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Increased levels of acetylcholine receptor α-subunit mRNA in experimental autoimmune myasthenia gravis

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To gain insight into the regulatory mechanisms underlying the blockade and loss of acetylcholine receptor (AChR) in myasthenia, we have followed AChR α-subunit mRNA levels in leg muscles of myasthenic and normal rabbits and rats. Northern blots of RNA preparations from normal and myasthenic animals were hybridized with a mouse AChR α-subunit cDNA probe. Our experiments indicate a specific increase (4–7-fold) in the levels of α-subunit mRNA in animals with experimental autoimmune myasthenia gravis (EAMG), in comparison with control animals. Actin mRNA levels were essentially unchanged. Our results thus suggest that EAMG is accompanied by an increased level of AChR gene transcription.

Acetylcholine receptor; Experimental autoimmune myasthenia gravis; mRNA

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a well characterized ligand gated ion-channel. It forms an oligomeric complex of four types of subunits present in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ [1,2]. Special interest in the immunological properties of the receptor stems from its involvement as the major autoantigen in the autoimmune disease myasthenia gravis (MG) and its animal model, experimental autoimmune myasthenia gravis (EAMG). The disease is accompanied by humoral and cellular anti-AChR immune responses and by receptor loss [3–5]. Although the observations indicate a net reduction of AChR levels, the molecular mechanisms underlying this process, as yet, are not clear. The decrease in AChR levels in myasthenic muscles may be reflected by similar changes in mRNA levels, or alternatively, receptor loss may trigger a cascade of compensatory mechanisms such as increasing the rate of protein biosynthesis from pre-existing mRNA and/or enhancing the levels of mRNA for the receptor subunits.

It has previously been demonstrated that denervation of rat diaphragm and mouse leg muscle as well as tetrodotoxin treatment of muscle cell cultures results in an increase in the levels of mRNA encoding for the AChR α-subunit [6–8]. This observation has also been extended to the mRNA transcripts of the other subunits. The mRNA levels of the AChR subunits were shown to increase in the course of development in mouse [9,10] and calf [11], and in response to muscle denervation in chick [12], rat and mouse [9].

In the present study we have followed the level of AChR α-subunit mRNA in myasthenic rats and rabbits. Based on reactivity with a ^{32}P-labeled cDNA probe of the mouse α-subunit, we could observe a 4–7-fold increase in the α-subunit mRNA in leg muscles of myasthenic animals.

2. MATERIALS AND METHODS

2.1. *AChR preparation*

AChR was purified from *Torpedo californica* electric organ (Pacific Bio-Marine, Venice, CA) as described [13].

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2.2. *Immunological procedures*

Rabbits weighing 2–3 kg were injected in the footpads and intradermally in three or four spots each with 100 μg purified AChR emulsified with complete Freund's adjuvant (CFA, Difco) [14]. Animals injected with CFA only were used as controls. EAMG was observed in the rabbits by muscular weakness, uncoordinated movements, paralysis of the limbs and trunk, and difficulties in breathing. Female Lewis rats aged approx. 10 weeks were each injected in the footpads with 40 μg purified AChR in CFA supplemented with 1 mg H_{37} RA mycobacterium (Difco). Rats were boosted intradermally at monthly intervals with 40 μg AChR in phosphate-buffered saline (PBS) until myasthenic symptoms were observed. Animals injected with CFA only were used as controls.

Rabbit and rat sera were tested for antibodies to purified *Torpedo* AChR by solid-phase radioimmunoassay (SP-RIA) using ^{125}I-labeled protein A to detect antibody binding [15].

2.3. *RNA isolation and Northern blot analysis*

Rabbits and rats exhibiting EAMG symptoms were killed and their leg muscles were frozen in liquid N_2 immediately after dissection. Total RNA was precipitated in LiCl/urea essentially as in [16], with several modifications. The tissues were homogenized in 3 M LiCl, 7 M urea. After 16 h at 4°C the pellet was removed by centrifugation at 10000 × *g* for 30 min and resuspended in 0.5% SDS, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 10 mM EDTA (1 ml/g tissue). An equal volume of a buffer containing 0.5% SDS, 10 mM Tris-HCl, pH 7.5, 0.35 M NaCl, 7 M urea and 10 mM EDTA was added prior to phenol extraction and ethanol precipitation. For Northern blot analysis 20 μg total RNA and 10 μg poly(A)$^+$ RNA obtained by purification on oligo-(dT)-cellulose [17] were denatured in formaldehyde at 65°C and subjected to electrophoresis on a 1% agarose gel in 16% formaldehyde. RNA was then transferred onto Gene Screen Plus membrane filters (NEN, Boston, MA). Prehybridization was performed at 42°C for 2 h in 50% formamide, 5 × SET (0.75 M NaCl, 0.1 M Tris-HCl, pH 7.4, 0.05 M Trizma base and 0.05 M EDTA), 5 × Denharts (0.1% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 1% SDS, 50 mM sodium phosphate buffer, pH 6.8, and 40 μg/ml heat-denatured salmon sperm DNA. Hybridization was then performed at 42°C for 16 h in 50% formamide, 5 × SET, 1 × Denharts, 20 mM sodium phosphate, pH 6.8, 10% dextran sulfate and 1% SDS.

2.4. *cDNA probes*

The AChR hybridization probe employed was a 900 bp NCO1 fragment (extending from within the 5′-end to amino acid 280) derived from a recombinant DNA plasmid (pMAR15) containing the cDNA coding for the α-subunit of mouse AChR [18] kindly provided by Dr S. Heinemann. The actin probe was a 570 bp insert from plasmid 749 coding for α-, β- and γ-actins [19] kindly provided by Dr U. Nudel. Both probes were labeled by nick translation with [α-^{32}P]dATP [20], to a specific activity of 1–3 × 10^8 cpm/μg.

3. RESULTS

EAMG was induced in rabbits and rats by injection of purified AChR from *Torpedo* electric organ. Rabbis displayed myasthenic symptoms of muscle weakness 3–4 weeks following a single immunization. Chronic muscle weakness in rats was apparent after 1–3 boosts of AChR at monthly intervals. Muscle tissue for RNA preparation as well as antisera were sampled from the above animals.

As shown in fig.1, both rabbits and rats had significant anti-AChR antibody titers when killed for RNA preparation. We then addressed the question of whether AChR gene expression is altered in myasthenic animals. To this end, RNA was extracted from leg muscles of normal and myasthenic

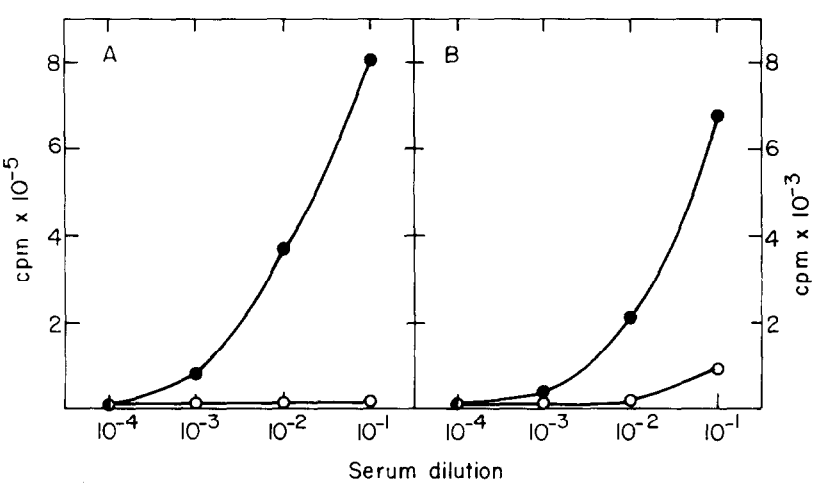

Fig.1. Binding (solid phase radioimmunoassay) of AChR to rabbit (A) and rat (B) antisera; ●, AChR injected animal; ○, CFA injected animal.

rabbits and rats. Poly(A)$^+$ RNA was prepared by passage through an oligo(dT)-cellulose column. The RNA preparations were then size fractionated on denaturing formaldehyde-agarose gels [21] and transferred onto Gene Screen Plus membrane filters. To detect AChR α-subunit-specific mRNA, Northern blots were probed with the ^{32}P-labeled 900 bp fragment of the mouse α-subunit cDNA clone. in both rabbits and rats a significant increase in abundance of α-subunit-specific mRNA was observed in myasthenic muscles as compared to their control counterparts (figs 2,3). Reactivity of both myasthenic and normal mRNA preparations with an actin-specific probe [19] was essentially similar, thus reflecting the selective increase

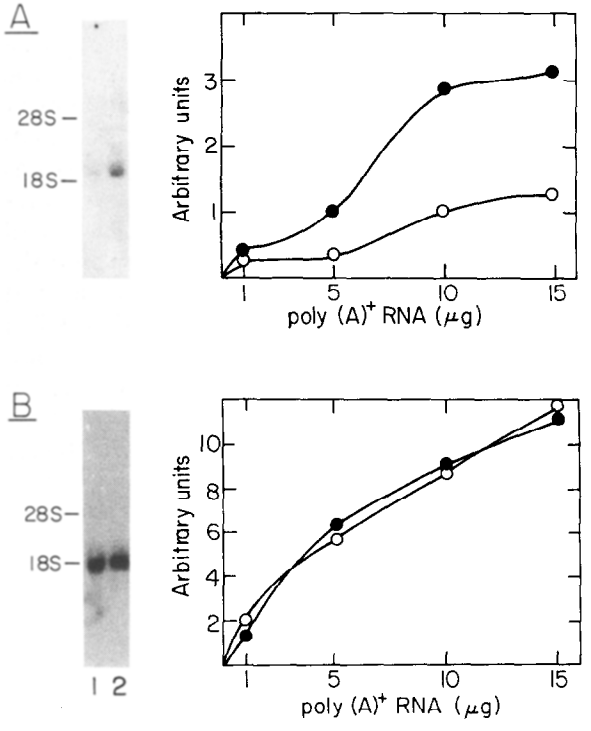

Fig.2. Northern blot analysis of AChR α-subunit transcripts in rabbit leg muscle. 10 μg each of poly(A)$^+$ RNA isolated from normal (lane 1) and myasthenic (lane 2) rabbit leg muscle were denatured in formaldehyde at 65°C, electrophoresed in 1% agarose gel containing 16% formaldehyde and transferred to Gene Screen Plus membrane filters. These blots were then hybridized with the α-subunit cDNA probe from mouse (A) and following stripping, with an α-actin probe (B) as described in section 2. For quantitation of the Northern blots densitometric scanning was performed. Signals were plotted in arbitrary units as a function of the amount of poly(A)$^+$ RNA applied to the gel. ●, AChR injected rabbits; ○, CFA injected rabbits.

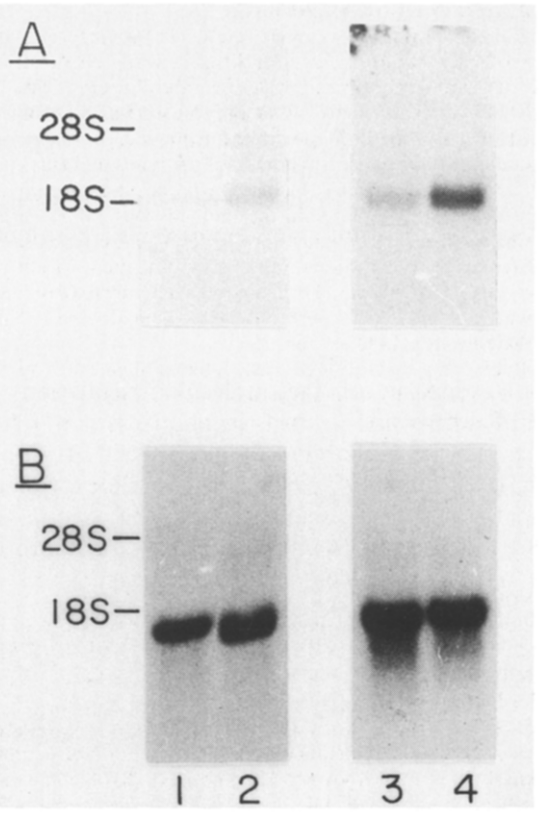

Fig.3. Distribution of the AChR α-subunit (A) and α-actin (B) mRNAs from normal (1,3) and myasthenic (2,4) rat leg muscle. Total RNA (20 μg each in lanes 1,2) and poly(A)$^+$ RNA (10 μg each in lanes 3,4) were subjected to blot hybridization as described in fig.2.

in AChR α-subunit mRNA in myasthenic muscle.

Fig.2 represents autoradiograms of blot hybridization analysis of leg muscle poly(A)$^+$ RNA from normal and myasthenic rabbits. Quantitation of mRNA levels was performed by densitometer scanning of the autoradiograms and plotting the integral of the scan versus the amount of mRNA applied to the gel. Elevation of AChR α-subunit mRNA was detected in myasthenic muscles at the different amounts of RNA tested (fig.2A). Calculated on the basis of densitometric analysis this increase amounts to 4-fold. On the other hand, the levels of actin mRNA were virtually identical in both myasthenic and normal muscles in all amounts of RNA tested (fig.2B).

We also examined the levels of AChR α-subunit mRNA in myasthenic rats [22], where the nature of

the disease is distinct from that in rabbits. As shown in fig.3, mRNA levels of AChR α-subunit increase significantly in myasthenic rat muscles as compared to normal ones. Based on densitometric scanning this increase was calculated to be approx. 7-fold. Again, an actin-specific probe revealed that actin mRNA levels remain unchanged in disease, reflecting the selective increase in α-subunit transcripts.

4. DISCUSSION

Investigation of the molecular regulation of AChR expression in myasthenia gravis is of great interest. The availability of specific cDNA probes for AChR subunits now makes it possible to follow AChR gene expression during the course of disease. Using an AChR α-subunit cDNA probe, we were able to demonstrate increased levels of AChR α-subunit mRNA in myasthenic leg muscles of both rabbits and rats (figs 2,3). The elevation of α-subunit mRNA seems to be selective, as actin mRNA levels remain unchanged.

Previous reports have indicated that the levels of surface membrane AChR are reduced in myasthenia [23,24]. In addition, accelerated degradation of AChR, mediated by anti-AChR antibodies was observed [25,26]. Although the final outcome of myasthenia is decreased surface receptor levels, it appears that at least with respect to AChR α-subunit there is increased mRNA transcription. This might reflect a compensatory mechanism for receptor loss. It is important to analyse the nature and fate of the newly transcribed AChR mRNA. One should investigate whether the observed increase in α-subunit mRNA is also seen for the other receptor subunits, whether the newly transcribed receptor mRNAs reflect the subunit stoichiometry in the protein (i.e. $\alpha_2\beta\gamma\delta$) and whether they are translated, and subsequently inserted into the membrane as functional receptor molecules. It is also important to determine whether the increase in mRNA is triggered by anti-AChR antibodies and whether a certain antibody specificity is required. In this respect it is noteworthy that preliminary experiments in our laboratory indicate that rabbits immunized with a denatured AChR preparation (reduced and carboxymethylated AChR [27]) also had increased levels of AChR α-subunit mRNA. As previously demonstrated in our laboratory [14,27] such rabbits displayed high anti-AChR antibody titers but had no myasthenic symptoms. These observations may indicate that anti-AChR antibodies may lead to increased AChR gene transcription without necessarily appearance of clinical symptoms of myasthenia. It should still be resolved whether the phenomenon of increased mRNA levels is directly connected to disease, or is a mere result of an immune response towards an autoantigen. Studies are now being performed in our laboratory to resolve the issues mentioned above.

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