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Developmental regulation of five subunit specific mRNAs encoding acetylcholine receptor subtypes in rat muscle

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The muscular content of the mRNAs encoding the five subunits of the nicotinic acetylcholine receptor was measured during postnatal development in the rat. Subunit specific mRNAs show differential regulation. The levels of the α-, γ- and δ-subunit specific mRNAs decrease steadily after birth, while the β- and ε-subunit mRNAs increase transiently and then decrease. The adult pattern of subunit specific mRNA levels is reached at 4–6 weeks postnatally. The content of γ- and ε-subunit mRNA changes in a reciprocal fashion during the first 2 postnatal weeks, supporting the view that differential regulation of γ- and ε-subunit mRNA during development is one mechanism mediating the appearance of the adult, ε-subunit containing, subtype of end-plate channel. Denervation of neonatal muscle increases the levels of all subunit-specific mRNAs during further development. It prevents the postnatal decrease in γ-subunit mRNA and enhances the initial increase in ε-subunit mRNA. This makes it appear that the ε-subunit gene is less sensitive to regulation by the nerve in the postnatal period than the γ-subunit gene.

Acetylcholine receptor; Development; Channel subtype; RNA blot hybridization

1. INTRODUCTION

The acetylcholine receptors (AChRs) of mammalian muscle undergo drastic changes in their metabolic stability and functional properties during development of the neuromuscular synapse (review [1]). Results obtained by combining electrophysiological and recombinant DNA techniques as well as immunological evidence, have suggested that mammalian muscle expresses two subtypes of end-plate channels, a low conductance fetal and a high conductance adult type. Both subtypes are comprised of the α-, β- and δ-subunits and either the γ- or the ε-subunit [2–4]. In bovine muscle the levels of the mRNA encoding the γ- and the ε-subunit change during development and correlate with the presence of the two AChR subtypes in fetal and adult muscle, respectively [2]. A number of important issues remain unresolved, including the time course in appearance of mRNAs encoding the γ- and ε-subunits and its relationship to changes in channel properties, as well as the relative roles of nerve and muscle in controlling the content of subunit specific mRNAs. In rat the conversion of end-plate channel properties occurs within a relatively short period during the second and third postnatal week [5–8]. Therefore we investigated developmental changes in the amount of subunit mRNAs with a time resolution of a few days and examined the importance of nerve and muscle in the developmental regulation, by comparing the postnatal changes in subunit-specific mRNA levels in normal and in denervated neonatal muscle.

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2. MATERIALS AND METHODS

2.1. *Isolation of lower leg muscle*

Wistar rats of various ages were decapitated and the triceps surae muscles were removed and frozen in liquid nitrogen. Denervation was performed under deep ether anesthesia by cutting the right sciatic nerve and ligating the proximal nerve stump to prevent reinnervation. The postnatal age, given in

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days after birth, is defined as follows: day 1 refers to the time within the first 24 h of birth. In each experiment where the time course of postnatal changes in RNA content was measured, muscles from littermates from two or three litters, born within one day, were used. Litters consisted of eight or more neonates.

2.2. *RNA isolation and RNA blot analysis*

Total RNA was extracted by the guanidinium isothiocyanate method as described [9]. Samples of RNA (10 μg each) were denatured in 1 M glyoxal and 50% dimethyl sulfoxide [10], electrophoresed on 1.5% agarose gels and transferred [11] to Biodyne nylon membranes (Pall). Hybridization was carried out according to Thomas [11] and as detailed by Mishina et al. [2]. All hybridization probes were labelled with [α-^{32}P]dCTP using the random primed labelling kit from Boehringer (Mannheim) which followed labelling procedures based on the method of Feinberg and Vogelstein [12]. The specific activities of the probes ranged from $1-2 \times 10^9$ cpm/μg DNA.

2.3. *Hybridization probes*

Probes for the α- and β-subunit mRNAs were derived from denervated rat muscle AChR cDNAs isolated from a λgt10 library. The α-subunit specific probe is a ~550 bp cDNA fragment from an α-subunit specific clone (unpublished). The rat specific nucleotide sequence is homologous to the published bovine cDNA from nucleotide 650 to 1150. The β-subunit specific probe is a ~300 bp cDNA fragment derived from a β-subunit specific cDNA clone. The homology to the bovine β-subunit cDNA extends from nucleotide 1033 to 1330 (unpublished). The γ- and ϵ-subunit specific probes have been described [3]. The δ-subunit specific probes were isolated from the λrACRγ25 genomic clone which also carries the γ-subunit specific sequences [3]. The δ-subunit specific probe is a genomic *Hin*dIII fragment of about 1500 bp and contains sequences corresponding to the putative protein coding region P7 plus additional 3'- and 5'-flanking sequences (unpublished). The chicken β-actin cDNA probe used was a gift from B. Paterson and represents the ~2000 bp *Pst*I fragment described by Cleveland et al. [13].

2.4. *Densitometric evaluation of autoradiograms*

Autoradiograms were scanned densitometrically using a Shimadzu dual wavelength TLC scanner (CS-910). Increasing amounts (2–15 μg) of total RNA from 7-day denervated rat muscle gave a linear increase in the specific hybridization signals for all five AChR-subunit specific probes. To follow the developmental changes in mRNA levels the same blots were used for hybridization with the five different probes. The subunit-specific changes and the differences in transcript sizes support the specificity of the observed hybridization signals and exclude the possibility of any significant cross-hybridization. The β-actin specific cDNA probe has been used to measure the actin-mRNA levels in the developing muscle. This probe does not discriminate between various actin forms such as α- and β-actin, which are developmentally regulated, but yields an estimate of total actin-mRNA levels.

3. RESULTS

Fig.1 (left column) shows autoradiograms of blot hybridizations of total RNA from lower leg muscle isolated at days 4, 8, 13, 23, and 42 of postnatal age (lanes 1 to 5) and from adult muscle (lane 6) using DNA probes specific for the α-, β-, γ-, δ- and ϵ-subunit mRNAs. The autoradiograms shown in fig.1 were obtained by hybridizing the same blot consecutively with the five subunit specific probes. The amount of each of AChR-subunit specific mRNAs shows characteristic changes during postnatal development. Whereas the α-, β-, δ- and ϵ-subunit mRNAs are present at all postnatal stages as well as in the adult muscle, the γ-subunit mRNA is present only at early postnatal stages and becomes undetectable in the adult muscle. These results show that the hybridization probes bind specifically to their corresponding mRNA species and that the muscular content of subunit mRNA levels is developmentally regulated. The autoradiograms shown in fig.1 and those of additional experiments were densitometrically evaluated and the results are summarized in fig.1 (right column), where the relative amount of each subunit-specific mRNA species is plotted as a function of time after birth. The α-subunit mRNA is present in much higher amounts at day 1 than in adult muscle. With development the level decreases gradually to reach the level of adult muscle after day 42. The β-subunit mRNA content shows a different time course, the level being low at day 1 and increasing during the following 10 days. Maximal amounts are detected about 2 weeks after birth and thereafter the β-subunit mRNA declines slowly to the adult level. The time course of changes in the amount of the other subunit specific mRNAs seems to follow roughly one of these two profiles. The γ- and δ-subunit mRNAs are comparable to the α-subunit mRNA being present at highest levels at birth and declining steadily to the lower adult level. The ϵ-subunit mRNA shows, like the β-subunit mRNA, a transient increase within the first 2 weeks before decreasing to the adult level.

A closer inspection of the time courses shown in fig.1 reveals characteristic differences between the individual subunit mRNAs. Whereas the α-subunit mRNA declines after a plateau of about two weeks, the γ- and δ-subunit mRNAs decrease earlier and more rapidly. The γ-subunit mRNA becomes undetectable, the δ-subunit mRNA does not disappear completely and even displays a

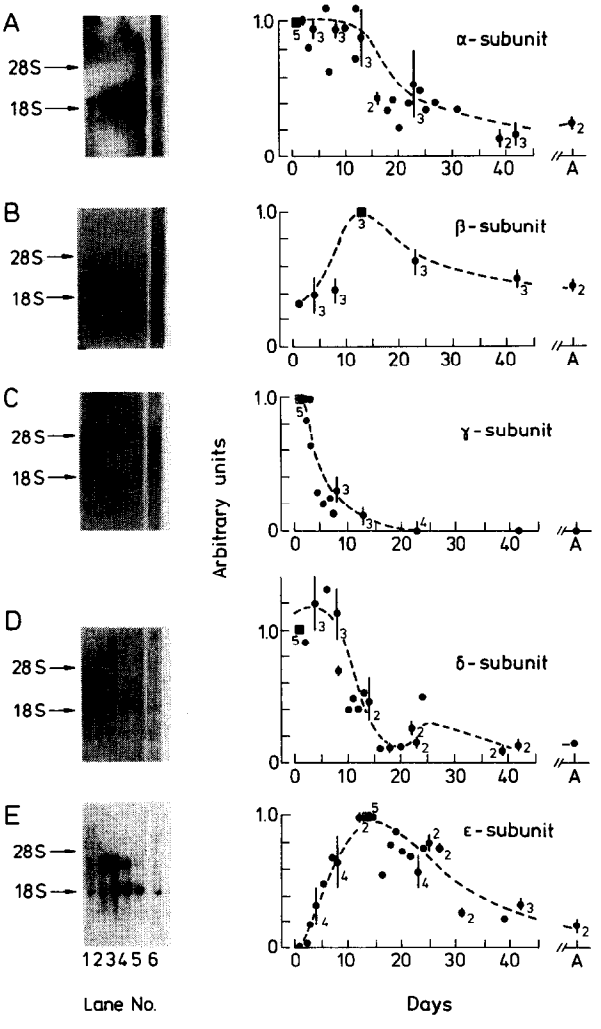

Fig.1. AChR-subunit mRNA levels during development of rat muscle. Left column of figs A–E shows autoradiograms of Northern blots where total RNA (10 μg each) from lower leg muscle was analyzed by blot hybridization using α-, β-, γ-, δ- and ϵ-subunit specific DNA probes. The subunit-specific mRNA, as indicated in each panel, was visualized upon autoradiography at −70°C with an intensifying screen. Exposure times ranged from 2 to 10 days. The positions of the rat ribosomal RNAs are shown. The numbers below the lanes correspond to the developmental stage of the muscle for which the RNA was extracted: 1, 2, 3, 4, 5 correspond to postnatal days 4, 8, 13, 23, 42. Adult muscle corresponds to lane 6. The apparent size of the five AChR-subunit mRNAs was as follows: α-subunit mRNA ~2.4 kb; β-subunit mRNA ~2.8–3.0 kb; γ-subunit mRNA ~2.2 kb; δ-subunit mRNA ~2.3 kb; ϵ-subunit mRNA ~1.9 kb. Distinct differences in transcript size and the subunit-specific developmental changes observed demonstrate the specificity of the hybridization probes and exclude the possibility of cross-hybridization. The larger RNA species detectable in some blots may represent incompletely spliced RNA (see [18]) and were not included for densitometric evaluation. The right column of figs A–E is a graphic representation of the relative changes of the AChR-subunit mRNA levels during postnatal development. Blot hybridization analysis was carried out as described above and the resulting autoradiograms were scanned densitometrically. The relative content of the respective subunit-specific mRNAs (indicated in each graph) was plotted as a function of days after birth, the value for adult muscle is indicated by A. The values for the α-, γ- and δ-subunit specific mRNAs were normalized with respect to those obtained at day 1 after birth. The values for the β- and ϵ-subunit specific mRNAs were normalized with the values measured at postnatal day 13. Each symbol represents an individual sample. The symbols with bars indicate mean values and the width of the bars the range of values observed. The values used for normalization are marked by open squares. The numbers represent number of independent experiments. Broken lines are drawn by eye.

moderate increase between days 20 and 30 before reaching the adult level. The levels of the β- and ϵ-subunit mRNAs both increase transiently with the increase being much more marked for the ϵ-subunit mRNA.

The rapid increase of ϵ-subunit mRNA and concomitant decrease of γ-subunit mRNA levels is of particular interest, since a correlation between the expression of adult type end-plate channels and the abundance of ϵ-subunit mRNA was previously found [2,3]. To investigate the relationship between the time course of changes in the level of the γ- and ϵ-subunit mRNAs and the conversion of channel properties, we analyzed the developmental changes of these mRNAs with a finer time resolution. Fig.2 shows that the γ- and ϵ-subunit mRNAs change rapidly within a few days after birth. The γ-subunit mRNA level decreases within 7 days to about one-fifth of the initial value. In contrast the ϵ-subunit mRNA increases rapidly from barely detectable amounts by about 10-fold. The levels of the α-, β-, and δ-subunit mRNA vary much less during this time. Thus the earliest and most conspicuous change following birth is a switch in the expression of the γ- and ϵ-subunit specific mRNAs. This switch precedes the conversion of end-plate channel properties [7,8] by several days.

To evaluate the changes in AChR subunit mRNA levels all values were compared with the actin-mRNA levels to correct for variations in the

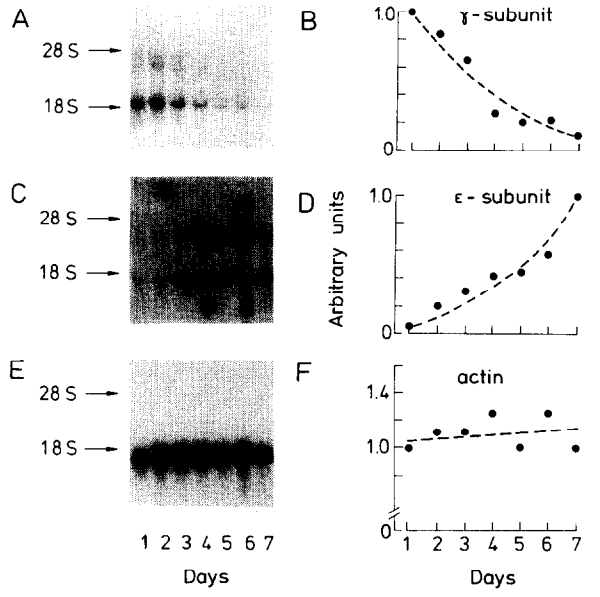

Fig.2. Changes of γ- and ϵ-subunit specific mRNAs and actin-mRNA levels during the first 7 days after birth at high time resolution. Total RNA (10 μg each) of lower leg muscle was subjected to blot hybridization analysis and densitometrically measured as described in the legend to fig.1. The values for the γ-subunit mRNA were normalized with respect to those obtained at day 1 after birth. The ϵ-subunit mRNA values were normalized with respect to those obtained at day 7 after birth. (A) Autoradiogram of the γ-subunit mRNAs and (B) densitometric evaluation; (C) autoradiogram of the ϵ-subunit mRNAs and (D) densitometric evaluation; (E) autoradiogram of the actin-mRNAs and (F) densitometric evaluation. Broken lines are drawn by eye.

amounts of total RNA analyzed (see e.g. fig.2E,F). A β-actin cDNA probe was used. Densitometric measurements of autoradiograms obtained upon hybridization analysis of mRNAs isolated at different postnatal stages reveals relatively constant amounts of actin-mRNA (fig.2). Only a moderate increase (less than 2-fold) in actin-mRNA is observed within the first 42 days of postnatal development (not shown). This shows that the total mRNA content does not change dramatically and indicates that the changes observed for the AChR-subunit mRNAs are specific.

The results described show that the mRNA pattern characteristic of adult muscle is reached within the first 4 to 6 weeks after birth as a result of differential regulation of subunit specific mRNAs. Previous experiments on adult muscle showed that following denervation all subunit-specific mRNA levels increased [14,15] to different extents, the ϵ-subunit mRNA level being less responsive than the γ-subunit mRNA [3]. To investigate the role of the motor nerve in this differential regulation we compared postnatal changes in subunit-specific mRNA levels in muscle denervated during the first day after birth with those of the innervated muscle. Since the earliest and most conspicuous postnatal change is the switch in γ- and ϵ-subunit mRNA content, we investigated whether innervation of the muscle is necessary for this switch to occur.

Total RNA was prepared from denervated and the contralateral, innervated muscles of littermates (RNA from 3–7 neonates was pooled) at 2- or 3-day intervals up to day 7 of age. Total RNA from neonatal muscle at day 1 was taken as a control. Fig.3A,C shows the result of hybridization analysis of total RNA from 1 day control, the innervated normal (N) and the contralateral denervated (D) muscles of the same animals at different postnatal ages. The levels of both γ- and the ϵ-subunit mRNAs are increased in denervated muscle as compared to the innervated muscle. The evaluation of the Northern blots shown in fig.3B,D, illustrates, however, that the muscle responds to denervation differently with respect to each of the two subunit mRNAs. The normally occurring developmental drop in γ-subunit mRNA content is not observed in denervated muscle (fig.3B). The developmental increase seen in ϵ-subunit mRNA in innervated muscle on the other hand persists in denervated muscle, and is even enhanced (fig.3D). In two experiments the γ- and ϵ-subunit specific mRNA content was compared between sham-operated and unoperated animals at day 7 of postnatal age. No significant difference was found in either the γ- or the ϵ-subunit specific mRNA indicating that the changes in mRNA levels in denervated muscle are a consequence of muscle denervation. Furthermore, the γ- and ϵ-subunit specific mRNA content of the muscles contralateral to the denervated muscle was not significantly different from that of muscles removed from unoperated littermates at the same age (two experiments, comparing muscles at day 7 of age).

Denervation of neonatal muscle produces an increased muscular content in the other subunit mRNAs as well, similar to what is seen in adult

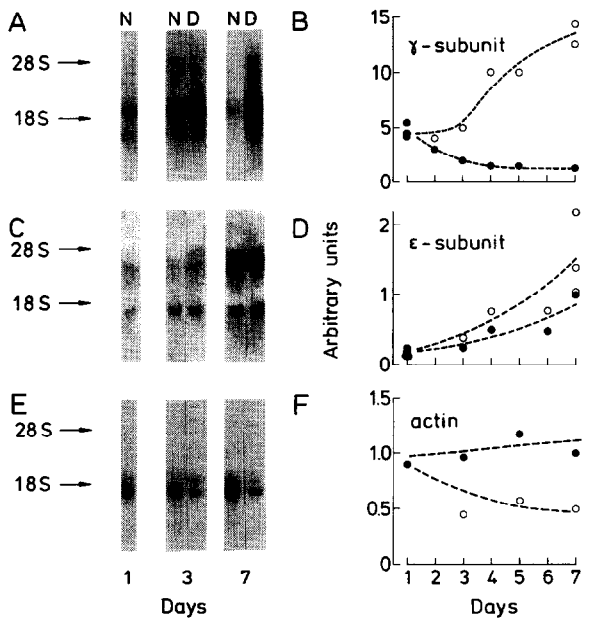

Fig.3. Effect of denervation on postnatal changes in muscular content of γ- and ϵ-subunit mRNA levels. Total RNA (10 μg each) from lower leg muscle was analyzed by blot hybridization and densitometrically measured as described in the legend of fig.1. All densitometric values were normalized with respect to the value measured at day 7 of normal development. (A) Autoradiograms of γ-subunit mRNA of normal (N) and denervated (D) muscle at postnatal days 1, 3 and 7, respectively. (B) Densitometric evaluation of changes in γ-subunit specific mRNA content. Filled symbols represent normal muscle, open symbols denervated muscle. Data from three experiments. (C) Autoradiograms of ϵ-subunit mRNAs and (D) densitometric evaluation of changes in ϵ-subunit mRNA. The larger RNA species detectable in some blots may represent incompletely spliced RNA (see [18]) and were not included for densitometric evaluation. Data from two experiments. (E) Autoradiograms of actin mRNAs and (F) densitometric evaluation. Days refer to days after birth.

muscle [14,15]. We compared the muscular content of α-, β- and δ-subunit mRNA at day 7 of postnatal age in denervated and normal muscle. In denervated muscle the level in α-subunit mRNA was about 50-fold higher than in innervated muscle and the values for the β- and δ-subunit mRNAs were 3- and 11-fold, respectively (one experiment).

To investigate the specificity of the AChR-subunit mRNA level changes after denervation we also examined changes in the muscular content of actin mRNA. Fig.3E,F show the autoradiograms of Northern blots and their densitometric evaluation indicating that 7 days after denervation the content of actin mRNA decreased to about one half of the control value. Thus denervation also produces changes in the expression of other muscle-specific genes.

4. DISCUSSION

The results reported here support the view that the appearance of two types of AChR channels in the end-plate depends on the muscular content of γ- and ϵ-subunit specific mRNAs [2,3]. They show that during rat muscle development at early postnatal ages the γ-subunit mRNA content is relatively high, whereas that of the ϵ-subunit mRNA is relatively low. By about 4 to 6 weeks of age the adult pattern of mRNAs, where the amount of ϵ-subunit mRNA is much higher than shortly after birth and the γ-subunit mRNA is no longer detectable, is reached. During this time the properties of end-plate channels change from fetal to adult type [5–8].

The switch in the abundance of γ- and ϵ-subunit mRNA, beginning within a day or two after birth, apparently occurs several days before the change in channel properties and the appearance of the ϵ-subunit in the end-plate. The AChRs already present at birth turn over slowly [7,16]. Therefore the appearance of receptors having adult properties may be expected to lag behind the appearance of mRNA encoding the ϵ-subunit although the number of AChRs in the end-plate increases several-fold postnatally [7]. We measured mRNA content in the triceps surae muscle, rather than only in the soleus muscle where the change in AChR-channel properties was determined. Thus the observation that the switch in mRNA content occurs earlier than the change in channel properties might partly reflect a difference in experimental conditions, because the change in channel properties is asynchronous in different muscles, being relatively late in the soleus muscle [17].

The observation that the reciprocal changes in γ- and ϵ-subunit mRNA and the change in channel properties occur at roughly the same developmental stage, could indicate that the change in channel properties is causally dependent on changes in levels of both subunit mRNAs. Because we measured only the relative changes of the two subunit mRNAs, it cannot at present be decided whether the levels of the two mRNAs are of equal

importance, or whether an increase in the level of the ϵ-subunit mRNA alone is sufficient to cause changes in channel properties. While the results suggest that replacement of the γ-subunit by the ϵ-subunit in the AChR is a mechanism underlying the conversion of channel properties, they do not exclude additional mechanisms contributing to channel conversion which occur on a faster time scale than receptor replacement [7,8].

The denervation experiments show that the normal developmental change in mRNA levels during the first postnatal week, in particular the reciprocal change in γ- and ϵ-subunit mRNA levels, does not occur when the motor nerve is cut shortly after birth. Instead there is an increase in the mRNA levels of all subunits. Denervation may upset normal developmental regulation within the muscle causing non-specific increase in the content of mRNA encoding all subunits. Alternatively the differential expression of AChR subtypes may be controlled directly by the nerve. This regulation is lost upon denervation. This latter type of control could govern the expression of the γ-subunit gene since the γ-subunit mRNA level does not decrease postnatally when the muscle is denervated early after birth. The ϵ-subunit mRNA on the other hand increases postnatally in denervated muscle, as it does in normal muscle, suggesting that this increase in ϵ-mRNA could be largely independent of continued innervation of the muscle, requiring perhaps only an initial induction by innervation.

The expression of subtypes of receptor-channel complexes with different subunit compositions as a consequence of differential regulation of subunit-specific mRNAs, could represent a general mechanism by which functional properties of synapses are modulated according to specific physiological requirements. One way by which differential regulation could be achieved would be a different sensitivity of individual subunit coding genes towards pre- and postsynaptic regulatory signals.

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