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Synapse-specific expression of acetylcholine receptor genes and their products at original synaptic sites in rat soleus muscle fibres regenerating in the absence of innervation

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Summary

To test the hypothesis that synaptic basal lamina can induce synapse-specific expression of acetylcholine receptor (AChR) genes, we examined the levels mRNA for the α- and ε-subunits of the AChR in regenerating rat soleus muscles up to 17 days of regeneration. Following destruction of all muscle fibres and their nuclei by exposure to venom of the Australian tiger snake, new fibres regenerated within the original basal lamina sheaths. Northern blots showed that original mRNA was lost during degeneration. Early in regeneration, both α- and ε-subunit mRNAs were present throughout the muscle fibres but in situ hybridization showed them to be concentrated primarily at original synaptic sites, even when the nerve was absent during regeneration. A similar concentration was seen in denervated regenerating muscles kept active by electrical stimulation and in muscles frozen 41-44 hours after venom injection to destroy all cells in the synaptic region of the muscle. Acetylcholine-gated ion channels with properties similar to those at normal neuromuscular junctions were concentrated at original synaptic sites on denervated stimulated muscles. Taken together, these findings provide strong evidence that factors that induce the synapse-specific expression of AChR genes are stably bound to synaptic basal lamina.

Key words: neuromuscular junction, acetylcholine receptor, basal lamina, muscle regeneration, rat.

Introduction

During development, innervation of mammalian skeletal muscle has two major effects on the expression of the acetylcholine receptor (AChR) (reviewed by Schuetze and Role, 1987). The first is to suppress the constitutive expression of 'fetal' AChRs, which are present all over the surface of embryonic muscle fibres and also appear at the surface of adult muscle after denervation. These AChRs are composed of α, β, γ- and δ-subunits in the stoichiometry $\alpha_2\beta\gamma\delta$ and are associated with ion channels with relatively low conductance and relatively long open times. Numerous studies indicate that muscle activity plays an important part in this suppression of fetal AChRs by causing the down-regulation of the mRNAs encoding their subunits (Klarsfeld and Changeux, 1985; Goldman et al., 1988; Witzemann et al., 1991). The second effect of innervation is to induce at the neuromuscular junction the appearance of 'adult' AChRs, in which the γ-subunit is replaced by another, termed ε, resulting in a subunit composition $\alpha_2\beta\varepsilon\delta$. These 'adult' AChR channels have a higher ion conductance and shorter open times than those of fetal AChRs (Mishina et al., 1986). The nature of neuromusclar transmission requires that adult AChRs are not sensitive to the down-regulating effects of normal muscle activity.

In innervated muscle fibres, expression of the genes for the subunits of the adult AChR channel occurs primarily in a subsynaptic population of myonuclei located within about 100 μm of the neuromuscular junction and is not suppressed by muscle fibre activity (Witzemann et al., 1991). It appears that this localized, synapse-specific expression of AChR genes is induced by the nerve early in neuromuscular junction formation (Brenner et al., 1990; Martinou and Merlie, 1991; Sanes et al., 1991). Recent studies have demonstrated that the neurons inductive influence on muscle fiber AChR genes persists after the neuron degenerates (Brenner et al., 1990; Witzemann et al., 1991). Because the synaptic basal lamina at mature neuromuscular synapses can induce the aggregation of AChRs (McMahan and Wallace, 1989), one explanation for the continued synapse-specific gene expression after denervation is that the factors that induce such expression are components of the synaptic basal lamina. Here we show that when rat soleus muscle fibres, damaged by a myotoxin regenerate within their original basal lamina sheaths, factors which survive muscle damage

induce the expression of α- and ε-subunit mRNAs of the AChR at original synaptic sites in the absence of innervation, even if the muscle is kept active by direct stimulation. We also show that these mRNAs lead to the expression in the muscle fibre membrane of AChRs with functional properties characteristic of normal adult neuromuscular synapses. During the course of our studies, Goldman et al. (1991) reported results of in situ hybridization experiments on damaged muscles that led them to conclude that the basal lamina induces regenerating muscle fibres to express ε-subunit mRNA. However, because of the experimental paradigm they used, they could not rule out, nor did they address, other equally plausible explanations for their findings. For example, the way in which they damaged muscles allowed Schwann cells to persist at synaptic sites on the muscle fibre basal lamina even though axon terminals and muscle fibres had degenerated. Schwann cells are known to synthetize maturation factors (Chapron and Koenig, 1989) and to release acetylcholine under certain conditions (Birks et al., 1960) and, thus, it cannot be ruled out that, when present in regenerating muscles, Schwann cells provide factors that induce muscle fibres to express ε-subunit mRNA. Accordingly, in one set of experiments, we damaged muscles by freezing them, which is known to result in degeneration and phagocytosis of all cells at the synaptic site, i.e. Schwann cells, persisynaptic fibroblasts and myosatellite cells as well as axon terminals and muscle fibres (McMahan and Slater, 1984). We found that as in the case of myotoxic damage, components of the original synaptic site directed regenerating muscle fibres to express locally ε-subunit mRNA. Since the only component of the original synaptic site that was present in these studies was the basal lamina, we demonstrate for the first time that indeed the basal lamina has stably bound to it molecules that can induce the expression of ε-subunit mRNA. Our electrophysiological finding described above indicates that ε-subunit is incorporated into the AChRs expressed in the muscle fibre surface and that they are identical in function to those at normal neuromuscular synapses. This provides direct evidence that basal lamina components not only induce the aggregation of AChRs on muscle fibres (McMahan and Wallace, 1989) as do motor neurons, but specify, again like motor neurons, the structure and function of those receptors. It may well be that all such basal lamina molecules are provided by the motor neurones.

Some of the results have been presented as an abstract (Brenner et al., 1992).

Materials and methods

Induction of muscle degeneration-regeneration, stimulation

Wistar or Sprague-Dawley rats (80-140g) were anaesthetised with Nembutal. 5 µg of *Nss* venom (Sigma) in 50 µl sterile saline was injected into the belly of the soleus muscle. In some rats, the muscle was denervated by removing approximately 5 mm of the sciatic nerve. To kill all cells in the central, synapse-containing region of the muscle, some muscles were exposed 41 hours after *Nss* venom injection and the central part was frozen and thawed twice by touching the blade of a screw driver precooled in liquid nitrogen.

Six to nine days after venom injection, electrodes were implanted into the hindleg of some animals as previously described (Brenner et al., 1987). Stimuli were given in 100 Hz trains of 1 second duration, applied once every 100 seconds for 7 to 10 days.

For acute experiments, animals were killed with CO_2 and the excised muscles were processed as descibed below or frozen in liquid nitrogen and stored at −70°C until use.

Cell cultures

Diaphragms were excised from rats, their extrasynaptic segments isolated under the dissecting microscope and satellite cells were isolated by incubating the minced segments in 0.2% trypsin (Type 1, Sigma) in Ca^{2+}-free MEM (GIBCO) for 2 hours at 37°C and by subsequent trituration. After rinsing, 4-6×10^6 cells were plated in a 100-µl drop on rat tail-collagen coated glass coverslips in MEM equilibrated with 5% CO_2 and supplemented with 10% horse serum (Amimed, Basel), 5% chick embryo extract (CEE; GIBCO) and 1% antibiotic/antimycotic solution (GIBCO). After the cells had been allowed to settle for 4 hours, 2 ml of medium was added. From the fourth to the fifth day, cytosine arabinoside (10^{-5} M, Sigma) was added to the medium. From the fourth day onward, the medium was without CEE. Within 4 days of plating, myotubes began to form and spontaneous twitching was observed after 5-6 days.

Cytochemistry

Freshly dissected muscles were frozen between small pieces of liver by immersion of isopentane cooled in liquid nitrogen. Transverse frozen sections 6 µm thick were reacted to demonstrate cholinesterase (Karnovsky and Roots, 1964) to reveal the position of neuromuscular junctions. Other sections were stained with haematoxylin-eosin to display cellular features.

Electron microscopy

Lightly stretched muscles were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1-2 hours at room temperature. To promote penetration of the fixative, muscles were teased into several bundles after being partially fixed. The bundles were then reacted to demonstrate acetylcholinesterase activity using the method of Karnovsky and Roots (1964) or of Strum and Hall-Craggs (1982). Small pieces containing neuromuscular junctions were cut out, osmicated and embedded in Araldite. Transverse ultrathin sections were examined in a Jeol 1200 microscope.

RNA isolation and northern blot hybridization analysis

Total RNA was extracted from 40-170 mg of muscle tissue following the guanidine isothiocyanate/phenol-chloroform method described by Chomczynski and Sacchi (1987). Samples containing 10 µg RNA were denatured in 1 M glyoxal and 50% dimethylsulfoxide (McMaster et al., 1977) and electrophoresed on 1.2% agarose (BRL) gels in recirculated 10 mM sodium phosphate buffer, pH 7.0. RNA was transferred to nylon membranes (Boehringer Mannheim) by diffusion in 10×SSC, pH 7.0 (Fourney et al., 1988) and crosslinked by baking at 120°C for 30 minutes. Equal loading of agarose gels was controlled by ethidium bromide staining.

Blots were hybridized with full length, digoxigenin-labelled antisense cRNA probes specific for rat AChR α- and ε-subunit mRNAs and labelled with digoxigenin. AChR subunit clones rAChRα and rAChRε3 (Witzemann et al., 1990) subcloned into the polylinker region of the transcriptional vectors pSP64 and pSP65 (Promega) were kindly supplied by Drs V. Witzemann and B. Sakmann, Heidelberg (FRG). Digoxigenin-labelled probes were

prepared using the DIG RNA Labelling Kit (SP6/T7) from Boehringer (Mannheim) and hybridization was carried out essentially as recommended. The actin probe used was the chicken β-actin cDNA *Pst*I fragment (Cleveland et al., 1980) labelled with digoxigenin by random primed labelling method (Feinberg and Vogelstein, 1983). Specific hybridization signals were detected by using an enzyme immunoassay (DIG Nucleic Acid Detection Kit, Boehringer) and the chemiluminescent AMPPD (Boehringer, Mannheim) as a substrate according to the producers prescription. Membranes were then exposed to Kodak-X-OMAT film for 30 minutes to 4 hours and scanned densitometrically on a Shimadzu Dual Wavelength TLC Scanner (CS 930). To test for the linearity of the signals, increasing amounts of total RNA (1-16 μg) isolated from denervated rat muscle were electrophoresed and hybridized with an α-subunit specific cRNA probe. Hybridization signals then increased in an approximately linear fashion.

In situ hybridization

^{35}S-labelled cRNA probes specific for rat AChR α-subunit and ε-subunit mRNAs were prepared as previously described (Brenner et al., 1990). Soleus muscles were fixed overnight at 4°C in 4% paraformaldehyde in PBS, stained for acetylcholinesterase by the method of Koelle and Friedenwald (1949) and embedded in paraffin. Longitudinal sections 8 μm thick were cut and mounted on aminoalkylsilane-treated glass slides. After deparaffinizing and rehydration, the sections were scanned for the position of AChE positive sites and their locations with respect to the scales of the microscope stage were recorded. Sections were then pretreated and hybridized essentially as previously described (Fontaine and Changeux, 1989; Brenner et al., 1990). The positions of silver grains in the autoradiographs were compared with the positions of AChE-positive sites, in the same sections, recorded before hybridization.

Myotube cultures 6 to 8 days-old were fixed in ice-cold 4% paraformaldehyde in PBS for 30 minutes, washed for 20 minutes in cold PBS and then hybidized as described above, except that prehybridization involved only 2-3 minutes of treatment with 20 μg/ml proteinase K and no acetylation was performed.

Analysis of ACh-induced current fluctuations

The gating properties of AChR channels were examined in regenerated muscles that had been stimulated for >7 days. One of them was superfused with 2.5 μM α-bungarotoxin for 30 minutes at the time of electrode implantation to preblock AChRs. For electrophysiological experiments, muscles were bathed in a solution consisting of: 40% Leibovitz L-15 medium and 60% of a solution containing (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2.5, glucose 5, Hepes 5 (pH 7.2). Synaptic sites in regenerated stimulated muscles were localized by iontophoretic application of ACh in pulses of 2-3 milliseconds duration from a constant current pump. The generation of brief depolarizations (time to peak 10-20 millisecond) was taken to indicate an original synaptic site. Subsequent staining for AChE showed deposits characteristic of original synaptic sites at such locations. Muscle fibres were voltage clamped to −60 to −90 mV membrane potential at such sites. The subtypes of AChRs expressed were assessed from the gating properties of their ion channels by analysis of the ACh-induced membrane current fluctuations as described (Brenner et al., 1987). The fraction of adult AChR channels was estimated from $A_1/(A_1 + A_2)$, where A_1 and A_2 are the amplitudes of the fast and slow components of the autocovariance function, respectively.

Results

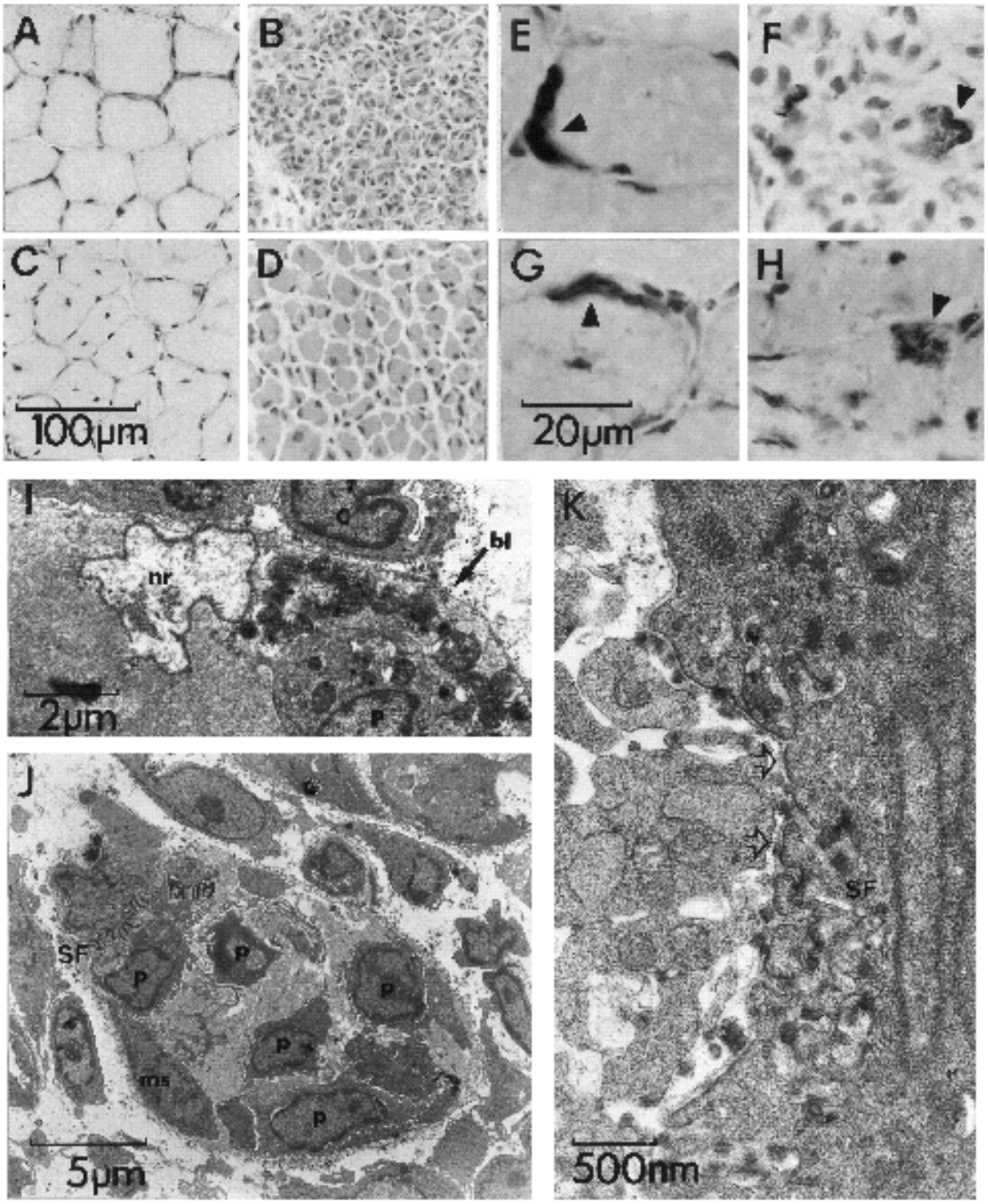
Muscle degeneration and regeneration

Rat soleus muscle fibres were damaged by an intramuscular injection of the venom of the Australian tiger snake (*Notechis scutatus scutatus*, *Nss*, Harris et al., 1975; Harris and Johnson, 1978). This procedure results within 24 hours in the breakdown of muscle fibres and their nuclei (Fig. 1A,B) but leaves intact the surrounding basal lamina sheaths (Fig. 1I), the myosatellite cells that normally share them with the muscle fibres (Fig. 1J), and the microvasculature (Fig. 1I) (Harris and Cullen, 1990; Sesodia and Cullen, 1991). In a series of 23 muscles damaged in this way, and studied in transverse section 1-4 days after venom injection, no surviving muscle fibres were present in 22 of them. In one muscle, 143 original muscle fibres were present, about 5% of the normal total. Thus, in the great majority of muscles, *Nss* venom injection leads to the destruction of all fibres in the rat soleus muscle in less than 24 hours.

It was important to establish that the loss of muscle fibres seen in the light microscope involved the destruction of all the original myonuclei. We therefore examined, with the electron microscope, more than 300 profiles of degenerating muscle fibres, defined by the presence of an intact basal lamina sheath, 24-48 hours after *Nss* venom injection (Fig. 1I,J). At 24 hours after injection, identifiable remnants of myonuclei were seen in about 20% of the 136 profiles examined (Fig. 1I). The nuclear membrane of these remnants was often disrupted and the nucleoplasm was in all cases pale and lacking in the internal structure typical of normal nuclei. In a sample of 171 profiles in 9 muscles studied 48 hours after venom injection, nuclear remnants were seen in only 3 fibres, all from one muscle. Thus, we found no evidence to suggest that any significant number of original myonuclei survive the breakdown induced by *Nss* venom, confirming a previous observation on the effects of muscle fibre damage in vitro (Bischoff, 1975).

At all stages of the response to *Nss* venom, a high activity of acetylcholine esterase (AChE) persists in association with the synaptic basal lamina and can be used to identify original synaptic sites (Fig. 1E-H,J,K). Our sample included 15 profiles containing the sites of original neuromuscular junctions. There was no indication of any preferential survival of muscle fibres and their nuclei at these synaptic sites.

Muscle fibre breakdown is followed by rapid proliferation of the myosatellite cells which normally represent about 5-10% of all the nuclei within the basal lamina sheath in rats of the age used in the present experiments (Kelly, 1978b). Of the cells that were present within the surviving basal lamina sheaths 48 hours after venom injection (Fig. 1J), 53% had the characteristics of phagocytes, including relatively sparse, dense cytoplasm, finger-like extensions enwrapping muscle fibre debris and dense intracellular vacuoles containing such debris. The remaining 43% had smooth contours, pale cytoplasm and large nuclei, features characteristic of activated myosatellite cells. By the beginning of the third day after *Nss* venom injection, a few new myotubes were seen and by the end of that day, they were present in most of the surviving basal lamina sheaths,



throughout the muscle. Nuclear counts made in the electron microscope indicate that by this time, the number of nuclei within the profiles of the new myotubes, i.e. cells containing recognizable contractile filaments, is already at least as great as in normal muscle fibres. This implies that more than 90% of the nuclei in these new muscle fibres are produced during the 3 days following venom injection.

If the nerve is left intact, these new muscle fibres are rapidly innervated (Grubb et al., 1991) and grow to nearly normal size within 2-3 weeks (Harris and Johnson, 1978; Fig. 1C). If the nerve is cut at the time of venom injection,

Fig. 1. Degeneration and regeneration of rat soleus muscles after treatment with *Nss* venom. (A-D) Transverse frozen sections stained with haematoxylin and eosin to show general features of the response to *Nss* venom. (A) Control muscle. (B) 2 days after venom injection. The muscle fibres are completely broken down and replaced by a mixture of proliferating myogenic cells and phagocytes. (C) 14 days after venom injection. New muscle fibres are present throughout the muscle and are distinguishable from those in control muscles mainly by the persistence of many central nuclei. (D) 14 days after venom injection, nerve cut at time of muscle damage. Many new fibres are present but in the absence of innervation and activity, they fail to grow. (E-H) Synaptic sites in muscles treated as in A-D. The synaptic sites are identified by a high density of reaction product for AChE (Strum and Hall-Craggs, 1982) (arrowheads). (E,F) The normal highly localised AChE activity becomes more granular during muscle fibre damage. (G) In innervated regenerating muscles, the normal AChE distribution is restored. (H) Even in the absence of innervation, AChE activity remains concentrated at the original synaptic sites and indications of folding can just be seen. (I,K) Ultrastructural features of degenerating and regenerating muscles. (I) 24 hours after venom injection occasional nuclear remnants (nr) can be seen. These remnants have fragmented membranes and electronluscent nucleoplasm when compared with the nuclei of phagocytes (p) and capillary endothelial cells (c). The original basal lamina (bl) persists. (J) 48 hours after venom injection. A single muscle fibre profile containing nuclei of 6 separate cells; 5 are phagocytes (p) while one is probably a myosatellite cell (ms). The persisting synaptic folds, filled with reaction product for AChE (sf), can be clearly seen. (K) Newly regenerated muscle fibre 4 days after venom injection and denervation. In the synaptic region, marked by the square crystals of AChE reaction product from the method of Karnovsky and Roots (1964), disorganised postsynaptic folding is present (sf), with increased membrane density at the tops of the folds (arrows).

its terminals degenerate within less than 24 hours and the new muscle fibres are never innervated. While the early stages of new muscle fibre formation are identical to those in which the nerve is left intact, very little subsequent growth of these denervated muscle fibres occurs (Fig. 1 D). However, when we kept such muscles active by direct electrical stimulation, their size and appearance was similar to that of muscles regenerating with an intact nerve supply (data not shown).

Within a day or so of new muscle fibre formation, elaborate infolding of the muscle fibre membrane occurs in the region of the original synaptic sites (Fig. 1 K). At the tops of the folds, the membrane is increased in density, presumably because of the high density of AChRs known to be present there (Slater and Allen, 1985).

Thus, a single injection of *Nss* venom causes destruction of all muscle fibres in the soleus muscle and their nuclei, and is followed within a few days by the production of a new population of muscle fibres exhibiting some morphological signs of postsynaptic specialization even in the absence of the nerve.

Changes in α- and ε-AChR mRNAs in regenerating muscle

The temporal pattern of expression of α- and ε-AChR mRNAs during muscle degeneration and subsequent regeneration was examined by northern blot hybridization of total RNA extracted from regenerating muscles. AChR subunit

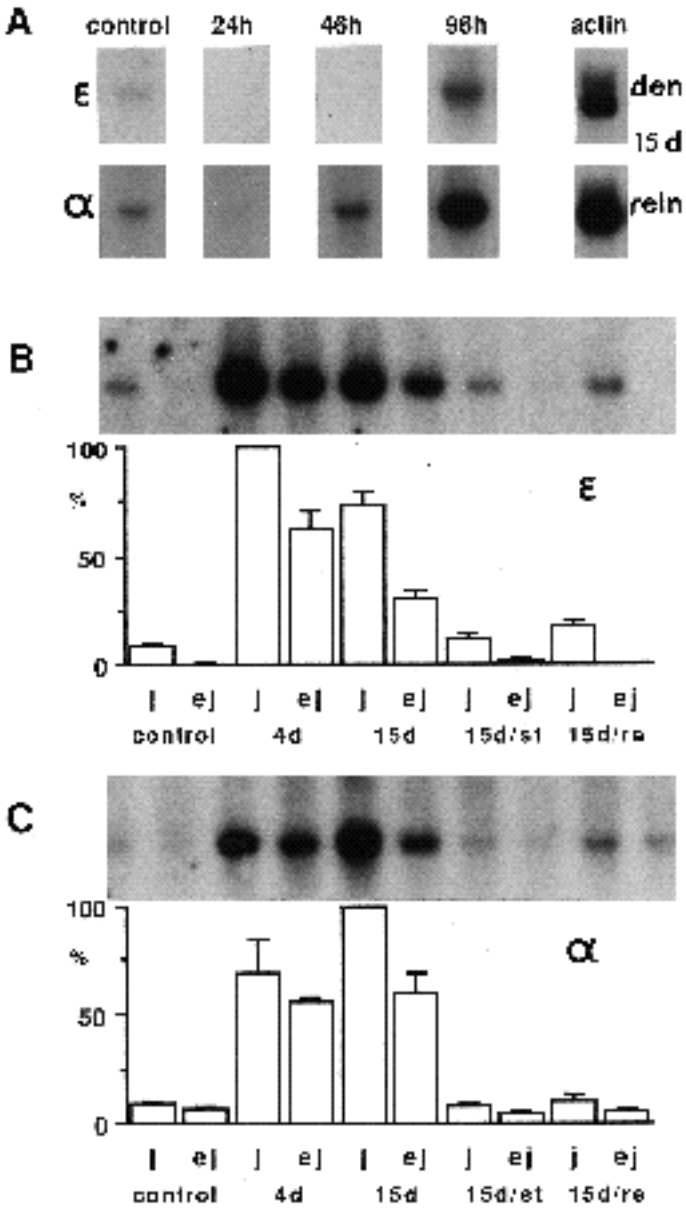


Fig. 2. Expression of α- and ε-AChR subunit mRNAs and of actin mRNA in total muscle and in synaptic and extrasynaptic muscle segments during different stages of regeneration. RNA blot analyses. (A) Expression of AChR subunit mRNAs and of actin mRNA in total muscle at the times after venom injection indicated. (B) Expression of ε-subunit mRNA in synaptic and extrasynaptic segments in regenerating muscle. Time course during regeneration and effects of reinnervation and of electrical stimulation. Bars represent densitometric quantification of abundance at the times and treatments indicated (control; 4d etc: days after denervation and *Nss* venom injection; rein: reinnervated; st: stimulated during the last 7-10 days). Means ± s.e.m. from 3-4 experiments are expressed as percentages of maximum value which for ε-subunit mRNA was reached in all experiments in the junctional segment at 4 days after *Nss* injection. For each experiment, abundance in extrajunctional segment (ej) is expressed relative to that in the junctional (j) segment from the same muscle. (C) Expression of α-subunit mRNA (treatments and times as in B) reaching its maximum at 15 days following *Nss* injection. For both B and C, the order of the lanes in the northern blots corresponds to that of the densitometric bar histograms.

mRNA-specific cRNA probes were used for hybridization. Relative contents of α- and ε-subunit mRNAs at different stages of regeneration are shown in Fig. 2. The degeneration of original soleus muscle fibres during the first 24 hours after *Nss* venom injection was accompanied by the loss of detectable α- and ε-AChR subunit mRNAs. When regeneration occurred in the absence of the nerve, the level of α-subunit mRNA began to increase within 48 hours of venom injection, by which time extensive satellite cell proliferation had occurred and the first new myotubes had formed, and then remained high during the entire postinjection period examined, i.e. 2-3 weeks. By contrast, ε-subunit mRNA could not be detected 48 hours after venom injection but was markedly increased 2 days later, when most new muscle fibres had formed, and then declined somewhat during the next 10 days (Fig. 2A).

When regeneration occurred in the presence of the nerve so that the new muscle fibres were reinnervated, both subunit mRNAs were markedly decreased, approaching the levels as observed in innervated controls by 15 to 17 days following venom injection. In contrast, the level of actin mRNA analysed with a β-actin probe was increased in reinnervated as compared to denervated regenerated muscles.

To differentiate between AChR-subunit mRNA expression in synaptic and extrasynaptic regions of the muscle, we compared AChR mRNA levels in central segments of muscles, which contain the neuromuscular junctions, and in segments at the ends of the muscle which are devoid of neuromuscular junctions (Fig. 2 B,C). At 4 days after *Nss* venom injection, the levels of both α- and ε-AChR subunit mRNAs were somewhat higher in the synapse containing segment, but were clearly present also in the extrasynaptic segments. This pattern was maintained up to 17 days after *Nss* venom injection. By contrast, in reinnervated muscles, both α- and ε-subunit mRNAs were greatly reduced or absent from the extrasynaptic segments at two weeks, but present at higher levels in the segments containing neuromuscular junctions.

One characteristic property of AChR expression at synaptic sites of normal muscle is that it is resistant to muscle activity even after the nerve has been removed. We therefore tested whether AChR mRNAs could be observed in synaptic segments of nerve-free muscles which had been kept active by electrical stimulation throughout most of the period of regeneration. In such muscles, the levels of both both α- and ε-subunit mRNAs were strongly reduced as compared with nonstimulated muscle but as in normal muscle, they remained higher in the synaptic than in the extrasynaptic segments. In fact, they were not distinguishable from those in innervated controls and in reinnervated regenerated muscle. Thus, the induction of activity-resistant expression of both α- and ε-subunit genes can take place in synaptic segments of the regenerating muscles in the absence of the nerve.

Spatial pattern of AChR mRNA expression

To examine at higher resolution the spatial distribution of the expression of α- and ε-subunit mRNAs in muscles regenerating in the absence of the nerve, we carried out in situ hybridization with specific ^{35}S-labelled cRNA probes followed by autoradiography. Synaptic sites were identified in longitudinal sections before autoradiography by their AChE activity (Figs 3-5) and their positions noted.

When sections of muscles removed 46 to 48 hours after injecting *Nss* venom were hybridized with the α-AChR mRNA probe, numerous clusters of grains associated with muscle nuclei were found scattered throughout the section. However, there was no obvious association of these clusters with the original synaptic sites (Fig. 3A,B). When hybridizing with the ε-subunit mRNA specific probe, we found no accumulations of grains either at more than 100 synaptic sites or in the extrasynaptic regions of 4 of 5 muscles (Fig. 4A,B). In one further muscle, a few myotubes had already formed and some of these contained strings of several labelled nuclei. However, their occurrence was too rare to establish a relationship to original synaptic sites.

At 4 days after venom injection, when northern analysis showed significant levels of ε-subunit mRNA in both synaptic and extrasynaptic muscle segments, occasional accumulations of this subunit mRNA were seen in both regions; in the synaptic segments, most AChE positive sites coincided with accumulations of silver grains (Fig. 4C,D), but not vice versa, suggesting that at this early stage of muscle regeneration, ε-subunit mRNA is focally expressed both at synaptic and non-synaptic sites (Fig. 5A). A similar distribution pattern was seen as early as 73 hours after *Nss* injection. By contrast, accumulations of α-subunit mRNA were much more frequent throughout the muscle (not shown).

In denervated muscles studied two weeks after damage, grains associated with α-mRNA were detected all along their length, and grain density was higher at many AChE positive sites (Fig. 3 C,D). When these muscles were stimulated via implanted electrodes, α-subunit mRNA was clearly reduced in the extrasynaptic region. At many (61%) of the original synaptic sites examined in these regenerated muscles (n=74; Fig. 3E,F), however, accumulations of grains remained even after stimulation. Small focal accumulations of grains were also observed in the extrasynaptic muscle segments. Many of these were clearly not associated with muscle fibres and may have been unfused myogenic cells (not shown).

By contrast, ε-subunit mRNA labelling was distributed in a similar way in stimulated and unstimulated muscles: it was low in the extrasynaptic region, but was accumulated at synaptic sites, with 85% of a total of 112 AChE positive sites examined in both types of muscle showing an accumulation of grains (Fig. 4 E-H). Thus, in situ hybridization indicates that the synthesis of both α- and ε-subunit mRNAs can occur at original synaptic sites in spite of intense muscle activity, confirming our data obtained with northern analysis.

The finding of some ε-subunit mRNA expression in extrasynaptic regions early in regeneration was of particular interest. To see whether that expression depended on the presence of the basal lamina or may be caused by the migration of perisynaptic satellite cells within the fibre, we examined its distribution in aneural myotube cultures derived from nonsynaptic satellite cells of adult rat diaphragm. Accumulations of grains coinciding with selective nuclei could indeed be observed (Fig. 5B) in such cultures, suggesting that neither of these mechanisms is

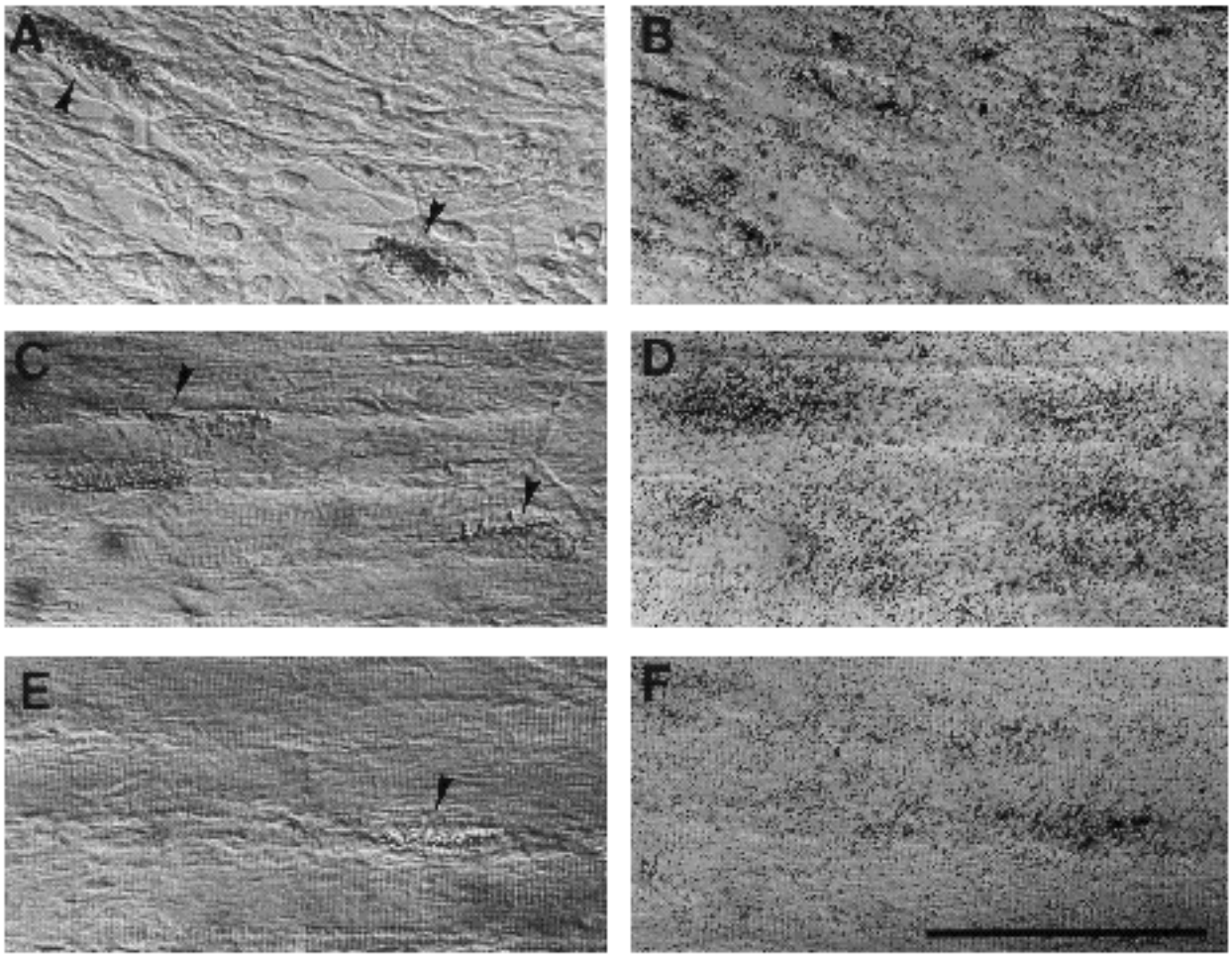


Fig. 3. Expression of α-AChR subunit mRNA is induced at original synaptic sites of muscle fibres regenerating within pre-existing basal lamina sheaths. Sections from same muscles as in Fig. 4 stained for AChE and hybridized with ^{35}S-labelled cRNA probe specific for α-subunit mRNA. For each muscle, the same section with AChE staining (left-hand column) and after hybridization (right-hand column) is shown using Nomarski optics. Sections from muscle at 43 hours (A,B), 14 days (C,D) after *Nss* venom injection. (E,F) From muscle at 15 days after *Nss* venom injection and stimulation from the 6th day onward. All sections were hybridized in the same experiment with the same of batch α-subunit mRNA specific probe. Calibration bar: 100 μm.

required to explain the early extrasynaptic expression of ε-subunit mRNA.

Expression of ε-subunit mRNA in muscles regenerating after damage to all cells in the synaptic region

While *Nss* venom causes destruction of all muscle fibres in rat soleus, it leaves intact the perisynaptic satellite cells (Kelly, 1978a), terminal Schwann cells and other cells in the vicinity of the neuromuscular junction which have been shown to have molecular properties that distinguish them from similar cells away from the synaptic region (Weis et al., 1991). To eliminate these cells as possible sources of the induction of synapse-specific AChR gene expression, we damaged some muscles a second time, 41 hours after *Nss* venom injection and denervation, by freezing the central third of the muscle with a metal probe precooled in liquid nitrogen. In an earlier study, this treatment was shown to destroy all cells intrinsic to the muscle, including all those in the vicinity to the neuromuscular synapse (McMahan and Slater, 1984). New fibres then regenerated from proliferating satellite cells invading from undamaged extrasynaptic muscle segments.

When muscles treated in this way were removed 5 hours after freezing and subsequently examined in the electron microscope (data not shown), the only intact cells found had the appearance of phagocytic cells (see above) which had presumably invaded the muscle in the 5 hours after freezing. All other cells showed signs of severe disruption of the plasma membrane and all internal organelles, including the nuclei. Regeneration in these freeze-damaged muscles was generally less effective than in muscles treated with *Nss* venom alone. However, in 6 out of 19 muscles examined 2-3 weeks after damage, numerous regenerating fibres were found and synaptic sites could be identified on many of them by staining for AChE. The distribution of ε-AChR subunit mRNAs in these 6 muscles was examined by in situ hybridization. The pattern of labelling was similar to that seen in muscles which had not been frozen; i.e. accumulations of ε-subunit mRNA coincided with original synaptic sites (Fig. 5C,D). In muscles where no stain

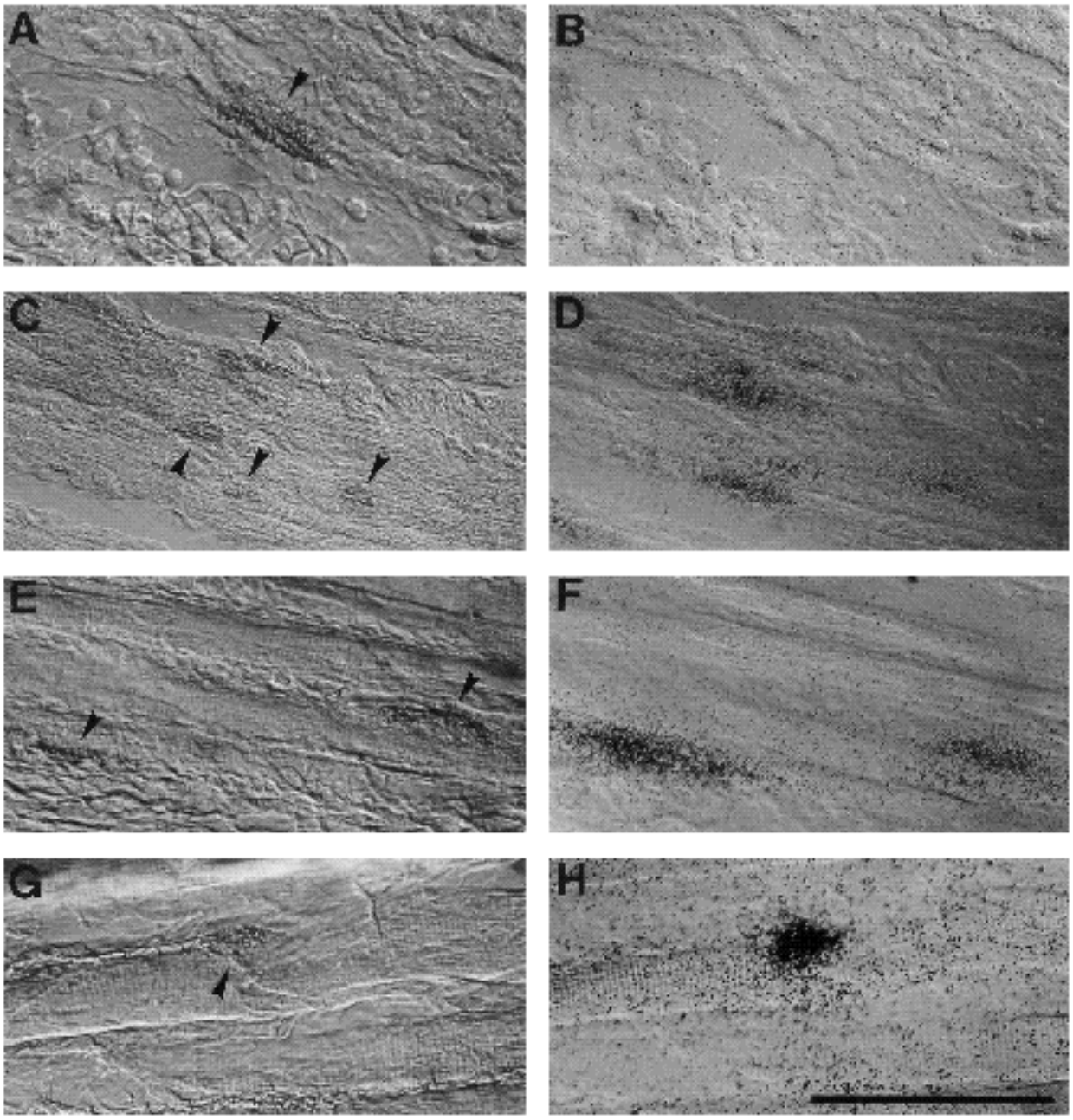


Fig. 4. Expression of ε-AChR subunit mRNA is induced at original synaptic sites of muscle fibres regenerating within pre-existing basal lamina sheaths. At various times after *Nss* venom injection, regenerating rat soleus muscle were fixed and stained for AChE, sectioned and hybridized with ^{35}S-labelled cRNA probe specific for ε-subunit mRNA. For each muscle, the same section with AChE staining (left-hand column) and after hybridization (right-hand column) is shown using Nomarski optics. Sections from muscle at 43 hours (A,B), 4 days (C,D), 14 days (E,F) after *Nss* venom injection. (G,H) from muscle at 14 days after *Nss* venom injection and stimulation from the 6th day onward. All sections except C,D were hybridized in the same experiment with the same batch of ε-subunit mRNA specific probe. Calibration bar: 100 μm.

deposits could be seen, clusters of grains were grouped in the 'synaptic' segment in a manner characteristic of the usual endplate distribution. In 3 of the 6 muscles examined, extrasynaptic clusters were also seen. These experiments suggest that none of the cellular components present in the original synaptic region are required to induce the synapse-specific expression of AChR ε-subunit gene in regenerating rat soleus muscle fibres.

Synaptic expression of adult AChR channels

At mature neuromuscular synapses, the focal expression of α-, β-, ε- and δ-subunit mRNAs leads to the accumulation of 'adult' AChR channels with short (1-2 milliseconds) apparent mean open times (Schuetze and Role, 1987). However, the activity-resistant expression of α- and ε-subunit genes at nerve-free, original synaptic sites of fibres regenerated after *Nss* treatment does not necessarily imply the

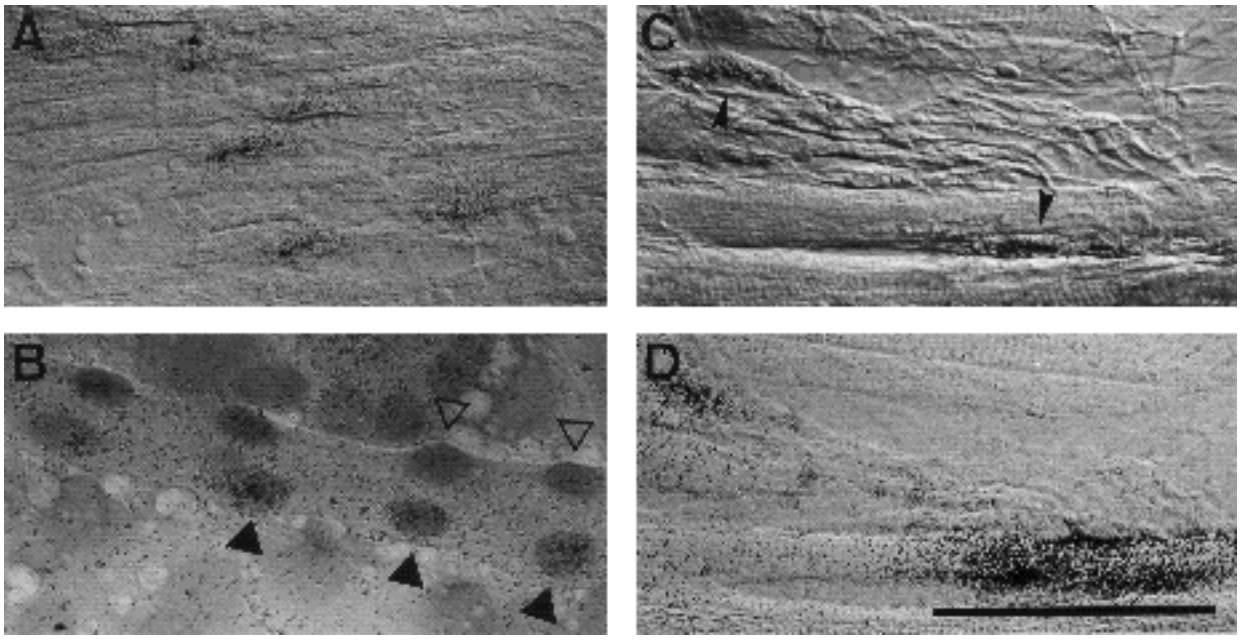


Fig. 5. (A) Focal expression of ε-AChR subunit mRNA in extrasynaptic segments of muscle fibres regenerating within pre-existing basal lamina sheaths 4 days after after *Nss* venom injection. (B) Expression of ε-AChR subunit mRNA by single nuclei in cultured myotube originating from satellite cells isolated from extrasynaptic region of adult rat diaphragm. Note accumulations of grains above selective nuclei (closed arrowheads), while at others of the same myotube, ε-subunit mRNA could not be resolved (open arrowheads). Culture was hybridized at 6 days after plating the cells. (C, D) Expression of ε-AChR subunit mRNA at synaptic sites identified by AChE staining (C) in muscle 17 days after *Nss* injection and 15 days after freezing twice its central synapse-containing segment. (D) Same section as in C after hybridization with ε-AChR subunit specific probe. Calibration bar: for A, 250 μm; B, 65 μm; C,D, 100 μm.

insertion of adult AChR channels in the regenerated muscle membrane.

To see if synaptic sites of such fibres contain adult channels in association with the accumulation of ε-subunit mRNA, we analysed the membrane current fluctuations induced by local application of acetylcholine (ACh). To localise synaptic sites in stimulated muscle fibres, we mapped the sensitivity to iontophoretically applied pulses of ACh (Fig. 6A). Most of the surface of the muscle fibres was not or only weakly sensitive to ACh, indicating the effectiveness of the stimulation in reducing the density of extrasynaptic AChRs. At single spots on 14 fibres in 4 muscles, sites were found at which application of ACh led to rapid depolarization of the muscle fibre membrane. At each of these sites, autocovariance functions (acfs) computed from membrane current fluctuations were doubly exponential (Fig. 6A), suggesting the presence of both AChR types. At membrane potentials of −60 to −90 mV, the channel open time estimated from the decay time constant of the fast acf component averaged 1.62 ± 0.06 milliseconds (s.e.m.) and was, therefore, similar to the apparent mean open times of adult AChRs at synapses in both normal muscles and in reinnervated, regenerated muscles (Fig. 6B). The time constant of the slow component was 5.49 ± 0.16 milliseconds (s.e.), i.e. similar to the mean open time of fetal AChRs. The relative sizes of the acf components indicated that about 80% of the AChRs at the regenerated endplates were of the adult type. In the extrasynaptic membrane of unstimulated fibres which were sensitive to ACh all along their length, singly exponential acfs with similar slow decays were observed (Fig. 6C), suggesting that 'fetal' AChRs predominate in extrasynaptic fibre segments. Similar results were obtained in one muscle which had been exposed to α-bungarotoxin at the time of electrode implantation to preblock AChRs possibly surviving attached to the synaptic basal lamina (Slater, 1990).

Discussion

We have shown that in rat soleus muscle fibres that regenerate in the absence of innervation, synapse-specific expression of AChR genes occurs selectively at the original synaptic sites. The characteristic 'synapse-specific' features which we have documented include the presence of a high local density of the mRNA for both the α- and ε-subunits of the AChR in muscles which are kept active by direct stimulation and the presence of functional, 'adult' type AChR-gated ion channels, demonstrating that both the localised expression of α- and ε-subunit mRNAs and of 'adult' type AChR channels are activity-resistant as they are at denervated endplates on undamaged muscle fibres. Furthermore, ε-subunit mRNA is accumulated at synaptic sites when, in addition to the muscle fibres themselves, all other cells in the synapse containing region of the muscle have been damaged. This shows that the factors inducing the expression of ε-subunit gene at synaptic sites in these muscles are associated with noncellular components of the synaptic region, very likely the synaptic basal lamina.

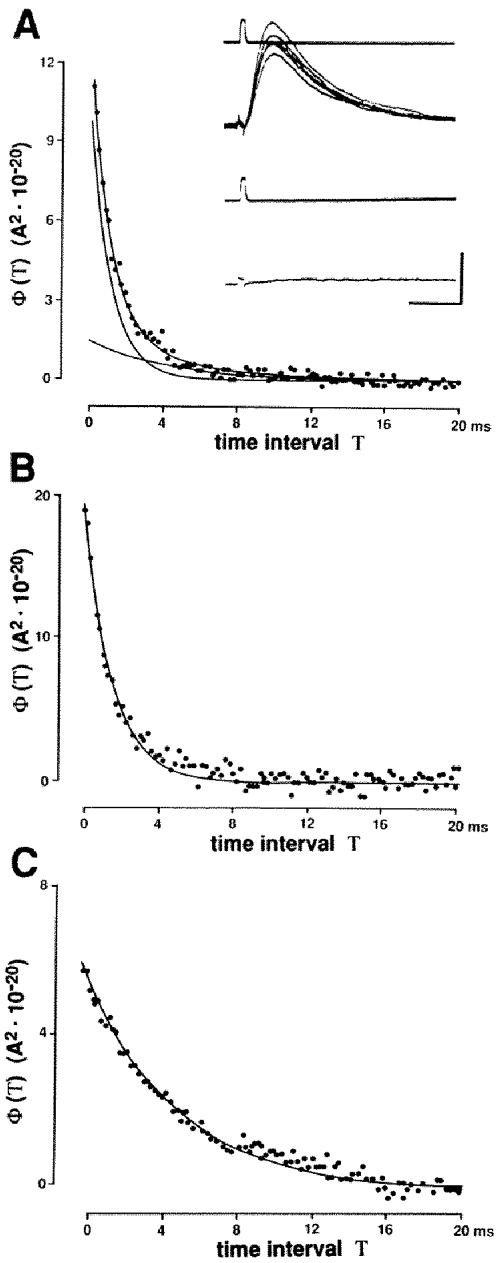


Fig. 6. Autocovariance functions (acf) of ACh-induced membrane current fluctuations recorded from original synaptic sites of muscle fibres which have regenerated within original basal lamina sheaths. Solid lines are visual fits describing double (A) or single (B,C) exponentials, respectively. (A) acf from original synaptic site in stimulated muscle fibre which has regenerated in the absence of the nerve. The acf is described by a double exponential whose components, with their time constants (τ_f = 1.6 mseconds; τ_s = 4.8 mseconds) are also shown. Holding potential, −60 mV, temperature 22°C. Inset: depolarizations of same muscle fibre in response to ACh pulses at synaptic (top) and extrasynaptic (bottom) sites. Calibrations: 2 mV, 200 nA, 20 mseconds. (B) acf at −70 mV from synaptic site in reinnervated, regenerated fibre (τ_f = 1.9 mseconds). The large, fast decaying components at synaptic sites (A,B) indicate the presence of a large fraction of adult ACh-gated channels; these are absent from the extrasynaptic membrane of unstimulated regenerated muscle (C, τ_s = 5.1 mseconds).

The method we have used to induce muscle fibre damage has the advantages of causing a single phase of degeneration in which all the muscle fibres and their nuclei are broken down within a matter of hours. Because the vascular supply remains largely intact, the invasion of phagocytes and the subsequent elimination of muscle fibre debris is rapid. It is followed by a similarly rapid phase of satellite cell proliferation and fusion which leaves new muscle fibres in the majority of original basal lamina sheaths within 3 days of venom injection. The completeness, speed and synchrony of the response to *Nss* venom injection make these muscles well suited to studies of changes in total RNA metabolism as well as for in situ hybridization.

Patterns of α- and ε-AChR mRNA expression in regenerating muscles

In regenerated nerve-free and thus inactive muscle, northern and in situ hybridization analyses revealed the presence of both α- and ε-AChR mRNAs along the entire length of the muscle, with a concentration at original synaptic sites. In the absence of more quantitative information, it is not certain whether these concentrations of mRNA result from an accumulation of myonuclei at synaptic sites, from a greater amount of mRNA associated with each synaptic nucleus, or both. Our preliminary studies suggest that an accumulation of nuclei does indeed occur in the absence of the nerve. However, as in normal muscles (Merlie and Sanes, 1985), this accumulation seems unlikely to be able to account for the full amount of synapse-specific AChR mRNA we observe.

An important feature of AChRs at normal neuromuscular junctions is that their presence is not abolished by muscle activity, contrasting with those AChRs present in the extrasynaptic regions of embryonic or denervated muscles. There is increasing evidence that this is due to locally restricted activity-resistance of AChR subunit gene expression (Witzemann et al., 1991). Suppression of AChRs by muscle activity is conferred upon the genes of all subunits except ε by the presence of *cis*-acting regulatory DNA elements (Merlie and Kornhauser, 1989; Dutton et al., 1991; Klarsfeld et al., 1991). To allow expression of these genes in the synaptic region in the face of normal activity, factors must be present locally which override the effect of those controlling elements (Martinou and Merlie, 1991; Witzemann et al., 1991). Our present findings suggest that such factors are present in the synaptic region even after the original nerve and muscle cells have been destroyed: in nerve-free, regenerated muscles kept active by stimulation, both northern and in situ hybridization analyses indicate that α-subunit mRNA is accumulated at original synaptic sites. Combined with the focal accumulation of ε-subunit mRNA at these same sites, this leads to the activity-resistent expression of functional adult-type AChR channels in the original synaptic portion of the regenerated muscle membrane.

While our results further support the notion that the expression of ε-subunit mRNA at the synapse results from a local effect of the nerve, they also show that this subunit mRNA can be present transiently in substantial amounts outside the synaptic region of regenerated muscle. Tran-

sient expression of ε-subunit mRNA independent of innervation has also been observed recently in regenerating rat soleus muscle (Goldman et al., 1991) and in a mouse muscle cell line derived from satellite cells (Pinset et al., 1991). While a similar pattern has been found during the first two weeks of postnatal life in rat leg muscle (Witzemann et al., 1989), it is not clear whether the two observations reflect the same developmental process. Another question remaining is how the regulation of ε-subunit mRNA expression in extrasynaptic fibre segments is related to its developmental accumulation at the synapse. Our finding that synapse-specific expression of ε-subunit mRNA could be observed at the earliest stage of myotube formation would be consistent with the idea that neural factors act by locally preventing its constitutive down-regulation during myogenesis.

In view of our previous observation (Witzemann et al., 1991) that ε-subunit mRNA in denervated adult muscle is not or only mildly dependent on muscle activity, it was striking to observe that the level of ε-subunit mRNA was significantly lower in reinnervated or stimulated than in unstimulated regenerated muscles. Since the level of actin mRNA was, if anything, increased under these conditions, this could indicate that, at least in muscle fibres regenerating from *Nss* damage, the level of ε-subunit mRNA is downregulated by muscle activity and, once down-regulation has taken place, becomes independent of activity.

We observed with in situ hybridization that ε-subunit mRNA was expressed selectively by small groups of extrasynaptic nuclei at early stages of regeneration. This could be due to the migration of myogenic cells originating from perisynaptic satellite cells (Kelly, 1978a) on which some previous neurotrophic influence cannot be excluded. On the other hand, we have also observed focal accumulation of ε-subunit mRNA in cultured myotubes prepared from extrasynaptic satellite cells, suggesting that the transient expression of this subunit mRNA could be constitutive rather than depend on a previous neural influence. The mechanism for its restriction to a minority of extrasynaptic nuclei remains unclear but is reminiscent of a similar selective expression pattern of α-subunit mRNA in denervated muscle and in cultured myotubes (Fontaine and Changeux, 1989). At later stages when northern analysis of mRNA from nerve-free muscle showed a decline in the extrasynaptic level of ε-subunit mRNA, clusters of this subunit mRNA could not be observed, suggesting that in the extrasynaptic region it is either made by a very small number of nuclei that escaped our detection, or by many nuclei at a low level.

In the present experiments, the ratio of α-subunit mRNA levels in synaptic compared with extrasynaptic segments of normal and of stimulated regenerated muscles was lower than observed previously in normal diaphragm (Merlie and Sanes, 1985). This may be due to the fact that endplates in the soleus are more widely dispersed than in the diaphragm such that the synaptic portion contains a greater proportion of non-synaptic fibre segments which in the soleus are known to be sensitive to ACh even in innervated muscle (Miledi and Zelena, 1966). Moreover, due to the small size of soleus muscles used, our extrasynaptic segments contained myotendinious junctions which are also known to be ACh sensitive (Miledi and Zelena, 1966).

Location of factors which induce synapse-specific gene expression

Both *Nss* venom and the procedures used by Goldman et al. (1991) eliminate the original nerve and muscle fibres as sources of those factors. However, they leave open the possibility that other cells, normally associated specifically with the synaptic region, may survive and act as such a source. Such cells might include perisynaptic satellite cells (Kelly, 1978a), Schwann cells and perisynaptic fibroblasts (Weis et al., 1991). By freezing muscles which have been previously damaged with *Nss* venom, we were able to destroy all cells intrinsic to the muscle. Although regeneration was impaired in such severely damaged muscles, when it occurred, accumulations of ε-subunit mRNA were found at original synaptic sites. This strongly suggests that factors which can induce ε-subunit mRNA expression are associated with the extracellular material which survives the destruction of the cells in the muscle. The presence of functional ACh-gated ion channels with adult properties at synaptic sites on muscles regenerating after venom injection indicates the expression of all the usual AChR subunits. Thus, it seems most likely that extracellular factors are also able to induce the synapse-specific expression of the β- and δ-subunits contained in the adult AChR subtype.

Since the effect of extracellular factors on AChR gene expression is limited to the site of the original neuromuscular junction, and since the basal lamina in this region is known to contain a number of molecular components which are not found in the extrasynaptic region (Sanes, 1983), the factors must be associated with the synaptic basal lamina. Studies in the frog have provided strong evidence that components of the synaptic basal lamina are able to influence the distribution of AChR and AChE molecules at the surface of regenerating muscles (McMahan and Wallace, 1989). Our results suggest that the effects of such extracellular factors reach to the interior of the muscle fibre to act on nuclear gene expression.

Possible basal lamina factors

The identity of the factors that induce the synapse-specific expression of AChR genes observed here is unknown. One well characterized component of the synaptic basal lamina which causes the accumulation of AChRs in cultured muscle fibres is agrin (Nitkin et al., 1987). This protein is synthesised in the motor neurone cell body, transported to the periphery in the motor axon and there probably secreted and incorporated into the basal lamina in the synaptic cleft (McMahan and Wallace, 1989). Agrin-mediated aggregation of AChRs is accompanied by phosphorylation of some AChR subunits (Wallace et al., 1991). It is possible that agrin also induces the modification of DNA binding proteins that mediate the regulation of AChR gene activity.

Recently, another protein purified from chick brain termed ARIA (AChR-inducing activity) has been proposed to act as the neurotrophic factor inducing adult type AChRs at the synapse (Martinou et al., 1991). When applied to cultured mouse myotubes it increases the levels of α- and ε-AChR mRNAs about two- and eight-fold, respectively. No

association with the basal lamina has been reported, however.

Control of AChR genes by stable basal lamina factors could explain 'imprinting' in normal development and the 'trace' in ectopic NMJ formation

A scheme in which a neurally derived protein becomes stably bound to the basal lamina, defining it as 'synaptic' and inducing synapse-specific gene expression, could help to explain the effects of transient nerve-muscle interaction on the subsequent development of synaptic properties during neuromuscular junction formation. In rats, both in normal development (Brenner et al., 1990) and during the formation of new, ectopic, synapses in adult muscles (Lømo and Slater, 1980), destruction of the nerve at an early stage in synaptogenesis appears to leave behind an 'imprint' or 'trace' which permits the subsequent differentiation of the synaptic region. In the case of normal development, this persisting effect results in the localised appearance of ε-AChR mRNA, even though the nerve is absent. In the analogous situation in ectopic neuromuscular junction formation in adults, brief contact (<3 days) of a denervated muscle with an implanted foreign nerve was found to be adequate to cause the persistence of adult AChRs at the presumptive synaptic site in spite of intense activity imposed by stimulation (Lømo and Slater, 1980; Brenner et al., 1987), and in the expression of α- and ε-subunit mRNAs (Brenner and Sakmann, unpublished data). Both these sets of findings could reflect the activity of a stable basal lamina factor, originating in the nerve, which has the dual effects of inducing the synthesis of synaptic AChRs and causing their subsequent aggregation.

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