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Protein synthesis is required for the denervation-triggered activation of acetylcholine receptor genes

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The effect of cycloheximide (CHX) on denervation-induced acetylcholine receptor (AChR) expression was investigated in chickens one day after nerve section, using probe excess solution hybridization to quantitate AChR α-subunit gene transcript levels and run-on analysis to measure subunit gene activity. The increase in α-subunit transcripts that normally follows denervation was prevented when drug treatment was commenced 2 h before or after denervation but was not blocked when CHX administration was begun 6 h after the operation. Drug-induced reduction of transcript levels results from decreased activity of genes coding for the α-, δ-, and γ-subunits; in contrast, the transcription rates of several non-receptor genes are not affected by CHX. The results suggest that the de novo synthesis of a transcriptional activator is required as a mediating event in the signalling pathway linking the plasma membrane and AChR gene expression.

Transcriptional activator; Subunit mRNA metabolism; Cycloheximide; Chick muscle

1. INTRODUCTION

Denervation of adult skeletal muscle induces expression of extrajunctional acetylcholine receptor (AChR). It is now well established that to a large extent this phenomenon is accounted for by a derepression of receptor synthesis upon cessation of electrical activity of the plasma membrane [1-3]. Recent analysis of the mechanisms underlying the increased receptor synthesis rate has revealed that denervation also results in a remarkable increase in mRNAs coding for the individual receptor subunits [4-11]. The increased message concentrations in turn are at least in part caused by enhanced transcription rates of receptor genes [1,12]; in the chicken a denervation signal reaches the genome and activates the α-, δ-, and γ-subunit genes within 12 h after nerve section [12]. The nature of this signal has remained elusive. For example, it is not known if denervation results in the appearance of an activating factor or in the loss of an inhibitor. Treatment of primary muscle cells with the sodium channel blocker tetrodotoxin results in an increase of AChR protein and α-subunit mRNA, and has been employed to analyze the effects of membrane electrical activity on receptor gene expression [5,11,13]. Using this in vitro model of denervation, Duclert et al. [14] have shown that inhibition of protein synthesis blocks the tetrodotoxin-induced rise in α-subunit message level and have postulated a requirement for continuous production of positive regulatory factors in the tetrodotoxin response. Their observation immediately prompts further questions: Does the putative regulatory factor control transcript levels by affecting gene activity or by transcript stabilization? Are other receptor subunit messages controlled similarly? What happens in vivo upon denervation of skeletal muscle?

We have investigated the metabolism of the AChR subunit messages in denervated chick skeletal muscle. Using in vitro transcript elongation ('run-on') analysis we show that a newly synthesized transactivator protein or proteins are required for the denervation-induced stimulation of AChR α-, γ-, and δ-subunit genes.

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Abbreviations: AChR, acetylcholine receptor; CHX, cycloheximide

2. MATERIALS AND METHODS

2.1 *Denervation*

White Leghorn hatchlings were purchased from Hall's Brothers Hatchery (North Brookfield, MA). Section of the sciatic nerve was performed as described previously [9]. At the desired time after denervation animals were killed by ether overdose, and the leg musculature below the knee removed and processed immediately.

2.2. *Genomic and complementary DNA probes*

Chicken AChR α-subunit: for transcription elongation experiments, 1.4 kb of genomic sequence including exons I and II were inserted into the polylinker of M13mp10 [12]. For riboprobe protection, pα7 which comprises exon VII and flanking intronic sequences was cloned into Bluescript pSK+ (Stratagene, La Jolla, CA); this probe has been used to quantitate both mature mRNA and a putative splicing intermediate [9,13,14] (see also Fig. 1). Chicken AChR γ- and δ-subunits: 0.5 kb of the 5′ region of the γ-subunit gene including exon I and 4.8 kb of the 5′ portion of the δ-subunit gene including exons I through IV were cloned into M13mp10 to produce complementary strands. In addition, the following clones were used: chicken β-actin: full-length cDNA in pBR322, linearized with *Hin*dIII; chicken β-tubulin: full-length cDNA in pBR322, linearized with *Bgl*II; chicken

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MyoD: clone CMD-1 containing a 1.5 kb cDNA insert in pKS+ (Stratagene), linearized with *Eco*RI; chicken c-*fos*: clone pTZ19R/ch-c-*fos*, 1.6 kb cDNA inserted into the pTZ19R (USB) vector, linearized with *Kpn*I.

2.3. *Quantitation of α-subunit mRNA*

RNA was extracted, and riboprobe protection assays were performed as described previously [11].

2.4. *Run-on analysis*

Nuclei were isolated and assayed for specific transcript elongation as described in a previous paper [12]. To assess the specificity of weak signals (such as those seen with AChR subunits in innervated muscle) probes were preincubated in solution with the non-radioactive driver DNA; such an experiment with α- and γ-subunit transcripts established that all detectable binding is specific.

2.5. *Drugs*

CHX and actinomycin D were products of Sigma (St. Louis, MO). CHX (0.2 mg/kg body weight) was administered i.p. at 4-h intervals; a 24-h treatment was survived by over 90% of the animals. For $t_{1/2}$ studies, single doses of actinomycin D (2 mg/ml) were administered i.p.

3. RESULTS AND DISCUSSION

Since denervation supersensitivity is largely, if not entirely, caused by the cessation of electrical activity of the plasma membrane, and since AChR induction, at least in part, is mediated by gene activation, a signalling pathway must link sarcolemma and genome. The earliest reported changes in receptor metabolism occur at about 10–12 h after motor nerve section when the α-, γ-, and δ-subunit genes become active above background level [12]. Transcription rates then increase and reach a peak about 36 h after denervation. This indicates that a minimal number of required transcription factors are present at threshold concentration 10 h after the initial inductive event. What is happening during that latency period? If cessation of action potentials were to result directly in activation of a pre-existing factor one might expect a faster response. More likely, the long delay reflects metabolism of a regulatory factor. Perhaps an inhibitory protein is not manufactured any longer and after 10 h falls below the minimal concentration required for function; alternatively (or additionally), the synthesis of a transactivating factor may be required. To distinguish between these mechanisms, the effects of the protein synthesis inhibitor CHX were investigated.

3.1. *CHX blocks denervation-induced rise in α-subunit gene transcripts*

When CHX administration is commenced at the time of denervation α-subunit message levels 22 h later are only 1/17 of drug-free control, suggesting that protein synthesis is required for the denervation response. In contrast, N-CAM mRNA levels (which are not affected by denervation - Neville, unpublished results) are only slightly reduced (ca 30%) by a 1-day treatment with CHX (data not shown). As a rule, message levels were determined 22 h after nerve section to allow for the development of a quantitatively adequate response. Omission of the first drug dose (given 2 h before denervation) did not significantly affect α-subunit mRNA levels 24 h later. However, when the start of the drug treatment was delayed from 2–6 h after nerve section α-subunit gene expression was no longer strongly inhibited, and later treatment had even less of an effect (Fig. 1). This permits the conclusion that a large fraction of the activator proteins is synthesized during the first 6-h period after denervation.

In principle, denervation-triggered receptor induction could result from the removal of an inhibitory factor. It has been shown, for instance, that much of the tissue specificity of the expression of the δ-subunit gene arises from the presence of a silencer-like element in the upstream region [15]. However, CHX would then either have no effect or even stimulate receptor gene expression which is obviously not the case. Rather, the CHX experiments reported here suggest that synthesis of an activator protein precedes the denervation-triggered receptor up-regulation. This agrees with the identification, in the promoter regions of the chick AChR α- [16–18], γ- (H.-T. Jia and J.S., unpublished), and δ- [15] genes, of positive-acting elements that may mediate receptor up-regulation not only during differentiation, but also following denervation [19].

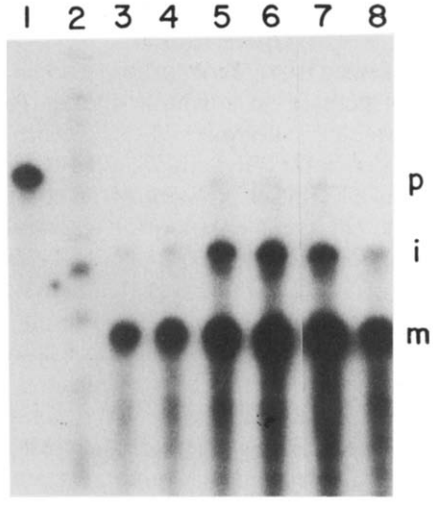

Fig. 1. Effect of CHX on the steady-state level of α-subunit mRNA. Nuclease protection analysis was performed as described in Materials and Methods, with 30 μg of total RNA and an excess of 10-fold or greater of radiolabeled riboprobe. Lane 1: intact α-subunit probe; 2: molecular weight markers (φX174/*Hae*III); 3: CHX (at 0.2 mg/kg body weight) administered i.p. in 20–100 μl of phosphate-buffered saline 2 h prior to denervation and followed by 4 more injections at 4-h intervals before sacrifice 22 h post-denervation; 4–6: muscle assayed 22 h post-denervation, with drug treatment begun 2 h (4), 6 h (5), and 10 h (6) after the operation; 7: no CHX treatment; 8: no denervation, no CHX. p, i, and m stand for primary transcript, putative splicing intermediate [9], and mature message, respectively. Exposure was extended to highlight the differences in the level of 'i'.

To gain insight into the origin and turnover of the transactivator we quantitated the amounts of α-subunit specific mRNA and precursor (splicing intermediate 'i' in Fig. 1) in chronically denervated muscle as a function of time after exposure to metabolic inhibitors. Results are shown in Fig. 3. Treatment with actinomycin D reveals that mature α-subunit mRNA has a half-life of about 4 h while the pre-mRNA, in agreement with an earlier assessment of α-subunit transcript processing rate [9] is much more short-lived. Upon exposure to CHX both transcripts decay more slowly, with a $t_{1/2}$ of one day, suggesting that the decline reflects the rate at which transcript replenishment, i.e. receptor α-subunit gene activity, diminishes. More precisely, the level of some protein required for receptor gene transcription decays with a $t_{1/2}$ of about 24 h. This finding argues against a model in which CHX depletes a labile pre-existing factor, because CHX treatment begun at the time of denervation should then reduce the 22-h denervation response by only about 50% instead of eliminating it altogether as is actually observed. In addition, we have observed that the basal levels of α-subunit mRNA in innervated muscle are not affected by repeated administration of CHX. Finally, the long latency period between the time of denervation and the onset of receptor subunit gene transcription (ca 10 h, see [12]) is difficult to reconcile with a transcriptional activator being targeted directly by a second messenger system. Clearly, the regulatory mechanism must comprise added variables and features such as de novo synthesis of the transactivator.

Further work will be required to outline the denervation response in greater detail. Nevertheless, it is safe to state at this point that, in vivo as well as in vitro, synthesis of an activator protein is required as a link between cessation of membrane activity and the derepression of acetylcholine receptor genes.

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3.2. *CHX blocks activation of AChR subunit genes*

The absence of a denervation response brought about by CHX treatment could arise from the failure to elaborate either a necessary transcription factor or a protein required to stabilize the message. The pronounced CHX effect on levels of α-subunit mRNA precursor strongly suggests that transcription is affected. As an additional test, run-on analysis was performed. Several genes including those coding for the AChR α-, γ-, and δ-subunits were examined. Among these, only the AChR subunit genes were significantly inhibited in CHX-treated animals. Under the same conditions total transcriptional activity, as measured by incorporation of [^{32}P]UTP, was not significantly affected. The other tested genes exhibited little change compared to the drug-free controls; as can be seen in Fig. 2, β-actin, β-tubulin, c-*fos*, and MyoD transcription are not much affected. These results indicate that the fall in the steady-state level or receptor messages is caused by reduced transcriptional activity rather than diminished message stability. It also suggests that the regulatory protein may be an activator not only of the α-subunit gene, but also of the genes coding for the γ- and δ-subunits, although the CHX-induced inactivation of the latter two genes is less severe. The notion of a common activator protein is strengthened by the recent identification of a consensus sequence for positive cis-acting elements in the α- and δ-subunit upstream regions [15] and by the frequent occurrence of CANNTG elements in functionally important regions of the α-, δ-, and γ-subunit genes ([15,16,18] and H.-T. J., unpublished observations). It is of course also possible that several trans-acting factors are involved; we have noticed that the chick AChR γ-subunit gene promoter displays only limited structural similarity with the α/δ consensus sequence (H.-T. J. and J. S., unpublished results). Finally, these experiments also indicate that CHX administration does not significantly reduce levels and/or activities of nonspecific components of the transcription machinery.

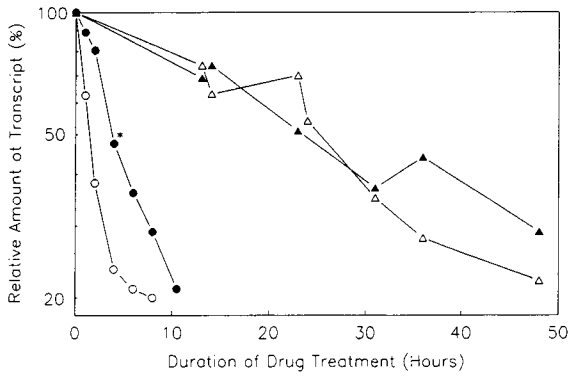

Fig. 3. Effect of metabolic inhibitors of α-subunit gene transcript levels. Intraperitoneal drug treatment of chicks was begun 5–7 days after sciatic nerve section. Animals received either a single dose of actinomycin D (circles) or multiple injections (repeated at 4-h intervals) of CHX (triangles). They were sacrificed at the indicated times, and total RNA prepared from the shank musculature was analyzed for α-subunit pre-mRNA (open symbols) and mature mRNA (filled symbols) as described in Fig. 1.

3.3. *Origin of transactivator*

Several regulatory mechanisms are compatible with the need for ongoing protein synthesis in the denervation response. Among them probably the simplest is mediation by a labile protein that doubles as the target for second messengers and as a transcription activator. Even in innervated muscle this protein would be maintained at a constant concentration by rapid synthesis and turnover; upon denervation it would undergo modification/activation to perform its task in the nucleus. CHX treatment would in such a case lead to depletion of the factor; consequently, denervation could not lead to activation of receptor genes any longer.

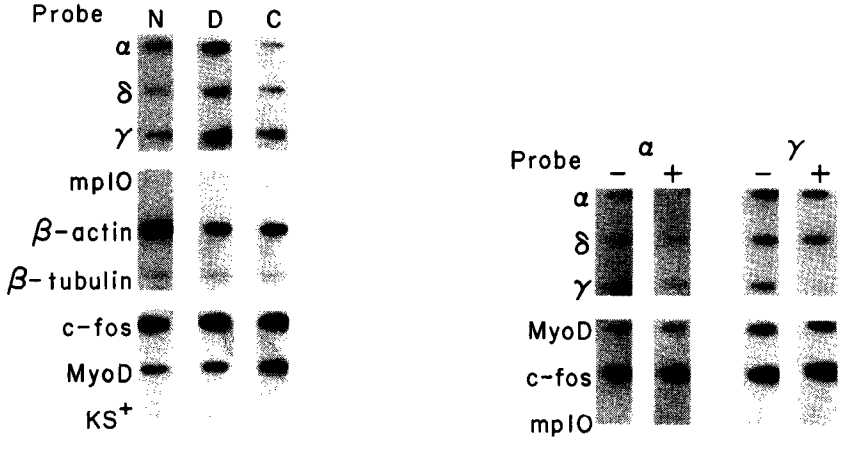

Fig. 2. Effect of CHX on receptor gene transcription. Transcription elongation was assayed, as described in the methods section, in nuclei isolated from innervated (N), denervated (D), and denervated/CHX-treated (C) muscle. 10^7 nuclei were used in each run-on assay, and 2×10^6 cpm ^{32}P-labeled RNA for each hybridization. Results shown are those obtained with α-, γ-, and δ-specific antisense DNA and the single-stranded vector alone, and with double-stranded probes specific for β-actin, β-tubulin, c-*fos*, MyoD, and with the double-stranded vector pKS+.