BoTX and TTX paralysis lasted for 5–7 d, α-BuTX paralysis for 3–4 d.

and extrasynaptic membranes after TTX and BoTX paralysis by electrophysiological techniques. Because synaptic AChRs in the soleus muscle retain their high metabolic half-life of >10 d for at least 8 d even after denervation (Bevan and Steinbach, 1983) and their number remains unchanged, up to 50% of the original synaptic AChRs present at the beginning of the paralysis are still present in the end-plate membrane after 8–14 d of TTX or BoTX paralysis, i.e., when the AChR properties were examined. Therefore, to detect the functional properties of the newly inserted AChR channels alone, we blocked the original AChRs with α-BuTX at the time when muscle paralysis was begun.

End-plate AChR Channels. In muscles chronically paralyzed by TTX and BoTX spontaneous miniature end-plate currents (m.e.p.c.s) were still recorded after block of preexisting AChRs. Fig. 13, *a* and *b* compares records of m.e.p.c.s from a normal soleus end-plate and one from an end-plate after 10 d of chronic TTX paralysis, both voltage clamped to −80 mV. The decay time course is characterized by a rapidly decaying time constant (τ_f) of <3 ms similar to that seen in control muscle. Most m.e.p.c.s could be fitted by single exponentials with time constants similar to the apparent mean channel open times of adult AChRs. Only a small minority of m.e.p.c.s contained a small second exponential component, with a longer time constant (τ_S) equaling the mean burst duration of elementary currents mediated by fetal AChRs. The amplitude of the fast component, which was taken as an estimate of the percentage of the adult AChRs accounted for >95% of the m.e.p.c. amplitudes (Fig. 13 *d*), suggesting that the vast majority of end-plate AChRs remained of the $AChR_\epsilon$ type in chronically TTX paralyzed

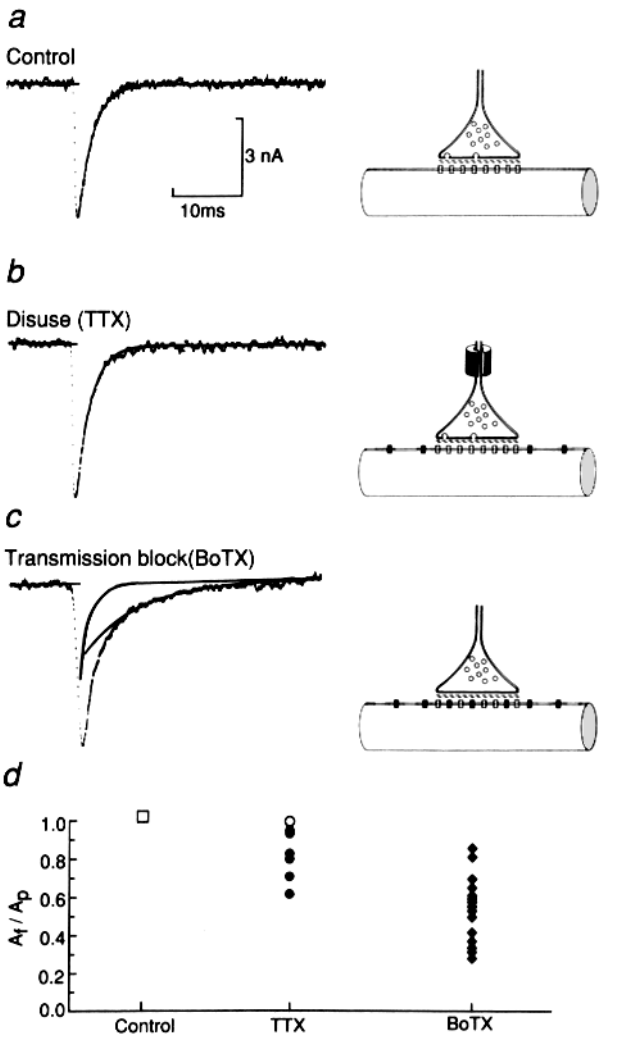

Figure 13. Gating properties of end-plate channels in normal, disused and blocked muscles. The decay time course of miniature end-plate currents (m.e.p.c.s.) is an estimate of the average duration of elementary currents. All experiments performed at −75–−80 mV holding potential, 20°C. (*a*) Time course of m.e.p.c. in normal muscle. M.e.p.c. decay is fitted (*continuous line*) by a single exponential function with a time constant of 2.5 ms. Schematic drawing of neuromuscular junction shows that only adult type AChR channels (*open symbols*) are present in the end-plate. (*b*) Time course of m.e.p.c. in 10-d TTX-blocked muscle. Decay is fitted (*continuous line*) by a single exponential function with a time constant of 2.8 ms. Same calibration as in *a*. Schematic drawing shows the mode of action of a TTX cuff causing action potential conduction block in the distal part of the motor nerve but leaving intact spontaneous vesicular release of ACh. The increase in the number of fetal type of AChR channels (filled symbols) is predominantly in extrasynaptic fiber segments. (*c*) Time course of m.e.p.c. in 8-d BoTX-blocked muscle. Decay is fitted by sum of two exponential components (*continuous lines*) with time constants of 2.5 and 8.8 ms, respectively. m.e.p.c. peak amplitude is 3.8 nA. Time calibration as in *a*. Schematic drawing illustrates the mode of action of BoTX inhibiting evoked as well as spontaneous vesicular release of ACh. Appearance of fetal type AChR channels in the end-plate as well as in extrasynaptic fiber segments. (*d*) Scatter histogram of relative size of fast decay components of m.e.p.c.s measured in normal muscle (*open square*) and in muscles paralyzed by TTX (*circles; open circle* represents mean of measurements from 24 end-plates) or by BoTX (*filled diamonds*). The ordinate indicates the amplitude of the fitted fast component (A_f) as a fraction of the peak m.e.p.c. amplitude (A_p) which is composed of the sum of the fast (A_f) and slowly (A_s) decaying components.

muscle. At some end-plates, the m.e.p.c. decays were not clearly doubly exponential or the fast component had time constants that were longer than that at the control end-plates with the longest τ_f of 3.1 ± 0.2 ms (n = 5) but shorter than expected from fetal AChRs. These data were not included in Fig. 13 *d* because it could not be decided whether the slow component was due to fetal AChRs or to a decrease in junctional acetylcholinesterase (AChE) activity that is seen in blocked muscle (Lømo et al., 1985). Analysis of ACh-induced current fluctuations also suggested the presence of a majority of $AChR_\epsilon$ type channels at end-plates of TTX-blocked muscle. At 17 end-plates the fast component of their autocovariance functions (acf's) contributed between 60 and 80% to their total amplitude.

A very different picture is observed in muscles paralyzed by BoTX. After 8–9 d of BoTX blockade the m.e.p.c. frequency in muscles was significantly lower (<1/min) than that in TTX-blocked muscles and many end-plates did not or only weakly respond to focal application of hypertonic sucrose solution. At −80 mV holding potential, the m.e.p.c.s at these end-plates were often higher in amplitude ("giant" m.e.p.c.s) than at control end-plates or at BoTX-treated end-plates responding to hypertonic sucrose solution as described (Cull-Candy et al., 1976). At one end-plate, a high frequency "shower" of normal-sized m.e.p.c.s was elicited by damage of the nerve terminal inflicted by the microelectrode impalement. The decay time course of m.e.p.c.s in BoTX-blocked muscles is characteristically different from that in control muscle (Fig. 13, *a* and *c*) since the decay is described by the sum of two exponential components. The contribution of the faster component to the total amplitude at 15 end-plates is on average <50% (Fig. 13 *d*). The faster time constants were similar as in TTX-blocked muscles but the time constants of both the fast and the slow component were somewhat slower than the apparent mean open times reported for fetal and adult AChR channels. We attribute this to a decline in AChE activity in the paralyzed muscle (Lømo et al., 1985). The important point here is, however, the presence of a slow m.e.p.c. decay component which indicates that paralysis by BoTX causes the expression of fetal type AChRs in the end-plate membrane. Sellin and Thesleff (1981) did not find doubly exponential m.e.p.c.s on BoTX-paralyzed rat muscle but only a twofold prolongation of the m.e.p.c decay. A possible reason for the discrepancy to the present results may be that they did not preblock the preexisting adult-type AChRs.

Extrasynaptic AChR Channels. With greatly reduced contents of γ-subunit mRNA in the TTX blocked muscles, the question arises whether the extrajunctional ACh sensitivity in the latter may be mediated by AChR channels lacking the γ-subunit (Jackson et al., 1990). AChR gating and conductance properties in hypersensitive soleus muscles paralyzed by denervation or by TTX block were compared by analysis of ACh-induced membrane current fluctuations and by single channel current analysis. The results revealed a predominantly slowly gating population of AChR channels in the extrajunctional membrane of both denervated and TTX-blocked soleus muscle. Both the apparent mean open times estimated from ACh-induced membrane current fluctuations and their single channel conductances estimated from the noise variance were similar in the two types of paralyzed muscle. The numerical values of mean duration and conductance of single channels obtained in 16–19 fibers in both muscle types are given in Table I. The data derived from noise analysis were confirmed by single channel current recordings. When measured with a pipette solution that would reveal the differences in conductance between αβδ-AChR and AChRγ channels (Table I) 11 patches of extrajunctional membrane (6 fibers from three TTX-blocked muscles) revealed a predominating class of channels with low conductances and long opening durations similar to the one observed in denervated muscle. Only in one patch from a TTX-paralyzed muscle were channel openings seen with a conductance of 124 pS, similar to the conductance of channels expressed in *Xenopus laevis* oocyte membrane after injection of only the α-, β-, and δ-subunit mRNAs of bovine AChR. In this preparation αβδ-AChR channels are assembled which lack the γ- or the ε-subunit (Jackson et al., 1990).

In conclusion, although the total level of γ-subunit mRNA in TTX-paralyzed muscle is ~10 times lower than in surgically denervated muscle mostly fetal type AChR channels comprising the γ-subunit are assembled in extrasynaptic fiber segments, whereas in the end-plate membrane this type of channel was not detectable.

Table I. Properties of Extrasynaptic AChR Channels in Surgically Denervated and in TTX Paralyzed Muscle

	Denervated muscle	TTX-paralyzed muscle
Current fluctuation analysis		
$\gamma(pS)$	57 ± 1.0 (16)	51 ± 2.0 (19)
$\tau(ms)$	2.8 ± 0.1 (16)	2.4 ± 0.1 (19)
Single-channel current measurement		
$\gamma(pS)$	69.3 ± 1.3 (10)	74.9 ± 1.3 (11) 124 (1)

Single-channel conductance (γ) and mean channel open time (τ) of extrajunctional AChR-channels were estimated from fluctuation analysis and single channel currents activated by ACh in extrasynaptic fiber segments. All experiments at membrane potentials of −70– −80 mV at 20–22°C. Means ± SD are given, *n* refers to number of experiments. Whole-cell currents were measured in normal bathing solution using iontophoretic ACh application. Single-channel current measurements were made in normal bathing solution, the pipette contained (in millimolar): CsCl, 100; EGTA, 10; Hepes, 10 (pH 7.2); and ACh, 1 μM. With this solution a characteristic difference in single channel conductance between AChR channel subtypes containing or lacking a γ-subunit (αβδ-AChR versus AChRγ) is found in bovine muscle (Jackson et al., 1990).

Discussion

Differential Regulation of AChR Subunit mRNAs by Myogenic and Neural Signals

In the present experiments, we have attempted to describe how neural signals maintain the α-, β-, δ-, and ε-subunit mRNAs of the nicotinic AChR locally accumulated in the end-plate region of adult skeletal muscle. The Northern blot and in situ hybridization experiments in normal, in surgically denervated, and in toxin-paralyzed adult muscles suggest three patterns of regulation that may underlie the synaptic accumulation of AChR mRNAs.

The level of ε-subunit mRNA is relatively independent of muscle innervation or usage of the neuromuscular junction and the mRNA is localized predominantly in the end-plate region irrespective of whether the muscle is innervated, disused or whether the nerve–muscle contact is altogether lost. This suggests that a neurotrophic imprinting signal (Brenner et al., 1990) acts on end-plate nuclei at early stages of synapse formation that renders the ε-subunit gene insensitive to further regulation.

In contrast, the level of γ-subunit mRNA is controlled very tightly both by neural and by electrical activity-dependent signals. In normally active fibers, the γ-subunit mRNA is decreased to undetectable levels and is only slightly elevated in muscles kept electrically inactive by mere disuse of the neuromuscular synapse. In muscle inactivated by surgical denervation or by presynaptic blockade of neuromuscular transmission, however, the γ-subunit mRNA is abundant along the entire fiber length and can be suppressed by direct electrical stimulation. Thus, downregulation of γ-subunit mRNA in the innervated fiber is the consequence of, firstly, repression by an inhibitory neural signal that presumably acts locally at the end-plate and is linked to Ca^{2+}-dependent secretory processes in the nerve terminal and, secondly, repression by a muscular signal which is linked to nerve-induced electrical activity and acts along the whole fiber length.

Finally, the level and the spatial distribution of the α-, β-, and δ-subunit mRNA is controlled by the combination of locally acting and more global signals. The level of total α-subunit mRNA in denervated muscle is strongly reduced by muscle activity comparable to the pattern seen for the γ-subunit mRNA. However, both Northern blot and in situ hybridization experiments on denervated/stimulated muscles show that the downregulation of total muscle α-subunit mRNA by electrical activity reflects a decline primarily in the level of extrajunctional mRNA, whereas at the former synapse, the α-subunit mRNA expression is resistant to the effects of electrical activity. This indicates that, over a period of weeks, the maintenance of the synaptic α-subunit mRNA accumulation, once it is induced, does not require the continued presence of the nerve terminal or putative anterograde neurotrophic factors. Thus, it is likely that similar to what is found for the ε-subunit mRNA, synaptic nuclei are also imprinted during synapse formation to express the α-subunit gene permanently; in the extrasynaptic fiber segments, however, the α-subunit mRNA is strongly repressed by a signal linked to electrical activity of the nerve and muscle. Although the changes in total β-subunit mRNA levels are small when compared to the changes of α-subunit mRNA the Northern blot and in situ hybridization experiments in adult muscle suggest that the β-subunit mRNA level is controlled in a comparable fashion. In synaptic regions it is largely independent of the state of innervation. In extrasynaptic regions, the mRNA levels are reduced by muscle activity. Finally the δ-subunit mRNA level could also be determined by imprinting of the nuclei of the synaptic region and downregulation by electrical activity in extrasynaptic regions. The observation that in adult fibers the total amount of α- and δ-subunit mRNAs on the one hand and that of the ε- and β-subunit mRNAs on the other follow similar patterns of regulation is reminiscent of the coordinate changes which these subunit mRNAs follow during postnatal development (Witzemann et al., 1989).

A simple view of a mechanism that could account for both the localized accumulation of α-, β-, δ-, and ε-AChR subunit mRNAs in innervated, adult muscle as well as their changes in abundance and spatial distribution after various types of paralysis would be that (*a*) the expression of each of the AChR-subunit genes depends on both neural factors and muscular signals, (*b*) neural factors override muscular signals near the end-plate, (*c*) two types of neural factors are acting on synaptic nuclei of normal muscle fibers, an early transient imprinting signal and a continuously acting inhibitory signal, (*d*) the different AChR subunit genes are differentially susceptible to these signals. According to this view the α-, β-, γ-, and δ-subunit genes are activated constitutively during myogenesis along the whole fiber. During synapse formation and maturation a positively acting neurotrophic factor "imprints" a few end-plate nuclei to express the genes of the α-, β-, and δ-subunits permanently and to render them independent of regulation by other signals. This imprinting signal also activates the ε-subunit gene in the subsynaptic nuclei. Later in postnatal development mRNA levels of α-, β-, and δ-subunits in extrasynaptic fiber segments and that of the γ-subunit in the entire fiber are reduced by a negative neural factor and possibly also by nerve-induced electrical muscle activity.

The basal level of the α-, β-, δ-, and ε-subunit mRNAs remaining in denervated/stimulated muscles localized in subsynaptic nuclei strongly supports the assumption that a permanent imprinting of synaptic nuclei occurs via a transient signal acting only briefly during synaptic maturation. The mRNA accumulation at the site of the former end-plate in denervated/stimulated muscles does not support a model that assumes that ongoing mRNA synthesis in synaptic nuclei of normal adult muscle requires release of "anterograde" factors from the nerve ending (Laufer and Changeux, 1989). In fact, the experiments reported here suggest that in adult muscle factors released from the terminal suppress synthesis of the γ-subunit mRNA and to some degree also that of the other subunits.

Imprinting of subsynaptic nuclei to express the ε-subunit gene occurs within the initial 3–4 d of synapse formation. The developmental stage at which the imprinting of subsynaptic nuclei to express permanently the α-, β-, and δ-subunit mRNA occurs is as yet unclear. For example the level of α-subunit mRNA in rat muscle, in contrast to chick muscle (Fontaine and Changeux, 1989), is high in both subsynaptic and extrasynaptic nuclei for >2 wk after initial stages of synaptogenesis and α-subunit mRNA is focally localized at the end-plates only after the second postnatal week (Witzemann et al., 1989; Brenner et al., 1990). This precludes the determination of the developmental stage at which imprinting occurs by in situ hybridization techniques. Also the question of whether imprinting occurs in the same synaptic nuclei for all AChR subunit genes is unclear at present.

Abundance of γ- and ε-Subunit mRNAs and AChR Channel Subtypes Are Correlated

The spatial distribution of the AChR mRNAs along the fiber and the local levels of the γ- and ε-subunit mRNAs are well correlated with the spatial distribution of functional AChR channel subtypes as determined electrophysiologically. Denervation and BoTX paralysis, which cause increases in the levels of all subunit mRNAs, in particular of the γ-subunit mRNA, also increase the proportion of fetal type of channels (AChRγ) consisting of α-, β-, γ-, and δ-subunits, in both the junctional and the extrajunctional fiber segments. In the end-plate region of denervated (Brenner et al., 1990) and probably also of BoTX-blocked muscle the ε-subunit mRNA remains highly concentrated and γ-subunit mRNA is strongly increased (Goldman and Staple, 1989). This leads to the appearance of both adult (AChRε) and fetal type (AChRγ) channels in roughly similar proportions (Brenner and Sak-

mann, 1983; Witzemann et al., 1987; Gu and Hall, 1988). Conversely, after disuse of the neuromuscular synapse after TTX block which leaves the γ-subunit mRNA level low, the proportion of fetal AChRγ in the end-plate remains small or is not detectable at all. In the extrajunctional fiber segments with their minor content of ε-subunit mRNA, even a low level of γ-subunit mRNA appears to be sufficient to have predominantly fetal AChRs expressed. Finally, the density of fetal AChR channels remains low both in the junctional and extrajunctional membranes of denervated muscle kept electrically active by direct stimulation (Brenner and Rudin, 1989) as is the level γ-subunit mRNA. Thus, the relative levels of γ- and ε-subunit-specific mRNAs along the fiber are determinants of the spatial distribution of the AChR subtypes expressed in the muscle membrane.

Neurotrophic Mechanisms Control the Developmental Switch of End-Plate AChR Channels

In situ hybridization experiments have shown that ε-subunit mRNA is focally induced at the neuromuscular contact from the very beginning of its appearance in neonatal muscle (Brenner et al., 1990) and that expression remains locally restricted at the end-plate after loss of terminal by early denervation. Combined with the present findings that γ-subunit mRNA and fetal AChRγ channels remain downregulated at disused synapses with TTX-blocked motor nerves, this suggests that the switch in the end-plate channel subtype from fetal AChRγ to adult AChRε in the developing neuromuscular junction (Schuetze and Role, 1987, for review) is regulated by diffusible neurotrophic factors and is not dependent on electrical activity. This view is consistent with previous experiments on embryonic amphibian muscle, if it is assumed that AChR channel conversion in developing amphibian end-plates is due to a similar switch in AChR subunit composition as in rat and calf. In developing *Xenopus laevis* muscle, immobilized by local anesthetics or by TTX, the level of γ-subunit mRNA declines in spite of the muscle inactivity (Baldwin et al., 1988) and in immobilized muscle AChR channel conversion takes place as in normally active control muscle (Kullberg et al., 1985).

The mechanism proposed here for the switch in AChR channel subtypes during postnatal development is in contradiction with the recently favored view that the prevalence of the adult AChR subtype at the mature end-plate is due to indiscriminate focal accumulation of both AChR types by neurotrophic effects and to subsequent selective depression of the fetal AChR subtype by the nerve-induced muscle activity (Brehm and Henderson, 1988). Rather, the present experiments show that in the presence of the nerve and in the absence of muscle activity, AChR insertion into the end-plate membrane is selective in favor of the adult AChR subtype. A similar conclusion was drawn previously from the analysis of ectopic end-plate formation in TTX-blocked nerve (Brenner et al., 1987).

Nature of Neurotrophic Signals and Role of Electrical Muscle Activity

Candidates for the early transient imprinting signal could be CGRP or other peptides present in motor nerve terminals (Fontaine et al., 1987; New and Mudge, 1986) and ARIA (Harris et al., 1988, 1989), which are derived from nerve tissue and which increase the levels of α-subunit mRNA and of AChRs in cultured chick muscle. However, their involvement in the regulation of AChR subunit gene expression during synapse formation and maturation remains to be demonstrated. Other candidates for the imprinting signal are Schwann cell–derived maturation factors (Chapron and Koenig, 1989), agrin like molecules (Nitkin et al., 1987) or ascorbic acid which has been shown also to lead to an increased synthesis of AChR-subunits (Horovitz et al., 1989). It is at present also unclear whether the imprinting signal affects transcriptional mechanisms directly or via an intermediate alteration of the local environment of subsynaptic nuclei, i.e., a neural "trace" on the muscle.

The continuous inhibitory neural factor that mediates activity-independent suppression of predominantly the γ-subunit and to a lesser extent of the other subunit mRNAs depends on the presence of an intact nerve terminal. The different sites of action of TTX and BoTX, i.e., block of nerve conduction versus interrupting neuromuscular transmission, and their different effects on γ-subunit mRNA levels allow some inferences to be made about the nature of this inhibitory signal. TTX abolishes only evoked transmitter release (i.e., evoked by motor nerve action potentials) but leaves the spontaneous quantal release of ACh unaffected or even increases it, whereas BoTX inhibits both the evoked and the spontaneous quantal release (Kao et al., 1976; Tsujimoto and Kuno, 1988; Thesleff, 1989*a*,*b* for review). During TTX-induced paralysis the end-plate is still exposed to quantally released ACh, whereas during BoTX paralysis this is not the case. As unlike with TTX-induced disuse, the effect of BoTX on γ-subunit mRNAs is a strong increase similar to that seen after surgical denervation, a simple interpretation of these findings is that the putative inhibitory neural factor is released from clear synaptic vesicles. This view is supported by the finding that BoTX inhibits only Ca^{2+}-dependent vesicular release but not the nonquantal ACh release (Stanley and Drachman, 1983). Thus nonquantally released ACh or substances released in a Ca^{2+}-independent way from dense-core vesicles such as CGRP (Matteoli et al., 1988) are not directly involved in the muscle activity independent downregulation of γ-subunit mRNA levels. The factor may be either ACh itself or other substances stored in the clear synaptic vesicles such as ATP or proteoglycan (Volknandt and Zimmermann, 1986; Stadler and Kiene, 1987), which are co-released with ACh. Since it has been suggested that elevated intracellular Ca^{2+} concentrations can reduce the number of AChRs (Shainberg and Burstein, 1982) and α-subunit mRNA levels in cultured embryonic muscle (Klarsfeld et al., 1989) the local Ca^{2+} inflow through end-plate channels during e.p.p.s (Miledi et al., 1980) might be responsible for the suppression of γ-subunit mRNA at the end-plate. The observation that in muscles immobilized by blockade of end-plate receptors with α-BuTX, the level of γ-subunit mRNA as well as that of the α-subunit mRNA is lower than in surgically denervated or BoTX paralyzed muscle would be consistent with such a mechanism assuming that these subunit genes have common regulatory elements sensitive to intracellular Ca^{2+} concentration. However, in view of the complete block by α-BuTX of spontaneous m.e.p.p.s and almost complete block of evoked e.p.p.s, which indicates that >95% of the end-plate receptors are inactive (Pennefather and Quastel, 1981) by α-BuTX treatment this seems an unlikely possibility.

An alternative mechanism could be that substances coreleased with ACh from clear vesicles act via their own cognate receptors in the end-plate membrane. For example ATP is coreleased with ACh and may bind to purinergic receptors activating the inositol-4,5-trisphosphate (IP_3) pathway (Haggblad and Heilbronn, 1987), which is known to be involved in regulation of gene expression in other cells (Sassone-Corsi et al., 1988).

The present experiments show that the localized synthesis of mRNAs encoding the adult AChR subtype at the end-plate is to a large degree regulated by neural factors. The effect of electrical muscle activity on mRNA synthesis seems to be restricted to extrasynaptic fiber segments where it acts in concert with inhibitory neural factors. However, at present neither the relative importance of the two mechanisms for repression of mRNA synthesis in extrasynaptic nuclei nor the functional role of AChR channels in extrasynaptic fiber segments are known.

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