Differential expression of nicotinic acetylcholine receptor genes in innervated and denervated chicken muscle

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Communicated by E.A. Barnard

We have studied the mRNAs encoding all four subunits of the acetylcholine receptor in 13-day embryonic, innervated and denervated chicken pectoral muscle. In all three states the transcript sizes of the α -, β -, γ - and δ -subunit mRNAs were approximately 3.2, 2.8, 1.8 and 1.9 kb respectively. Denervation was found to result in a large increase in the steady-state levels of each mRNA compared with those in innervated muscle. This increase was 8- to 9-fold for the β and δ subunits and ~12-fold for the α subunit. The evidence obtained shows a coordinate regulation of the acetylcholine receptor genes in response to denervation. Interestingly, no γ -subunit transcript was detected in innervated muscle, while significant levels were detected in embryonic and denervated tissue. This evidence suggests that there exists in a non-mammalian muscle acetylcholine receptor an additional subunit, analogous to the bovine ϵ subunit, and shows that the extra-junctional receptor of adult denervated muscle is produced by the same set of mRNAs which produce the major form of the receptor in embryonic muscle.

Key words: acetylcholine receptor/chicken muscle/denervation/gene expression

Introduction

The nicotinic acetylcholine receptor (AChR) of fish electric organs and of the neuromuscular junction of vertebrate skeletal muscle is a membrane-bound oligomeric glycoprotein composed of four different—but homologous—subunits, α , β , γ and δ , with a stoichiometry $\alpha_2\beta\gamma\delta$ (reviewed by Dolly and Barnard, 1984; Popot and Changeux, 1984; Stroud and Finer-Moore, 1985). Each polypeptide has been shown to be encoded by a distinct mRNA, by the cloning of all of the corresponding cDNAs from *Torpedo* electroplaque (Noda *et al.*, 1982, 1983a,b; Sumikawa *et al.*, 1982a; Devillers-Thiery *et al.*, 1983) and from calf muscle (Noda *et al.*, 1983c; Takai *et al.*, 1984; Tanabe *et al.*, 1984; Kubo *et al.*, 1985). Regulation of the expression of these genes *in vivo* in the assembly of the receptor structure is a matter of great interest but, as yet, little information is available on it. The availability of cDNA clones (α : Barnard *et al.*, 1986; β and δ : S.J. Moss, M.G. Darlison and E.A. Barnard, unpublished) and genomic clones (α : Klarsfeld and Changeux, 1985; γ and δ : Nef *et al.*, 1984) for the chicken muscle AChR and the accessibility of the chicken to experimental manipulation and investigation make this a favourable animal for the study of gene transcription.

After chronic denervation of muscle, either in birds or in mammals, a considerable amount of extra-junctional AChR appears. This has been shown to result from an increase in the biosynthesis of new receptor molecules (Fambrough, 1979); indeed it has recently been shown (for the case of mouse muscle cells in culture), that this is a consequence of increased gene transcription (Buonanno and Merlie, 1986). In mouse muscles the steady-state level of AChR α -subunit mRNA has been observed to increase 50- to 100-fold after denervation (Merlie *et al.*, 1984; Goldman *et al.*, 1985). However, denervation of rat diaphragm muscle resulted in only a 7-fold increase in mRNA coding for the α subunit (Goldman *et al.*, 1985). In the only such experiment reported on denervated chicken muscle (Klarsfeld and Changeux, 1985), a 17-fold increase in AChR α -subunit mRNA was found in leg muscles of 10-day-old chicks that had been denervated for 4 days, without a change in the level of the actin mRNA. We present here data on the transcript sizes and changes in the steady-state levels of all four AChR mRNAs in innervated and denervated chicken pectoral muscle. The results have implications in relation to the biosynthesis and structure of this receptor in chicken muscle.

Results

Transcript sizes of AChR mRNAs in 13-day embryonic muscle

Prior to investigating the effects of denervation on the steady-state levels of AChR α -, β -, γ - and δ -subunit mRNAs in chicken pectoral muscle, the sizes of these transcripts were established. Total poly(A)⁺ RNA extracted from 13-day embryonic muscle, the stage in development at which the expression of AChR mRNA overall is at or close to its maximum (unpublished data), was analysed in Northern blots, probing at high stringency with subunit-specific cDNA or genomic clones. Using an α -subunit cDNA fragment a major transcript of 3.2 kb was detected (Figure 1A, lane E). A size of ~2.8 kb has previously been reported by Klarsfeld and Changeux (1985). On overexposure of the autoradiogram, or when larger quantities of poly(A)⁺ RNA are used in each blot, two additional transcripts of 4.2 and 5.9 kb become apparent (Figure 2, lane E). These presumably represent small amounts of incompletely-processed forms of the mature mRNA. Single bands of 2.8, 1.8 and 1.9 kb are detected when Northern blots are hybridized with the β -subunit, γ -subunit and δ -subunit probes respectively (Figure 1B–D, lane E). These differences in sizes permit the unambiguous identification of subunit-specific transcripts and exclude the possibility, in denervation experiments, of cross-hybridization.

Detection of AChR mRNAs in innervated and denervated muscles

Northern blots of poly(A)⁺ RNA from 13-day embryonic or denervated or contralateral innervated (8-week-old) pectoral muscle were probed with radiolabelled chicken muscle AChR α -, β -, γ - and δ -subunit-specific probes. Strong hybridization signals, corresponding to the receptor subunit transcripts, were

Fig. 1. Northern blot analysis of AChR subunit transcripts in chicken muscle. Ten micrograms each of poly(A)⁺ RNA isolated from 13-day embryonic (lane E), contralateral innervated (lane C) and denervated (lane D) pectoral muscle were denatured (McMaster and Carmichael, 1977), electrophoresed in 1% (w/v) agarose gels and transferred to GeneScreen. Blots were hybridized with cDNA or genomic probes encoding either the α subunit (A), the β subunit (B), the γ subunit (C) or the δ subunit (D) of the chicken muscle AChR as described in Materials and methods. *Escherichia coli* 16S and 23S and (as indicated) bovine 18S and 28S ribosomal RNAs were used as size markers. The sizes of the AChR subunit transcripts are also shown.

Fig. 2. Detection of higher mol. wt AChR α -subunit transcripts in chicken muscle. The autoradiogram of the Northern blot, shown in Figure 1A, has been deliberately overexposed to indicate the presence of additional transcripts at 4.2 and 5.9 kb. The size of the major transcript (3.2 kb) is indicated. Size markers as in Figure 1. Note that no additional transcripts were observed for any of the other AChR subunits.

detected in blots of embryonic (Figure 1A–D, lane E) and denervated muscle mRNA (Figure 1A–D, lane D). Quantification of each of the four subunit mRNAs, by SP6 RNA polymerase-directed dot-blot analysis, shows that the steady-state levels of the α - and β -subunit mRNAs do not change significantly from day 10 to day 14 of development. However, the γ - and δ -subunit levels increase by factors of ~ 1.9 and 2.7 respectively during the same period (data not shown). The hybridization signals observed from contralateral innervated muscle poly(A)⁺ RNA (Figure 1A–D, lane C) were much weaker than those for denervated muscle, for all of the subunits; indeed, no γ -subunit transcript could be detected at all (Figure 1C, lane C). In the case of the denervated or contralateral innervated muscles, the same transcript sizes, for the α , β , γ and δ subunits, were detected as in the embryonic muscle. No larger, presumably incompletely-

Fig. 3. Quantitation of a dot-blot hybridized with a chicken muscle AChR α -subunit probe. Dilutions of poly(A)⁺ RNA (2–20 μ g) isolated from 13-day embryonic ($\circ$), denervated ($\triangle$) and contralateral innervated ($\square$) pectoral muscle were spotted onto GeneScreen as described in Materials and methods and hybridized with a chicken muscle AChR α -subunit probe. Signals were quantified by densitometric scanning of the autoradiogram and plotted, in arbitrary units (ordinate), against the amount of poly(A)⁺ RNA (in μ g) applied to the membrane (abscissa). For all three muscles this relationship is linear.

processed, transcripts were observed for any of the AChR subunits, other than for the α subunit as previously mentioned.

Quantitation of the mRNA differences

To confirm that the relationship between signal intensity and mRNA concentration was linear at the concentrations used in this study, dot-blot (Anderson and Young, 1985) of varying amounts of mRNA were probed with the α -subunit cDNA probe. These showed that linearity holds for the amounts of mRNA

Table I. Effects of maturation and of denervation on AChR subunit mRNA levels

Subunit mRNA probed	Innervated (I)	Embryonic (E)	Denervated (D)	D/I	D/I corrected
	(1540 ± 88)	(15283 ± 1320)	(13204 ± 711)		
α	0.12 ± 0.01	1.16 ± 0.20	1.0 ± 0.11	8.3	11.6
β	0.17 ± 0.02	1.59 ± 0.19	1.0 ± 0.09	5.9	8.3
γ	ND	1.46 ± 0.25	1.0 ± 0.18		
δ	0.15 ± 0.03	1.47 ± 0.21	1.0 ± 0.11	6.7	9.4

In each Northern blot all three tissues were compared simultaneously (as in Figure 1). The innervated muscle used here was the contralateral control pectoral from the denervated bird. After hybridizing with each of the four subunit probes in turn, scanning densitometry of each lane provided the integrated density value for each of the subunit transcripts. The values (in arbitrary units of area) are illustrated, for the α-subunit mRNA, in parentheses. Each determination was the mean (± SD) of three scans for each subunit. Using those mean values, all of the other values given for the muscles have been normalized to that for the denervated muscle, which for each subunit is assigned a relative value of 1.0. The ratio of the steady-state mRNA levels, denervated to innervated muscle (D/I), is also shown. In the last column, the D/I ratio has been corrected for the fact that the contralateral muscle itself shows a small increase over a true control innervated muscle (Figure 4A), so that it is now expressed for true innervated muscle. Note that essentially the same results were obtained in a repeat, independent series of blots (not shown). ND indicates that no signal above background was detectable. Under the conditions used, the lower limit of sensitivity for the detection of RNA was between 1 and 5 pg.

analysed from all three muscles (Figure 3). Also, the yields of poly(A)⁺ RNA relative to the amounts of tissue and total RNA were measured and appeared to be constant for the embryonic, contralateral innervated and denervated muscles, so that each lane should correspond to the same amount of tissue. This was confirmed by probing one of the blots with a chicken α-actin cDNA probe. No significant difference in the actin hybridization signal was observed in the embryonic, innervated or denervated muscle mRNA blots (data not shown).

The mRNA steady-state levels of the contralateral innervated, embryonic and denervated pectoral muscles were quantified by densitometric scanning of autoradiograms of Northern blots, with integration of the subunit-specific peaks (Table I). For the α, β and δ subunits of the chicken muscle receptor, denervation resulted in a 5.9- to 8.3-fold increase in the respective mRNA levels. This augmentation was even greater in the case of the γ-subunit mRNA; the magnitude of this increase could not be estimated, however, since γ-subunit transcripts could not be detected at all in innervated pectoral muscle. Interestingly, the ratio of the mRNA levels in embryonic to 8-day-denervated muscle did not differ significantly between the β, γ and δ subunits (Table I, column E), suggesting that similar regulatory processes control AChR gene expression in the two states. The same is true for β and δ (γ being absent) in the denervated/innervated ratio.

Differences between contralateral innervated and normal innervated muscles

It is known that unilateral denervation of a bilateral muscle, such as the pectoral, results in some compensatory changes in the contralateral side, due to the increased load on the latter; for example, a mild hypertrophy of the contralateral muscle occurs (Holly *et al.*, 1980), and this suggests that some increase in many mRNA species may occur there. We therefore looked for such effects on the steady-state level of the α-subunit mRNA, by Northern blot analysis of contralateral innervated and normal age-matched innervated pectoral muscle. Quantitation of autoradiograms (Figure 4A) as before revealed a 1.4-fold increase in α-subunit mRNA in contralateral muscle compared with normal innervated muscle. Assuming that this is the case for all the AChR subunit mRNAs, then the true increase in transcript levels, in denervated compared with innervated muscle, is in the range 8.3- to 9.4-fold for β and δ and ~12-fold for α (Table I).

Concurrent changes in muscle actin mRNA levels

For comparison, the level of the α-actin mRNA was quantitated

Fig. 4. Northern blot analysis of the AChR α-subunit transcript and the α-actin transcript in normal innervated, denervated and contralateral innervated chicken muscle. Poly(A)⁺ RNA isolated from denervated (lane D), contralateral innervated (lane C) and normal age-matched innervated (lane I) pectoral muscle was denatured (McMaster and Carmichael, 1977), electrophoresed in a 1% (w/v) agarose gel and transferred to GeneScreen as described in Materials and methods. Blots containing 20 μg poly(A)⁺ RNA were hybridized with an AChR α-subunit probe (A) and blots containing 10 μg poly(A)⁺ RNA were hybridized with a chicken α-actin cDNA probe (B). Size markers as in Figure 1. The sizes of the AChR α-subunit transcript and the α-actin transcript are indicated.

as for AChR-subunit mRNAs, on Northern blots in parallel with those described above but probed with a chicken α-actin cDNA. The actin mRNA signals were not significantly different in the innervated, denervated and contralateral innervated muscles (Figure 4B). The ratios for these, derived by densitometric scanning of the blots, were 1.0:1.08:1.0 respectively.

Discussion

The results presented here are relevant to several aspects of muscle acetylcholine receptor gene regulation. Firstly, denervation of the chicken pectoral muscle results (after 8 days) in an 8- to 12-fold increase in the steady-state mRNA levels of the

α , β and δ subunits. A large increase in the γ -subunit mRNA is also observed. This latter augmentation cannot be quantified as a ratio because no γ -subunit transcript was detected in innervated muscle (discussed below). Thus, a significant increase in the steady-state levels of all four AChR subunit transcripts occurs. These increases in the receptor mRNAs are consistent with the ~30- to 40-fold increase in the acetylcholine receptor protein content (as determined by α -bungarotoxin-binding) observed in 7- to 10-day-denervated, compared with innervated, pectoral muscles of chickens in the age range used here (Sumikawa *et al.*, 1982b). This is not due to a general increase in gene activity, since the level of actin mRNA did not change significantly upon denervation.

Secondly, the increases upon denervation in the mRNAs for the α , β and δ subunits are essentially the same, and there is also a co-ordination of the levels of the β -, γ - and δ -subunit mRNAs, as could be seen by comparing them with the embryonic muscle levels (Table I, 3rd and 6th columns). The level of α -subunit mRNA in embryonic muscle, compared with denervated muscle, is lower than that for the β , γ and δ subunits. However, this value is an underestimate since the α -subunit mRNA has been shown to peak slightly earlier during embryonic development than the other three messengers (unpublished observations).

Thirdly, the excess loading of the contralateral innervated pectoral muscle, when unilaterally denervated, leads to a modest but significant increase, 1.4-fold, in AChR mRNA synthesis as reflected in the α -subunit mRNA steady-state levels. A compensatory mild hypertrophy is present in the contralateral muscle, but this does not involve a general increase in the steady-state levels of all mRNAs, since the α -actin mRNA level in normal innervated and contralateral innervated muscle (Figure 4B) was unchanged. It will be of interest to know if the AChR mRNAs are one of a limited class which respond in this way to the muscle stress represented in this state.

The 8.3-fold increase in the α -subunit mRNA level reported here for denervated chicken muscle, when compared with the contralateral innervated muscle, is less than half that observed by Klarsfeld and Changeux (1985), who measured the α -subunit mRNA in the same species. This discrepancy may be due to differences in the period of denervation prior to mRNA extraction, namely 8 and 4 days respectively, or in the age of the chickens when denervated, 8–12 weeks versus 6 days. Alternatively, the fact that they used leg muscles and we used the pectoral muscle may be important. Denervation of different muscles may result in differential increases in the transcription of AChR genes, since with rat diaphragm or mouse leg muscle (Merlie *et al.*, 1984; Goldman *et al.*, 1985) increases of 7-fold or 50- to 100-fold respectively have been observed in the mRNA encoding the α -subunit after a 5-day denervation. Clearly, more extensive studies are required to determine the factors involved in these differences, which could also include differential stabilities of the various mRNAs in different situations.

A fourth point raised by the present study is the possible existence in the chicken of a fifth AChR subunit, as has been demonstrated for the bovine receptor (Takai *et al.*, 1985). As noted above, γ -subunit transcripts could not be detected in contralateral innervated chicken pectoral muscle, but were present in embryonic muscle (Figure 1C, lanes C and E). This is consistent with the results of Mishina *et al.* (1986) who showed that bovine γ -subunit mRNA was abundant in fetal skeletal muscle but was essentially undetectable in adult muscle. In parallel with this decline in γ -subunit mRNA during development, an increase in an mRNA encoding another subunit type, ϵ , was reported. It was

concluded that two forms of the bovine skeletal muscle AChR exist, a fetal form having the structure $\alpha_2\beta\gamma\delta$ and an adult form having the structure $\alpha_2\beta\delta\epsilon$. Also noteworthy is that, as we have shown in denervated muscle, the γ subunit is again produced.

Using combinations of subunit-specific mRNAs, derived from the cloned cDNAs, and the *Xenopus* oocyte *in vivo* translation system, Mishina *et al.* (1986) showed that the two types of receptor ($\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\delta\epsilon$) have different channel conductance and gating properties, which resemble the receptor channels found in fetal and adult muscle respectively. A marked decrease in the mean channel open time of the AChR during development (which is reversed upon denervation) has also been reported for rat muscle (Sakmann and Brenner, 1978; Fischbach and Schuetze, 1980), but no such change can be detected in the maturation of chicken muscle (Schuetze, 1980). Presumably, therefore, should a chicken muscle ϵ subunit exist, a receptor containing this polypeptide (i.e. $\alpha_2\beta\delta\epsilon$) would have channel characteristics indistinguishable from a receptor containing instead the γ subunit (i.e. $\alpha_2\beta\gamma\delta$). A second, less likely, interpretation of our results is that the adult chicken receptor comprises just α , β and δ subunits. Since the size and charge of the chicken AChR protein purified from innervated or denervated pectoral muscle are the same (Sumikawa *et al.*, 1982b), this would require two copies of both α and of another subunit in the oligomer. Full-length cDNA clones encoding all of the chicken muscle AChR subunits, which are currently being isolated and expressed in this laboratory, should clarify these present ambiguities in the structure of muscle AChRs.

A final point that requires comment concerns the possibility that more than one mRNA species may encode a single receptor subunit. Goldman *et al.* (1985) reported that two mRNAs, which differed in their 3' untranslated regions due to the existence of more than one polyadenylation site, encoded the mouse α subunit. This heterogeneity was also observed on Northern blots, a broad band being observed at ~2 kb. In the results presented here, there is no evidence for multiple transcripts encoding chicken receptor subunits other than for the α subunit as noted. We concede, however, that very small differences in mRNA sizes would go undetected.

Materials and methods

Denervation of chicken pectoral muscle

New Hampshire chickens (Davis line 412), bred at Imperial College, London, were anaesthetized using halothane. Denervation was performed unilaterally on the pectoral muscles of 8- to 12-week-old animals by removal of segments from both of the afferent nerve trunks. Muscles were removed 8 days after denervation and stored under liquid nitrogen. The contralateral muscles were also removed and used as controls. Unoperated animals of the same age provided unperturbed innervated control pectoral muscles.

RNA isolation and Northern blot analysis

Total RNA was extracted from innervated (8-week-old), 13-day embryonic, denervated and contralateral innervated pectoral muscle by the method of Chirgwin *et al.* (1979). Poly(A)⁺ RNA was purified by two cycles of oligo(dT)-cellulose chromatography (Aviv and Leder, 1972), denatured in 1 M glyoxal and 50% (v/v) dimethyl sulphoxide (McMaster and Carmichael, 1977), fractionated in a 1% (w/v) agarose gel, then transferred directly to GeneScreen (Du Pont). Blots were prehybridized in 50% (v/v) formamide, 10% (w/v) dextran sulphate, $5 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$, 0.015 M sodium citrate), 0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpyrrolidone 360, 0.1% (w/v) bovine serum albumin, 0.5% (w/v) sodium dodecyl sulphate (SDS), 10 mM EDTA, pH 8 and 100 $\mu\text{g/ml}$ denatured salmon sperm DNA, at 42°C, 2 h. Hybridization was in the same solution at 42°C, 18 h. Radiolabelled probe (see below) was added to a final concentration of 0.5 ng/ml. Blots were washed in $0.2 \times \text{SSC}$, 0.5% (w/v) SDS at room temperature for 60 min, then at 65°C for a further 60 min, followed by exposure to prefogged Fuji X-ray film at -70°C. Signals were quantified by densitometric scanning of autoradiograms (with subtraction of background from an adjacent empty lane) and digitized integration of the peaks.

RNA dot-blots

Dilutions of poly(A)⁺ RNA (2–20 µg) in sterile distilled water, including yeast tRNA to give a total of 20 µg RNA, were glyoxylated as for Northern blots and spotted onto GeneScreen using a Bio-Rad Bio-Dot apparatus. After washing twice with 20 × SSC and once with 1 M ammonium acetate, filters were dried and baked at 80°C for 2 h under vacuum. Prehybridization, hybridization, washing of filters, autoradiography and quantitation of signals were as described for Northern blots.

Hybridization probes

Probes for the α -, β - and δ -subunit mRNAs were derived from chicken muscle AChR cDNAs isolated in this laboratory. Specifically these were: α [an ~725-bp *EcoRI*–*HincII* fragment extending from within the 5' untranslated region to amino acid 217 of the mature subunit (Barnard *et al.*, 1986)]; β [an ~350-bp fragment extending from within the prepeptide to amino acid 100 of the mature polypeptide] and δ [an ~640-bp fragment encoding from amino acid 248 to 462 of the mature polypeptide (S.J.Moss, M.G.Darlison and E.A.Barnard, unpublished)]. The ~1280-bp γ -subunit genomic probe used, p9 (a generous gift from Dr Marc Ballivet), extends from within exon 2 to beyond exon 6 of the chicken muscle gene (Nef *et al.*, 1984). A chicken muscle α -actin cDNA probe (H.Lehrach and J.C.Perriard, unpublished) was used as a control. These were radiolabelled to high specific activity (~10⁹ c.p.m./µg) using the random hexanucleotide method (Feinberg and Vogelstein, 1983).

Note added after submission

Since this paper was submitted, an interesting report by Evans *et al.*, *J. Biol. Chem.*, **262**, 4911–4916 (1987) on denervated rat and mouse muscles has appeared. This shows that the steady-state levels of all four AChR mRNAs in rat diaphragm muscle and in mouse hindlimb muscle increase on denervation. However, the β -subunit mRNA level increases dramatically in mouse but only slightly in rat. The reason for this difference is at present unclear.

Acknowledgements

We thank Dr Marc Ballivet, University of Geneva, for his generous gift of the γ -subunit genomic clone and for helpful discussions. We also thank Dr John Rogers, Medical Research Council Laboratory of Molecular Biology, Cambridge, for the chicken α -actin cDNA probe. S.J.M. and D.M.W.B. acknowledge receipt of Science and Engineering Research Council studentships. This work was supported by the Medical Research Council.

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Received on April 9, 1987; revised on September 16, 1987