Volume 282, number 2, 242–246 FEBS 09662 May 1991

ADONIS 001457939100369I

Regulation of acetylcholine receptor gene expression in rats treated with α-bungarotoxin

Orna Asher, Carlo Provenzano and Sara Fuchs

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 25 February 1991

Regulation of acetylcholine receptor (AChR) gene expression was analyzed in α-bungarotoxin (α-BTX) treated rats. A reduction in available ^{125}I-α-BTX binding sites was accompanied by an increase in the various AChR transcripts. The increase in the AChR α-, β- ε- and δ-subunit mRNAs was similar to that observed in rats with experimental autoimmune myasthenia gravis (EAMG). Unlike in EAMG, the γ-subunit transcripts reappeared following α-BTX treatment. The quantitative differences in the levels of AChR transcripts between α-BTX treatment and EAMG on one hand and denervation on the other hand, support the notion that the regulation of AChR gene expression is controlled by muscle activity and by neuronal factors as well. We also demonstrate in this report that myogenin transcripts increase following α-BTX treatment as well as following denervation, whereas MyoD1 transcripts remain stable.

α-Bungarotoxin; Acetylcholine receptor transcript; Experimental autoimmune myasthenia gravis; Denervation; Myogenin; MyoD1

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) of the vertebrate neuromuscular junction (NMJ) is a major component of the end-plate post-synaptic membrane. It contains the acetylcholine (ACh) binding sites, the ion channel and all the structural elements required for regulation of its opening by ACh [1–4]. It is composed of four homologous transmembrane subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ during early embryonic stages or after denervation. In the adult form of the receptor an ε-subunit substitutes the γ-subunit. Denervation results in a large increase of newly synthesized AChRs, incorporated mostly into the extrajunctional plasma membrane, which have biophysical and biochemical properties distinct from those of junctional AChRs [5]. Northern blot hybridization experiments have shown that the expression of the γ- and ε-subunits correlates with the presence of the extrajunctional and junctional forms of the receptor respectively, suggesting that the exchange of a single subunit can account for the different properties observed after denervation of during development [5,6].

Recently, a new family of proteins involved in regulation of myogenic lineage and containing the helix-loop-helix motif has been isolated, among which are MyoD1 and myogenin [7,8]. MyoD1 is expressed in both myoblasts and myofibers [7], while myogenin is absent in myoblasts and is expressed during differentation into myofibers [8]. Two MyoD1 and myogenin binding sites, in muscle-specific enhancers of the chicken AChR α-subunit gene, were found to be functional for activity of the α-subunit in transfected myotubes [9], implying that myogenic factors may regulate the AChR α-subunit promotor.

AChR is the major autoantigen in myasthenia gravis (MG) and in its animal model experimental autoimmune myasthenia gravis (EAMG) [10]. The immune attack at the NMJ leads to a decreased AChR content in the post-synaptic membrane and impairs signal transduction from nerve to muscle. Similarly, administration of sublethal doses of α-bungarotoxin (α-BTX) into animals causes a block of the AChR, leading to muscle weakness and an electrophysiological picture which resemble those of EAMG [13]. In contrast to denervation, in both myasthenia and α-BTX treatment the nerve remains in contact with the muscle.

We have previously demonstrated that the reduction in AChR content observed in EAMG is accompanied by an increase in AChR transcripts coding for the adult type of the receptor ($\alpha_2\beta\varepsilon\delta$) [11,12]. In this report, we have analyzed the effects of α-BTX treatment on AChR gene expression using Northern blot hybridization with ^{32}P-labelled DNA probes for the different AChR subunits. In addition we have studied the regulation of MyoD1 and myogenin gene expression during α-BTX treatment and following denervation.

2. MATERIALS AND METHODS

2.1. *Animal models*

Ten-week-old female Lewis rats were intraperitoneally injected with αBTX (27 μg/kg body weight/day) in phosphate-buffered saline

Correspondence address: S. Fuchs, Department of Chemical Immunology, The Weizmann Institute Of Science, Rehovot 76100, Israel. Fax: (972) 466966.

Published by Elsevier Science Publishers B.V.

(PBS) for 5 consecutive days, while control animals were injected with PBS alone. Animals were sacrificed from day 1 to day 15 after the first injection, their hindlimb muscles were immediately removed, frozen in liquid nitrogen and stored at -70°C until use. Aliquots of the muscles were used to measure residual α-BTX binding sites or to isolate RNA. EAMG was induced in rats by injecting them monthly with 40 μg of purified *Torpedo californica* AChR in complete Freund's adjuvant (CFA) until myasthenic symptoms were observed [11]. For denervation, animals were anesthetized and 1 cm of the sciatic nerve was surgically removed, and after two weeks, they were sacrificed and muscles were removed and stored as for α-BTX-treated rats.

2.2. *AChR extraction*

Muscle AChR preparations from the hindlimb of rats were performed essentially as described previously [14]. In brief, muscle aliquots were homogenized at 4°C in PBS, centrifuged at 20,000 × *g* and the pellets extracted with 1% Triton X-100 in PBS. The supernatant obtained following centrifugation for 60 min at 100,000 × *g* was used. The residual membranal AChR content was determined by measuring the binding of ^{125}I-α-BTX as previously described [15].

2.3. *Northern blot analysis*

Total muscle RNA was extracted as previously described [11]. Following 1% agarose formaldehyde gel electrophoresis, RNA was transferred to GeneScreen Plus (NEN, Du Pont) filters [11]. Northern blots of total RNA were hybridized to cDNA probes specific for the different AChR subunits: the α-, β- and δ-subunit probes were *Eco*RI fragments derived from the respective mouse recombinant DNA (kindly provided by Dr. S. Heinemann), the ε-subunit probe was an *Eco*RI fragment and the γ-subunit probe was a *Xba*I/*Sal*I fragment, both derived from rat recombinant DNA (kindly provided by Dr. V. Witzemann). The α-actin-specific probe (kindly provided by Dr. U. Nudel) was a 600 bp rat cDNA. The MyoD1 probe (kindly provided by Dr. A.B. Lassar) was a rat cDNA fragment and was cleaved out of the vector pEMC11S with *Eco*RI. The myogenin probe (kindly provided by Dr. W.E. Wright) was a rat cDNA fragment and was cleaved out of the pESVMGN vector with *Eco*RI. Autoradiograms of the Northern blots were scanned through a Molecular Dynamics 300A Computing Densitometer using the ImageQuant Software to get volume integration (total O.D. in the area of the signal). Averages of the results from 3 different blots were calculated.

3. RESULTS

In our previous reports [11,12] we analyzed AChR gene expression in EAMG in rabbits, rats, and mice and found that an increase in AChR transcripts correlates exclusively with the expression of the disease and with a significant reduction in membranal AChR content. The moderate increase of AChR gene expression observed in EAMG corresponds mainly to an adult junctional receptor ($\alpha_2\beta\varepsilon\delta$). In order to further understand the mechanism of AChR gene regulation, we have analyzed the effect of a specific blockade of ACh-induced muscle activity on AChR gene expression, using a system other than EAMG. For this purpose we have chosen to use α-BTX, which is a specific antagonist of AChR [16], and its effect mimics in several aspects those observed in EAMG. Rats were injected with α-BTX (27 μg/kg of body weight/day) for 5 consecutive days and clinical symptoms resembling EAMG were observed: rats were weak, decreased their spontaneous movements in the cage and lost weight (up to 25–30%). The symptoms disappeared in a few days after the end of the treatment, and on day 9 or 10 experimental and control rats were indistinguishable in their behavior or body weight. The clinical picture was accompanied by a progressive decrease of residual detectable ^{125}I-α-BTX binding to muscle extracts (Fig. 1). From day 6 on detectable α-BTX binding increased gradually, and reached about 60% of its level before treatment (day 0) from day 10 to day 15 (Fig. 1).

Northern blot analysis of total RNA preparations (Fig. 2) showed maximal levels of the specific mRNAs coding for the α-, β-, ε- and δ-subunits on day 5, reaching 7.5-, 5-, 4.5- and 6.5-fold increases respectively. The γ-subunit mRNA was not detectable in control animals, while it could be detected between day 4 and day 9 in treated rats, with a peak on day 6. Pretreatment levels of all AChR subunit transcripts were reached again between days 8 and 10. No significant variation in α-actin mRNA was recorded, reflecting the selective increase in all AChR mRNAs in α-BTX treated rats. Control rats, receiving PBS alone, did not show any clinical symptoms, nor any change in mRNA levels or in membrane AChR content.

The increase of α-subunit mRNA, following α-BTX treatment is comparable to the increase observed in EAMG (5–7 fold) and is about one order of magnitude lower than the increase observed following denervation (Fig. 3). The differences in the regulation of the α- and the γ-subunit mRNA in EAGM, α-BTX treatment and denervation, may imply that different mechanisms are controlling AChR gene expression in these systems.

Since MyoD1 and myogenin were shown to affect AChR gene expression during myogenesis in chick myotubes [9], we compared the changes of AChR α-subunit mRNA levels with those of MyoD1 and myogenin mRNAs following α-BTX treatment (Fig. 4). For comparison we have also measured the levels of these transcripts following denervation. In both α-BTX

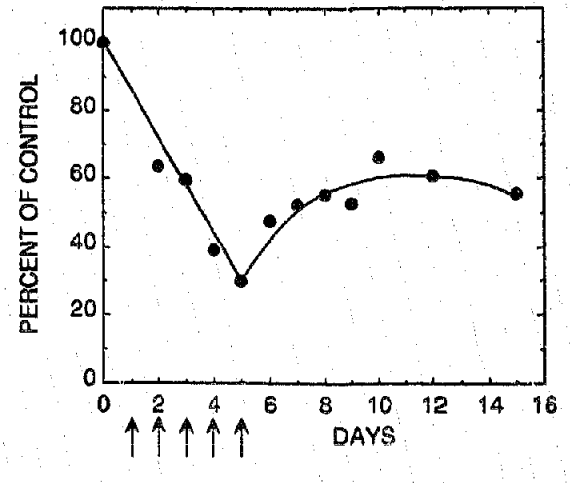

Fig. 1. Residual ^{125}I-α-BTX binding sites in rat muscle extracts following α-BTX treatment. The arrows indicate the days of injection of α-BTX.

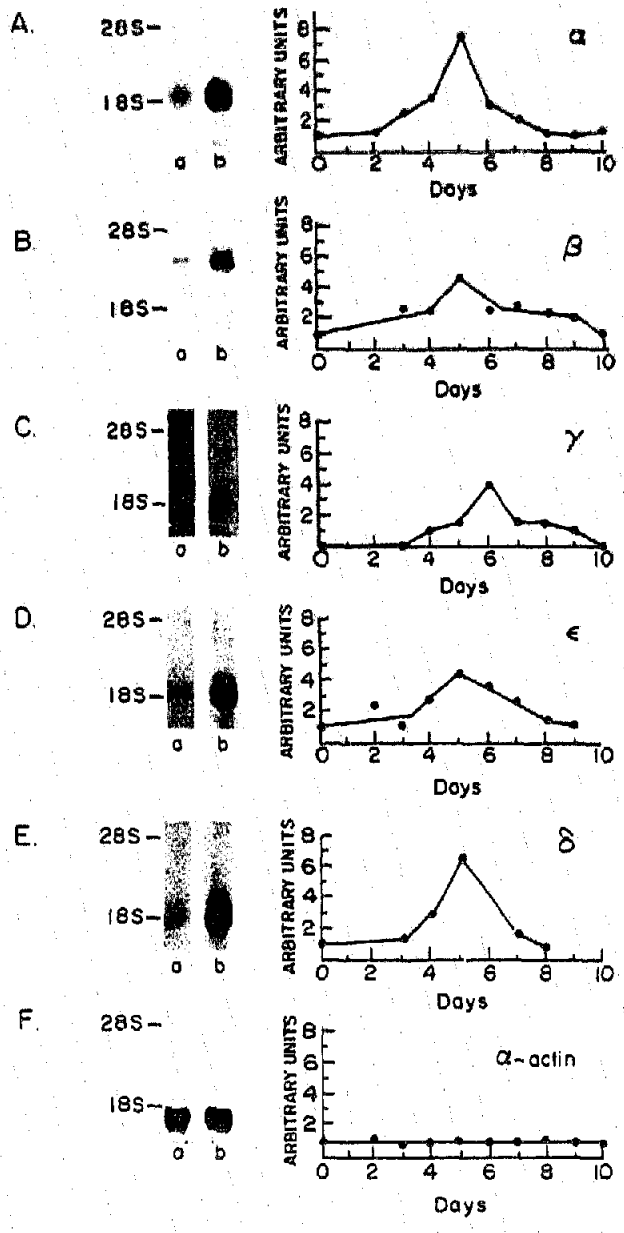

Fig. 2. Northern blot analysis of AChR mRNAs following α-BTX treatment. Total RNA samples (30 μg/lane) were hybridized with cDNA probes specific for: (A) α-subunit, (B) β-subunit, (C) γ-subunit, (D) ε-subunit, (E) δ-subunit and (F) α-actin. The relative content of the respective subunits mRNAs was plotted assigning the arbitrary value of 1 for control non-treated rats (day 0). The left inserts show the autoradigrams of Northern blots, where (a) corresponds to control (day 0) and (b) to the peak for each of the subunits. The blots were hybridized with the AChR cDNA probes and following stripping, with the α-actin probe.

treatment and in denervation MyoD1 mRNA levels remained stable, while those of myogenin were significantly elevated. Moreover, the changes in myogenin as well as in α-subunit mRNAs after denervation are much higher than those observed following α-BTX treatment. The relative increase in the myogenin

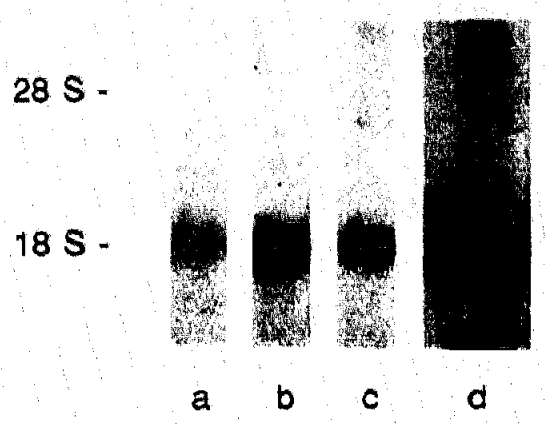

Fig. 3. Northern blot analysis with total RNA (30 μg/lane) of rat AChR α-subunit mRNA in (a) control, (b) α-BTX treatment, (c) EAMG, (d) denervation.

transcripts in α-BTX treated rats preceded that of the α-subunit mRNA. For instance, on days 3 and 4 the levels of α-subunit mRNA were rather low, whereas that of myogenin was high and reached almost its maximal level (Fig. 5).

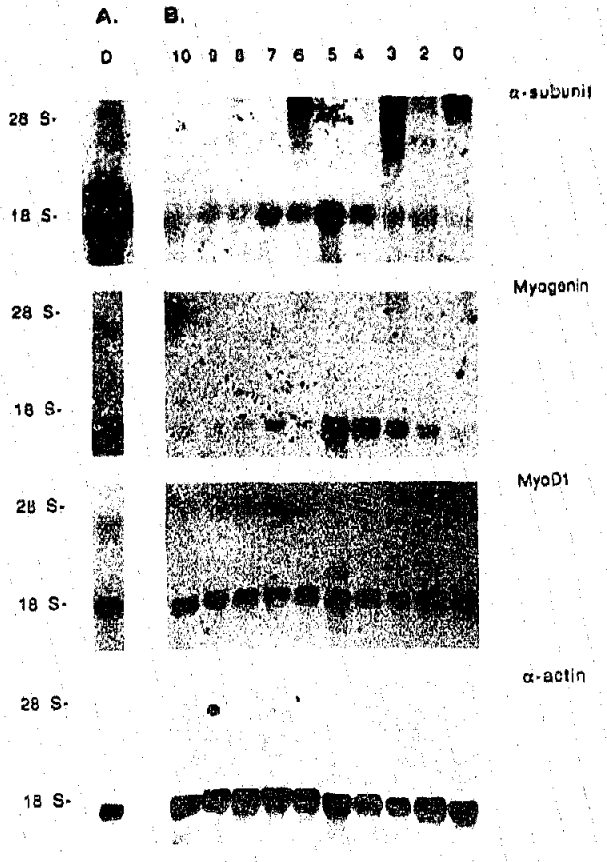

Fig. 4. Levels of transcripts specific for AChR α-subunit, myogenin, MyoD1 and α-actin following (A) denervation and (B) α-BTX treatment; numbers indicate days after the first injection. Total muscle RNA samples (30 μg/lane) were subjected to Northern blot hybridization analysis as described; the autoradiograms represent one blot which was stripped and hybridized each time with the different cDNA probes.

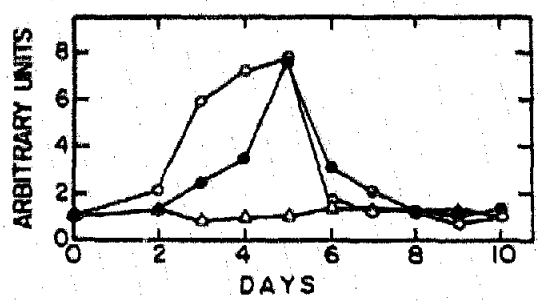

Fig. 5. Levels of specific transcripts for α-subunit (●), myogenin (○) and MyoD1 (△) following α-BTX treatment. The relative values were determined by densitometric scanning, assigning an arbitrary value of 1 for control non-treated rats (day 0).

4. DISCUSSION

α-BTX has been used widely as a specific antagonist for the AChR with a high affinity and specificity, both in vivo and in vitro [18]. When injected in vivo, its binding to AChR causes muscular weakness and an electrophysiological picture similar to those observed in MG and EAMG [13]. In this report we have shown that a blockade of cholinergic neuromuscular transmission by α-BTX causes a moderate increase in all AChR transcripts. The time-course for the increase in AChR mRNAs is similar for all subunits, except for a slight variation observed with the γ-subunit (Fig. 2).

It has already been demonstrated that denervation of adult rat muscle elicited an increase of the steady-state level of the receptor subunit mRNAs [3,17–19]. The newly synthesized receptor following denervation, is mainly of the embryonic, extrajunctional type ($\alpha_2\beta\gamma\delta$) [5,6,21]. The γ-subunit mRNA is not detected in normal innervated adult muscles, while upon denervation, a large increase in its level is observed [5,19]. On the other hand, in EAMG the increase in AChR subunit mRNAs corresponds mainly to the adult, junctional form of the receptor ($\alpha_2\beta\epsilon\delta$) [11,12]. The levels of AChR α-subunit mRNA in myasthenic rats and α-BTX-treated rats show a similar increase, 5- to 7-fold, which is about one order of magnitude lower than that observed in denervated muscles (Fig. 3). However, it should be noted that the γ-subunit transcript reappears following α-BTX treatment; this is qualitatively similar to denervation and distinct from EAMG. Although muscle activity is impaired in both EAMG and α-BTX treatment, the contact between nerve and muscle is not affected as is the case in denervation. Thus, the moderate increase in AChR transcripts following α-BTX treatment and in EAMG suggests that ACh-induced muscle activity is sufficient for the regulation of AChR gene expression. Nevertheless, the large increase in receptor transcripts observed following denervation supports the notion that the regulation of AChR gene expression is controlled not only through muscle activity, but also by neuronal factors other than ACh. AChR transcripts were shown to be elevated only beyond a threshold loss of available AChR binding sites. Later on, an inverse correlation between ^{125}I-α-BTX binding sites and AChR transcripts is observed (Fig. 2). Thus, the elevation in AChR subunits mRNA was observed between day 3 and day 9, when the membrane receptor was below 60% of its value before treatment. A similar effect was reported in EAMG, where the rise in AChR transcripts was triggered only beyond a certain threshold loss in receptor content [12].

In this study we have observed that α-BTX treatment and denervation induced an elevation of myogenin transcripts, while MyoD1 mRNA levels remained constant. Myogenin is transiently expressed following a stimulation to differentiate, while MyoD1 is continuously expressed in myoblasts as well as in myofibers [8]. In addition myogenin is expressed in at least one muscle cell line (L_6) in which MyoD1 could not be detected, and in myotomes at least 2 days before MyoD1 [8]. MyoD1 and myogenin belong to the same DNA-binding protein superfamilly (the helix-loop-helix), both share a domain with a very high degree of homology and may function in conjunction with one another. Nevertheless, it is possible that some of the effects of myogenin might be independent from MyoD1. It was proposed recently that MyoD1 and myogenin may regulate certain muscle-specific genes such as muscle creatine kinase [20] and AChR genes [9]. Our data indicate that there is a good correlation between the relative increase in myogenin mRNA and that of α-subunit mRNA in denervation and following α-BTX treatment (Fig. 4). In addition, the fact that the increase in myogenin mRNA precedes that of the AChR α-subunit (Fig. 5), along with previous data [9], indicates that myogenin can be implicated in controlling AChR α-subunit gene expression. Since we did not observe a change in MyoD1 transcripts in these systems we cannot conclude whether MyoD1 participates with myogenin in controlling α-subunit gene expression.

Acknowledgements: We thank D. Zuk, Drs D. Yaffe and U. Nudel for fruitful discussions, and Drs S. Heineman, V. Witzemann, A.B. Lassar and W.E. Wright for the cDNA probes. C.P. was a recipient of an EMBO long term fellowship, and was also supported by the Consiglio Nazionale delle Ricerche. This research was supported by grants from the Schilling Foundation, the Los Angeles Chapter of Myasthenia Gravis Foundation, the Muscular Dystrophy Association of America, the Association Francaise Contre les Myopathies (AFM), and the United States-Israel Binational Science Foundation (BSF).

REFERENCES

[1] Popot, J.L. and Changeux, J.-P. (1984) Physiol. Rev. 64, 1162–1184.
[2] Stroud, R.M. and Finer Moore, J. (1985) Annu. Rev. Cell Biol. 1, 317–351.
[3] Hucho, F. (1986) Eur. J. Biochem. 158, 211–226.
[4] Laufer, R. and Changeux, J.-P. (1989) Mol. Neurobiol. 3, 1–52.
[5] Witzemann, V., Barg, B., Nishikawa, Y., Sakmann, B. and Numa, S. (1987) FEBS Lett. 223, 104–112.

[6] Witzemann, V., Barg, B., Criado, M., Stein, E. and Sakmann, B. (1989) FEBS Lett. 242, 419–424.
[7] Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Cell 51, 987–1000.
[8] Wright, W.E., Sasoon, D.A. and Lin, V.K. (1989) Cell 56, 607–617.
[9] Piette, J., Bessereaux, J.L., Huchet, M. and Changeux, J.-P. (1990) Nature 345, 353–355.
[10] Fuchs, S. (1979) Curr. Topics Microbiol. Immunol. 85, 1–29.
[11] Asher, O., Neumann, D. and Fuchs, S. (1988) FEBS Lett. 233, 277–281.
[12] Asher, O., Neumann, D., Witzemann, V. and Fuchs, S. (1990) FEBS Lett. 267, 231–235.
[13] Takamori, M. and Iwanaga, S. (1976) Neurology 26, 844–848.
[14] Aharonov, A., Tarrab-Hazdai, R., Silman, I. and Fuchs, S. (1977) Immunochemistry 14, 129–137.
[15] Souroujon, M.C., Mochly-Rosen, D., Gordon, A.S. and Fuchs, S. (1983) Muscle Nerve 6, 303–311.
[16] Lee, C.Y. (1972) Annu. Rev. Pharmacol. 12, 265–286.
[17] Evans, S., Goldman, D., Heinemann, S. and Patrick, J. (1987) J. Biol. Chem. 262, 4911–4916.
[18] Moss, S.J., Beeson, D.M., Jackson, J.F., Darlison, M.G. and Barnard, E.A. (1987) EMBO J. 6, 3917–3921.
[19] Shieh, B.H., Ballivet, M. and Schmidt, J. (1988) Neuroscience 24, 175–187.
[20] Lassar, A.B., Buskin, J.N., Lockshan, D., Davis, R.L., Apone, S., Hauschka, S.D. and Weintraub, H. (1989) 58, 823–831.
[21] Brenner, H.R., Witzemann, V. and Sackmann, B. (1990) Nature 344, 544–547.