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Differential regulation of muscle acetylcholine receptor γ- and ϵ-subunit mRNAs

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The contents of the mRNAs encoding the γ- and ϵ-subunits of the nicotinic acetylcholine receptor as well as the single-channel properties of the receptor have been assessed in innervated, denervated and reinnervated rat muscle. The changes in abundance of the γ- and ϵ-subunit mRNAs correlate with the changes in relative density of two classes of acetylcholine receptor channels. The results support the view that a switch in the relative abundance of the γ- and ϵ-subunit mRNAs is a major mechanism in regulating the properties of acetylcholine receptor channels in muscle.

Acetylcholine receptor; Denervation; Reinnervation; RNA blot hybridization analysis; Patch-clamp analysis; (Rat diaphragm)

1. INTRODUCTION

Acetylcholine receptors (AChR) are distributed throughout the developing skeletal muscle cell surface, but become highly concentrated at the neuromuscular junction in the innervated adult muscle. Concomitantly, AChRs become metabolically stable and their channel properties change [1,2]. In bovine muscle, a developmentally regulated switch has been found in the expression of two types of AChR channels, which have different functional properties and which are composed of the α-, β-, γ- and δ-subunits or of the α-, β-, ϵ- and δ-subunits [3]. Denervation results in a large increase of newly synthesized AChRs, which become mostly incorporated into extrasynaptic regions. These AChRs have distinct biophysical properties and appear to be biochemically different from junctional AChRs [1,2]. To investigate the mechanism by which the motor nerve controls the expression of the different forms of AChR channels, we have now measured the contents of the mRNAs encoding the γ- and ϵ-subunits of the AChR in innervated, denervated and reinnervated rat muscle. The changes in abundance of the γ- and ϵ-subunit mRNAs are found to correlate with the changes in relative density of the two classes of AChR channels.

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Abbreviations: AChR, acetylcholine receptor; ACh, acetylcholine

The nucleotide sequence presented has been submitted to the EMBL/GenBank database under the accession number Y00696

2. MATERIALS AND METHODS

The rat genomic DNA library used was a collection of recombinant phages that carried rat liver DNA fragments generated by partial digestion with *Sau*3AI and joined to the arms of λEMBL3 [4] digested with *Bam*HI. Phages were screened [5] by hybridization in situ at 60°C. The hybridization

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probe used for initial screening was a mixture of the *Pst*I(−100)/*Hin*dIII(1181) fragment (numbers indicating the 5′-terminal nucleotide generated by cleavage) excised from the plasmid pSPcγ [6,7] carrying the bovine γ-subunit cDNA and the *Bam*HI (on vector)/*Eco*RI(1444) fragment derived from the plasmid pSPcϵ [7] carrying the bovine ϵ-subunit cDNA. Hybridization-positive clones were isolated by repeated plaque purification. Fragments of the genomic DNAs isolated were subcloned in the plasmid pUC19 [8]. The *Aat*I(530)/*Rsa*I(757) fragment of the bovine γ-subunit cDNA [6] and the *Eco*RV(520)/*Acc*II(737) fragment of the bovine ϵ-subunit cDNA [7] were used as probes corresponding mainly to exon P7 of the human γ-subunit gene [9]. DNA blotting analysis was carried out as in [10]. All hybridization probes were labelled with [α-^{32}P]dCTP by nick-translation [11]. DNA sequencing was carried out by the method of Maxam and Gilbert [12].

Diaphragms of female Wistar rats (150–300 g) were frozen in liquid nitrogen immediately after dissection. Total RNA was extracted by the guanidinium isothiocyanate method as detailed in [13]; in some experiments, poly(A)$^+$ RNA was isolated as in [13]. Samples of RNA (15 μg each) were denatured with 1 M glyoxal and 50% dimethyl sulphoxide [14], electrophoresed on 1.5% agarose gels and transferred [15] to Biodyne nylon membranes (Pall). Hybridization was carried out according to Thomas [15] and as detailed in [3]. The specific activity of the ϵ-subunit-specific probe (~ 2–4×10^8 dpm/μg) was about 2-fold higher than that of the γ-subunit-specific probe (~ 1–2×10^8 dpm/μg). Increasing amounts (2–15 μg) of total RNA isolated from 7 day denervated rat diaphragm showed, using the same blot for hybridization with the γ- and ϵ-subunit-specific probes, a linear increase in specific hybridization signals. The chicken β-actin cDNA probe used was a ~ 2 kilobase pair (kb) insert excised with *Pst*I [16]. The size markers used were rat and *Escherichia coli* ribosomal RNAs.

Denervation was performed by cutting the phrenic nerve innervating the left hemidiaphragm. In some experiments, hemidiaphragms denervated for 7 days were divided into three parts: a central part containing the former end-plates (end-plate-rich region) and two adjacent parts extending to the tendinous and rib insertion (end-plate-free region); each part was 5–8 mm in width. The extent of the end-plate-rich region was assessed in control strips by staining for acetylcholine esterase activity. The time course of reinnervation following mechanical crushing of the left phrenic nerve by a pair of forceps was examined in 13 experiments which were performed between days 5 and 30 after nerve crushing. A fibre was considered as being reinnervated when spontaneously occurring miniature end-plate potentials were observed at a rate >0.5/min. A strip was regarded as reinnervated when at least 90% of all fibres examined within the strip were reinnervated. Separate muscle preparations were used for electrophysiological experiments and RNA blot hybridization analysis.

Electrophysiological measurements on normal fibres were made on omohyoid muscle (female Wistar rats, 120–150 g). End-plates were identified visually at $\times 250$ magnification and all records were made on a perisynaptic membrane located within 20 μm of the ovoid-shaped end-plates. Measurements on denervated fibres were made on the left hemidiaphragm on the end-plate-free region 5–8 mm away from the former end-plates. Muscles were denervated by cutting the phrenic nerve 7–9 days before the experiment. Muscles were dissected to a sheet consisting of 3–5 layers of fibres and then incubated for 2–3 h in minimal essential medium (Gibco) containing 0.5 mg/ml collagenase (Sigma, type I). For current measurements, muscles were maintained in rat Ringer's solution (in mM): 140 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (pH 7.2). The patch pipette solution contained (in mM): 150 NaCl, 1 $BaCl_2$, 10 Hepes (pH 7.2). In all experiments, 0.5 μM acetylcholine (ACh) was added to the pipette solution to activate AChR channels. All experiments were performed at 20 ± 1°C. Conventional patch-clamp techniques [17] and standard methods for analysis were used. For the determination of average current durations, the time course fitting method [18] was employed. In all records, the distribution of current durations was characterized by two components. The amplitude and time constant of the two components were fitted using the maximum likelihood method [18]. The average current durations given in the text refer to the slower, main component of the distributions and were measured between -75 and -95 mV membrane potential.

3. RESULTS AND DISCUSSION

A rat genomic DNA library, constructed in the bacteriophage λ derivative EMBL3 [4], was screened for phage carrying AChR γ- or ε-subunit gene sequences by hybridization with the corresponding bovine muscle cDNAs [6,7]. From ~1.5 × 10^6 plaques, five hybridizable clones were isolated. One of these clones (λrACRγ25), carrying a ~19-kb DNA insert, hybridized with the bovine γ-subunit cDNA probe, and another (λrACRε1), carrying a ~20-kb DNA insert, hybridized with the bovine ε-subunit cDNA probe. A 4.1-kb *Bam*HI fragment and a 4.3-kb *Hin*dIII fragment from clone λrACRγ25 and a 2.4-kb *Bam*HI fragment from clone λrACRε1 were shown by DNA blotting analysis [10] to hybridize strongly with the bovine γ- and ε-subunit cDNA probes, respectively. These three fragments were subcloned in the plasmid pUC19 [8] for further analysis.

A relatively large and highly conserved protein-coding exon of the human γ-subunit gene is exon P7 [9]. DNA blotting analysis of the 4.1-kb *Bam*HI fragment from λrACRγ25 using a bovine γ-subunit cDNA probe corresponding mainly to exon P7 of the human γ-subunit gene showed that a 249-base pair (bp) *Hin*fI fragment hybridized intensively with the probe. Similar analysis of the 2.4-kb *Bam*HI fragment from λrACRε1 using a bovine ε-subunit cDNA probe corresponding approximately to the bovine γ-subunit exon P7 probe showed a 453-bp *Dde*I fragment to be strongly hybridizable. The nucleotide sequences of these *Hin*fI and *Dde*I fragments and adjacent regions, together with the amino acid sequences deduced from the exonic sequences, are given in fig.1. On the basis of comparing the nucleotide and the deduced amino acid sequences in the region of exons P7 and P8 with the corresponding sequences for the mammalian AChR γ- and ε-subunits and their coding DNAs hitherto known (see fig.1 legend), we conclude that λrACRγ25 and λrACRε1 carry rat genomic DNA sequences encoding the AChR γ- and ε-subunits, respectively. Thus, the *Hin*fI fragment from λrACRγ25 and the *Dde*I fragment from λrACRε1 were used as probes for RNA blot hybridization analysis as described below. Dot hybridization analysis showed that these probes did not cross-hybridize with each other.

Fig.2A and C represents autoradiograms of blot hybridization analysis of total RNA from innervated and denervated (7 days after cutting the nerve) rat diaphragms. In innervated diaphragm, the ε-subunit mRNA with an estimated size of ~1900 nucleotides is observed (fig.2A, lane a), whereas the γ-subunit mRNA is not detectable (fig.2C, lane a). The situation changes dramatically following denervation of the muscle. The content of the ε-subunit mRNA increases only slightly (fig.2A, lane b), whereas an enormous increase is observed in the content of the γ-subunit mRNA with an estimated size of ~2200 nucleotides (fig.2C, lane b). The variations in the contents of the γ- and ε-subunit mRNAs in rat diaphragms denervated for different lengths of time (2–7 days) were estimated densitometrically from autoradiograms of blot hybridization analysis (fig.2B,D). Assuming similar hybridization efficiencies for the γ- and ε-subunit-specific probes and correcting for the difference in the specific radioactivities of the two probes, the data suggest that in 7 day denervated diaphragm the γ-subunit mRNA increases to levels about one order of magnitude higher than those of the ε-subunit mRNA. The results indicate furthermore that the synthesis of the γ-subunit mRNA is switched on within the first 2 days after denervation. In contrast, the content of the ε-subunit mRNA increases only about 2–3-fold. Some blots were hybridized with a chicken β-actin cDNA probe; under the hybridization conditions used this probe is assumed to hybridize with various actin mRNAs and the strong band seen probably represents muscle type α-actin mRNA (see [29] and references therein). The results presented in fig.2E and F show little variation in actin mRNA content, indicating that denervation exerts specific and differential effects on the levels of the ε- and γ-subunit mRNAs.

Merlie and Sanes [30] have reported that the α- and δ-subunit mRNAs are concentrated in the synaptic regions of innervated mouse diaphragm muscle. The spatial distribution of the γ- and ε-subunit mRNAs was examined by analysing poly(A)$^+$ RNA from end-plate-rich and end-plate-free muscle strips from denervated diaphragm. Both mRNAs are detected in the end-plate-rich (fig.3A, lanes b,d) as well as in the end-plate-free region (fig.3A, lanes a,c), being more abundant in the end-plate-rich region. In both regions, the con-

Fig.1. Nucleotide sequences of rat AChR γ- (A) and ϵ-subunit gene regions (B) containing exons P7 and P8 together with the deduced amino acid sequences. The sequence of the message strand is shown. The positions of exons P7 and P8 assumed by comparison with those of the human AChR γ-subunit gene [9] and the relevant restriction endonuclease sites are indicated. The fragments used as hybridization probes are underlined. The percentage homology of amino acid sequences and that of exonic DNA sequences (in parentheses) in the region of exons P7 and P8 are as follows: rat γ/mouse γ [19,20], 96 or 97 (92 or 93); rat γ/bovine γ [6], 95 (90); rat γ/human γ [9], 93 (90); rat γ/bovine ϵ [7], 63 (64); rat ϵ/bovine ϵ, 89 (82); rat ϵ/mouse γ, 61 or 62 (64); rat ϵ/bovine γ, 63 (66); rat ϵ/human γ, 61 (65). The homology between the γ- or ϵ-subunit and the other subunits is considerably lower: rat γ/mouse or bovine α, β or δ or human α [21–27], 35–53 (53–64); rat ϵ/mouse or bovine α, β or δ or human α, 38–50 (48–59). The calculation of homology is based on the sequence alignment reported previously [23,28]; a continuous stretch of gaps has been counted as one substitution regardless of its length.

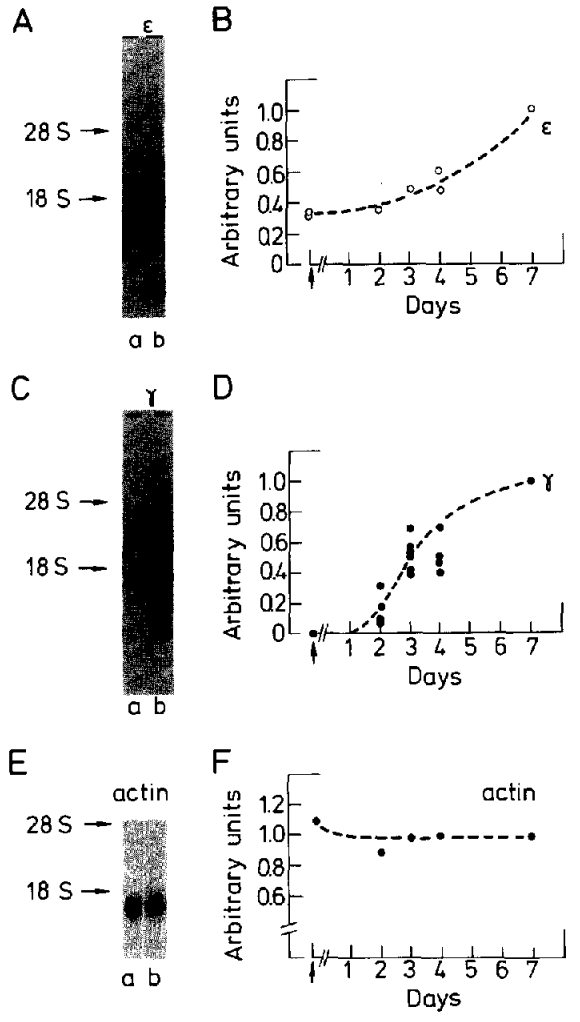

Fig.2. Effect of denervation on the contents of the AChR ϵ-subunit mRNA (A,B), γ-subunit mRNA (C,D) and actin mRNA (E,F) in rat diaphragm. (A,C,E) Autoradiograms of RNA blot hybridization analysis using the ϵ- (A) and γ-subunit-specific probes (C) and an β-actin cDNA probe (E). Total RNA from innervated rat diaphragm (lane a) and from rat diaphragm denervated for 7 days (lane b) was analysed. Autoradiography was performed at −70°C for 5 days (A,C) or 20 h (E) with an intensifying screen. The positions of rat ribosomal RNA are shown. (B,D,F) Changes in the contents of the ϵ-subunit mRNA (B), γ-subunit mRNA (D) and actin mRNA (F) at different times after denervation. The arrows indicate the values measured for innervated tissues. Total RNA from innervated and denervated rat diaphragms was subjected to blot hybridization analysis and the resulting autoradiograms (exposed for 20 h–5 days) were scanned densitometrically. The relative contents of the respective mRNAs thus evaluated have been plotted against the time that elapsed after denervation; the values are normalized with respect to those obtained for the diaphragms denervated for 7 days. The larger RNA species hybridizable with the γ- and ϵ-subunit-specific probes (<10% of the total signal), which presumably represent incompletely spliced RNA [3,6], were not included for densitometric evaluation. The symbols represent individual samples. In each case, a diaphragm denervated for 7 days served as standard. For the analysis of the γ- and ϵ-subunit mRNAs, five separate preparations of diaphragms denervated for 7 days were used; in the case of actin mRNA, two separate preparations were analyzed.

tent of the γ-subunit mRNA is higher than that of the ϵ-subunit mRNA. The uneven distribution of AChR subunit mRNAs in denervated muscle supports the notion that they are synthesized preferentially in synapse-associated nuclei. A nonuniform distribution of extrajunctional AChRs after denervation has been found using electron microscopic autoradiography [31].

When the left phrenic nerve is crushed 5–8 mm from its entry into the muscle, reinnervation, as measured by recording miniature end-plate potentials (see section 2), begins 7–9 days later. The proximal two-thirds of the hemidiaphragm are reinnervated completely on day 15–18 following nerve crushing (not shown). Fig.3B shows autoradiograms of blot hybridization analysis of total RNA from rat diaphragms excised at different times of reinnervation. 7 days after nerve crushing, the abundance of the γ-subunit mRNA is high, being comparable to that observed after denervation by cutting the nerve (lane d). The amount of the γ-subunit mRNA thereafter decreases as the hemidiaphragm becomes reinnervated, and on day 30 after the crush the γ-subunit mRNA is undetectable (lanes e,f). Assuming that reinnervation occurs on day 8 after nerve crushing, the increased expression of the γ-subunit mRNA is switched off within 22 days following reinnervation. For the ϵ-subunit mRNA, slightly elevated levels are measured on days 7 and 14 after nerve crushing, again being comparable to the levels observed after 7 days of denervation (lanes a,b).

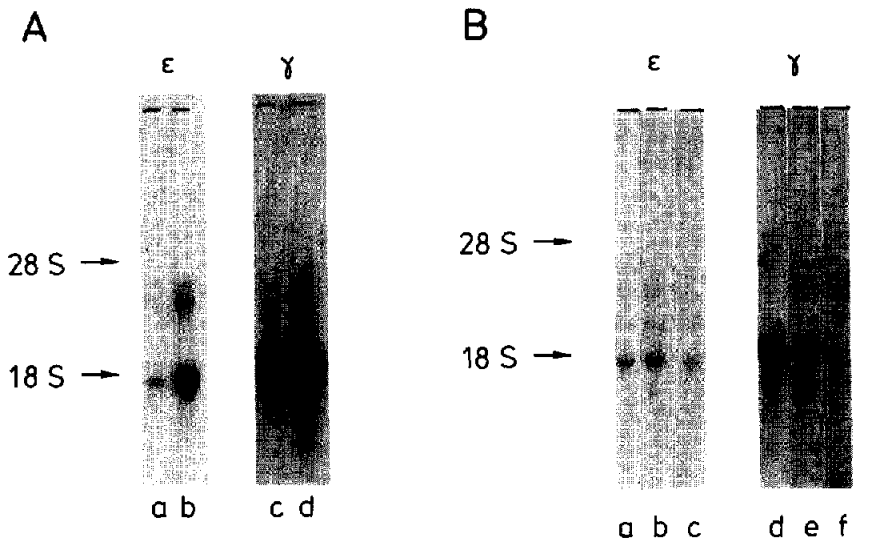

Fig.3. (A) Distribution of the ϵ- (a,b) and γ-subunit mRNAs (c,d) in 7 day denervated rat diaphragm. Poly(A)$^+$ RNA (15 μg each) from the end-plate-free region (lanes a,c) and from the end-plate-rich region (lanes b,d) was subjected to blot hybridization analysis. The resulting autoradiograms (exposed for 24 h) are shown. Qualitatively similar results were obtained with total RNA (15 μg each), although the hybridization signals were weaker. (B) Changes in the contents of the ϵ- and γ-subunit mRNAs in rat diaphragm during nerve regeneration. Total RNA (15 μg each) from diaphragms excised 7 days (lanes a,d), 14 days (lanes b,e) and 30 days (lanes c,f), after crushing the nerve was subjected to blot hybridization analysis. The resulting autoradiograms (exposed for 3 days) are shown.

Later during reinnervation the amount of the ϵ-subunit mRNA falls back to a level representative of normal innervated muscle (lane c). Thus, restored synaptic function leads to complete repression of γ-subunit mRNA expression reminiscent of the change observed during development of bovine muscle [3].

Innervated and denervated muscles were examined for the presence of different types of AChR channels. In innervated muscle, the ACh sensitivity is restricted to the region lying within 200 μm of the end-plate. Fig.4A shows single-channel currents recorded from a normal fibre which represent openings of a single class of high-conductance (62 ± 3 pS, mean $\pm$ SD, $n = 8$) AChR channels (fig.4B,C). The mean duration of the currents is 1.74 ± 0.14 ms ($n = 5$). In all patches examined ($n = 8$), amplitude histograms are characterized by a single peak suggesting that only a single class of high-conductance AChR channels is present in innervated muscle. In denervated muscle, ACh sensitivity has spread over the whole fibre. Fig.4D shows currents recorded from a denervated fibre in the end-plate-free region. The vast majority of elementary currents are of smaller amplitude than those recorded from normal fibres and represent openings of low-conductance (42 ± 3 pS, $n = 11$) AChR channels (fig.4E,F). These lower amplitude elementary currents also have longer average durations which vary widely between different patches (9.22 ± 4.11 ms, $n = 11$; range between 4.6 and 18 ms). In 13 of 23 patches, the distribution of current amplitudes showed an additional smaller peak (fig.4E) indicating that in the end-plate-free region of denervated fibres high-conductance channels (63 ± 3 pS, 2.26 ± 0.17 ms average duration, $n = 4$) are also present though at a very much lower density (fig.4F). Their average frequency of occurrence was $6.8 \pm 1.9\%$ of the total number of currents observed (mean $\pm$ SE, range 0–27%, 23 patches). Thus in denervated fibres, the low-conductance channel is much more abundant than the high-conductance channel.

The large increase in rate of AChR synthesis upon denervation is well documented [1,2]. Several reports confirmed this finding, demonstrating a significant increase in AChR subunit mRNA levels [19,29,32–34]. Our results indicate that the ϵ- and

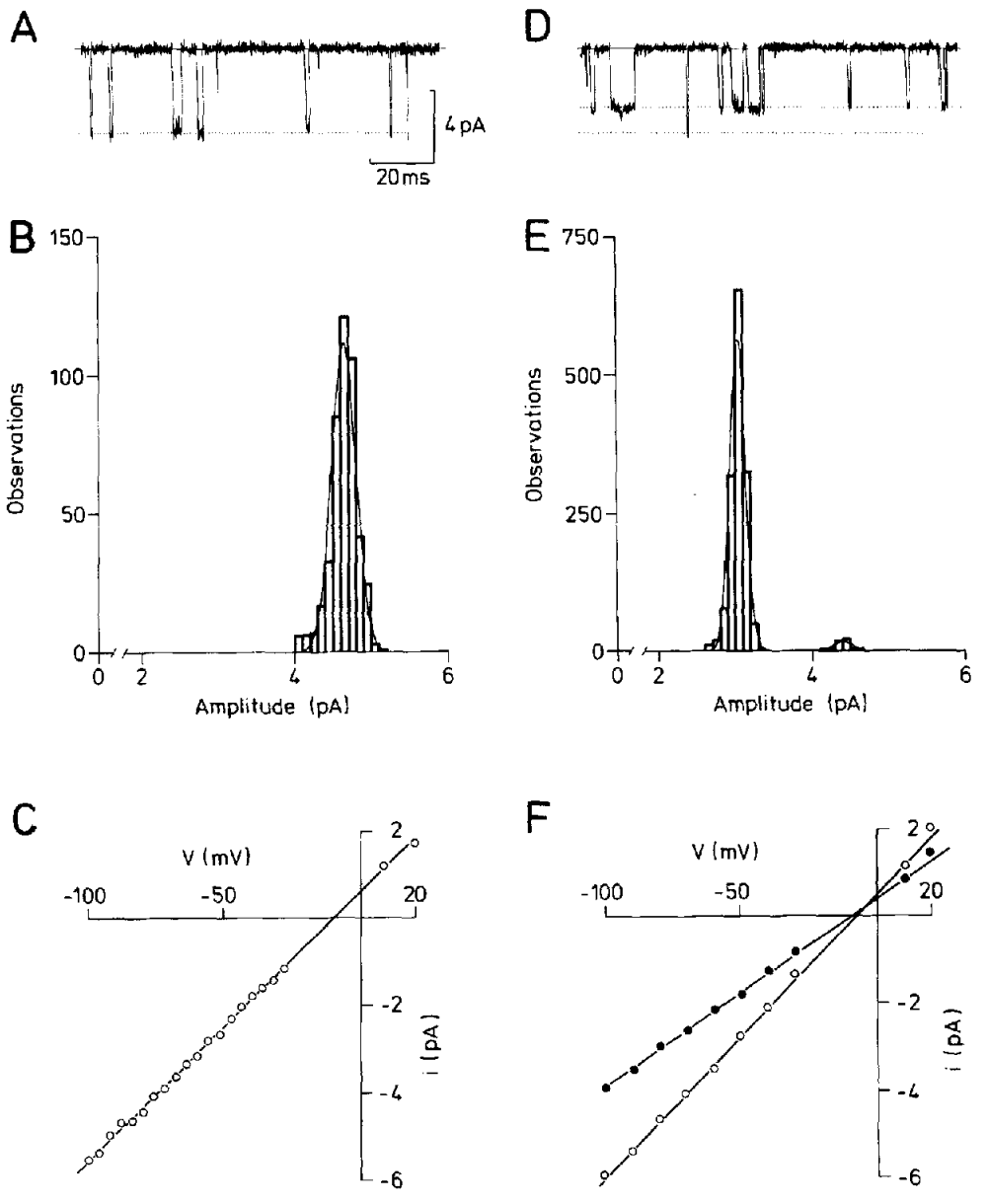

Fig.4. Functional properties of AChR channels in normal and denervated muscle. (A) Single-channel currents activated by ACh in perisynaptic membrane of normal muscle. Membrane potential −81 mV. Dotted line indicates average current amplitude of 4.4 pA. (B) Amplitude distribution of ACh-activated currents in normal fibre. −81 mV. Gaussian fit indicates mean amplitude of 4.65 ± 0.16 pA. (C) Current-voltage (*i-V*) relation of ACh-activated channels in normal fibre. The conductance of the end-plate channel (62 pS) is obtained by linear regression as indicated by the straight line. (D) Single-channel currents activated by ACh in denervated fibre (7 days after cutting phrenic nerve). Recording from end-plate-free region. Membrane potential −80 mV. Dotted lines represent average sizes of two classes of currents of 3.1 and 4.4 pA, respectively. Same scales as in (A). (E) Amplitude distribution of ACh-activated currents in denervated fibre. −80 mV. Two well-separated peaks fitted by Gaussians have mean amplitudes of 3.05 ± 0.10 and 4.41 ± 0.13 pA, respectively. (F) Current-voltage relations of ACh-activated channels in denervated fibre. Filled symbols represent the *i-V* relation of the more frequently occurring, low-conductance channel. Open symbols represent the *i-V* relation of the less frequently occurring, larger conductance channel. Linear regression lines indicate channel conductances of 43 and 65 pS, respectively.

γ-subunit mRNAs are regulated differentially. The ϵ-subunit mRNA level is relatively independent of denervation or reinnervation of adult muscle, whereas the γ-subunit mRNA level is under tight control of the motor nerve, being low when the muscle is innervated and being high when it is not innervated. A major mechanism that determines the expression of the two different classes of AChR channels in muscle is a switch in the relative abundance of the γ- and ϵ-subunit mRNAs. In innervated muscle where only the ϵ-subunit mRNA is found, the high-conductance channel ($\sim$60 pS) is exclusively observed. In denervated muscle, both the γ- and ϵ-subunit mRNAs are present, but the γ-subunit mRNA is much more abundant. Concomitantly, the low-conductance channel ($\sim$40 pS) predominates.

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