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Research Article

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Regulation of Acetylcholine Receptor Gene Expression in Human Myasthenia Gravis Muscles

Evidences for a Compensatory Mechanism Triggered by Receptor Loss

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Abstract

Myasthenia gravis (MG) is a neuromuscular disorder mediated by antibodies directed against the acetylcholine receptor (nAChR) resulting in a functional nAChR loss. To analyze the molecular mechanisms involved at the muscular target site, we studied the expression of nAChR subunits in muscle biopsy specimens from MG patients. By using quantitative PCR with an internal standard for each subunit, we found that the levels of β -, δ -, and ϵ -subunit mRNA coding for the adult nAChR were increased in severely affected MG patients, matching our previous data on the α -subunit. Messenger levels were highly variable in MG patients but not in controls, pointing to individual factors involved in the regulation of nAChR genes. The fetal subunit (γ -chain) transcripts were almost undetectable in the extrajunctional region of MG muscle, suggesting that gene regulation in MG differs from that in the denervation model, in which nAChR γ -subunit mRNA is reexpressed. Nicotinic AChR loss mediated by monoclonal anti-nAChR antibodies in both the TE671 muscle cell line and cultured normal human myotubes induces a similar increase in β - and δ -subunit mRNA levels, suggesting the existence of a new muscular signaling pathway system coupled to nAChR internalization and independent of muscle electrical activity. These data demonstrate the existence of a compensatory mechanism regulating the expression of the genes coding for the adult nAChR in patients with MG. (*J. Clin. Invest.* 1998; 102:249–263.) Key words: myogenic factors • anti-AChR monoclonal antibody • quantitative polymerase chain reaction • autoimmune disease • TE671 muscle cell line

Introduction

The muscle nicotinic acetylcholine receptor (nAChR)¹ is a well characterized ligand-gated ion channel of 270 kD that forms a pentameric complex of four homologous subunits with a molar stoichiometry of α_2 , β , ϵ , and δ (1, 2). nAChR mediates synaptic transmission at the vertebrate neuromuscular junction. In mammalian muscle, regulation of nAChR subunit mRNAs and the distribution of nAChRs along the fiber are developmentally regulated. Transcript levels increase during myogenic differentiation and are repressed during muscle innervation; denervation results in reaccumulation (3). The nAChR ϵ - and γ -subunits are also under developmental control, with expression of two types of channel in mammals. In early embryonic stages and upon denervation, a low-conductance, long open-time channel composed of α -, β -, δ -, and γ -subunits, and distributed throughout the fiber length predominates (4, 5), whereas in adult fibers, a high-conductance, brief open-time channel containing α -, β -, δ - and ϵ -subunits is expressed exclusively at the motor endplate (6).

A family of skeletal muscle-specific transcription factors that plays a central role in myogenic determination and/or differentiation (7) is composed of four members known as MyoD1, myogenin, Myf5, and muscle regulatory factor (MRF4), also referred to as herculin, in mice (8–11); and Myf3, Myf4, Myf5, and Myf6 in humans (10, 12, 13). Binding assays and transfection experiments have shown that both myogenin and MyoD1 transactivate the nAChR α - and γ -subunit genes (14, 15). Consistent with the notion that myogenic factors may regulate nAChR gene transcription in vivo is the fact that changes in myogenin, MRF4, and MyoD1 levels during innervation and after denervation of skeletal muscle precede changes in nAChR mRNA levels (16).

nAChR is the main autoantigen target in myasthenia gravis (MG), a human disorder of neuromuscular transmission, in which autoantibodies directed against nAChR at the neuromuscular junction are found in 85% of patient sera and cause functional nAChR loss at the endplate postsynaptic membrane, leading to muscle weakness and fatigability of voluntary muscle (17–19). The pathogenicity of anti-nAChR antibodies has been clearly demonstrated by their ability to transfer dis-

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1. Abbreviations used in this paper: ARIA, acetylcholine receptor-inducing activity; EAMG, experimental autoimmune myasthenia gravis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ¹²⁵I- α -Bgt, ¹²⁵I-labeled α -bungarotoxin; MG, myasthenia gravis; MIR, main immunogenic region; MRF4, muscle regulatory factor; nAChR, nicotinic acetylcholine receptor; RT, reverse transcriptase.

ease to normal animals (20) and to cause loss of nAChR expression in a myotube culture system (21, 22). The pathogenic mechanisms of anti-nAChR antibodies directed against the endplate nAChR are well established (23, 24), but little is known of the capacity of muscle cells to regulate nAChR expression after autoimmune attack. The α -subunit contains the main immunogenic region (MIR) recognized by autoantibodies in MG (25, 26). To elucidate the regulation of nAChR gene expression in human MG, we studied the transcriptional regulation of nAChR subunit genes. In a previous report, we described an increase in nAChR α -subunit mRNA levels in muscle samples from severely affected MG patients, suggesting the existence of a compensatory mechanism that attenuates the loss of nAChR in human MG endplates (27). However, a defect in nAChR expression could involve one of the other subunits involved in the structural organization of the receptor if it is synthesized in limited amounts. We thus investigated

whether the other subunit genes and myogenic factors were also upregulated. We did so by quantifying nAChR β , δ , and ϵ mRNA levels by reverse transcriptase (RT)-PCR with an internal standard for each subunit in muscle biopsy specimens from MG patients and controls. Finally, we analysed the compensatory mechanism of regulation triggered after nAChR loss by monoclonal anti-nAChR antibodies in the TE671 muscle cell line showing no spontaneous electrical activity. Evidence for a nAChR-coupled signaling pathway involved in up-regulation of nAChR subunit messengers is discussed.

Methods

Patients

MG was diagnosed on the basis of clinical features, an electromyographic decrement, and the positive effect of anticholinesterase. We studied sternocleidomastoid muscle samples from 27 MG patients (18

Table I. Characteristics of the Patients: Clinical Data and mRNA Levels of AChR β -, δ - and ϵ -Subunits

Patient	Sex	Age	Treatment*	Disease severity**	Anti-AChR Ab titer	Thymic histology	Onset of disease	AChR mRNA subunits (n)		
								β	δ	ϵ
		yr			nM		Mo			
1	F	36	A	IIA	33.7	Hyperplasia	39	12.00	ND	ND
2	F	56	A	IIA	0	Normal	4	3.30	5.60	0.15
3	F	31	A	IIA	26.9	Hyperplasia	6	5.60	ND	0.02
4	M	16	A	IIA	> 14.5	Normal	13	27.70	3.90	0.21
5	F	25	A	IIA	0	Normal	86	16.40	10.70	0.11
6	F	46	A	IIA	16.5	Hyperplasia	16	6.90	6.40	ND
7	M	49	A	IIA	0	Normal	7	7.20	ND	ND
8	M	33	A	IIA	5.4	Normal	40	6.50	ND	ND
9	F	36	A	IIA	7.6	Hyperplasia	24	16.50	10.90	0.25
10	F	21	A	IIA	> 9.5	Hyperplasia	44	18.50	5.50	0.20
11	F	24	A	IIA	142.2	Hyperplasia	14	ND	3.80	ND
12	F	26	A	IIB	8	Hyperplasia	12	8.20	7.30	0.09
13	M	81	A	IIB	> 22.7	Normal	4	10.50	6.00	ND
14	F	16	A	IIB	> 23.7	Hyperplasia	51	12.60	3.06	0.11
15	M	64	A	IIB	6.9	Normal	3	4.20	ND	ND
16	F	24	P	IIB	0	Normal	1	7.00	2.40	0.05
17	F	19	A	IIB	7.9	Thymoma	10	20.30	22.30	0.48
18	F	41	A-P	IIB	0	Normal	3	86.00	ND	ND
19	F	22	A	IIB	5.8	Hyperplasia	3	33.40	20.70	0.23
20	M	32	A	IIB	90.9	Hyperplasia	8	10.00	ND	ND
21	M	42	S-P	IIB	12.7	Normal	152	17.60	ND	ND
22	M	45	A-S-IVIG	IIB	6.5	Normal	12	34.00	8.15	0.13
23	F	56	A-S	IIB	0	Normal	518	14.60	6.50	0.89
24	F	34	A	IIB	0	Hyperplasia	18	ND	9.90	0.24
25	M	26	A-S	IIB	> 18.6	Hyperplasia	2	ND	11.80	0.20
26	F	28	A	IIB	3.2	Hyperplasia	24	ND	7.10	0.19
27	F	23	A	IIB	325	Hyperplasia	13	ND	ND	0.22
MGa	F	41	A	IIA	3	Normal	48	ND	ND	ND
MGb	M	61	A	IIA	> 11.3	Thymoma	18	ND	ND	ND
MCCc	F	30	A	IIA	22.8	Hyperplasia	84	ND	ND	ND
MGd	M	20	A	IIB	21.8	Hyperplasia	6	ND	ND	ND
MGe	M	51	A	IIB	6.5	Thymoma	9	ND	ND	ND
MGf	F	24	A	IIB	0	Normal	12	ND	ND	ND

*MG treatment; A, Anticholinesterase; P, Plasmapheresis; S, Steroids; IVIG, Intravenous gamma globulin. ND, not done. **According to Osserman's classification. No. of β -, δ - and ϵ -subunit mRNAs are expressed as number of molecules per μ g of total RNA $\times 10^3$.

women and 9 men) and 10 sex- and age-matched healthy controls. Clinical and immunological data are summarized in Table I. The anti-nAChR antibody titer was determined by using human muscle nAChR complexed with ¹²⁵I-labeled α -bungarotoxin (¹²⁵I- α -Bgt) as antigen (28). The severity of MG was graded according to Osserman's classification (29). Patients with severity grade IIA have moderately impaired functional activity and weakness of limb or eye muscles. Patients with grade IIB MG have a marked functional impairment and marked weakness of the limb and bulbar musculature. At the time of muscle sampling, 11 patients had a mild form (IIA) and 16 had a severe form (IIB). Three groups of patients were distinguished according to the antibody titre: (a) titre > 100 nM; two patients, one mild case and one severe case; (b) intermediate titre (10–100 nM), 10 patients; 5 mild cases and 5 severe cases; (c) titre < 10 nM, 15 patients; 5 mild forms and 10 severe forms. In this latter group, seven patients had negative and eight had low serum autoantibody titers. Treatment (steroids, intravenous gamma globulin, and plasmapheresis) is indicated in Table I. Thymic hyperplasia, defined according to Levine and Rosai (30) as the presence of germinal centers, was present in 14 patients. One patient had a thymoma.

Muscle biopsy samples

Sternocleidomastoid muscle biopsy specimens (100–200 mg) were obtained during thymectomy from patients with MG and during chest surgery from patients without MG at the Marie Lannelongue Hospital (Le Plessis-Robinson, France). The controls had no clinical signs of muscle disease. Each muscle biopsy sample was tested for the presence of motor endplates by the modified Koelle–Friedenwald acetylcholinesterase cytochemical staining method (31). After 1 h of staining, small bundles containing 10–15 muscle fibers were carefully microdissected from the surrounding connective tissue. Only two samples contained motor endplates. Segments enriched in motor endplates or without junctional regions were cut from these two samples and placed in separate tubes before performing total RNA extraction and quantitative RT-PCR. All the quantitative analyses of muscle from MG patients and controls were thus performed on samples without junctional regions. All biopsy specimens (with or without endplates) were flash-frozen in liquid nitrogen and stored at –80°C in RNase-free conditions.

Cell cultures

The TE671 cell line was a gift from A. Bloc (Genève, Switzerland). Cells were grown to confluence in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (EUROBIO, les Ulis, France), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (all from GIBCO BRL). The cells were cultured in 50-

ml culture flasks at 37°C in humidified air containing 5% CO₂ and subcultured every 4 d when cells were confluent. Cells were harvested using trypsin (0.1%) and replated onto 35-mm Petri dishes (0.3 \times 10⁶ cells per plate) for toxin-binding studies and quantitative analysis of nAChR β -subunit mRNA levels.

Normal human embryonic myoblasts were grown and fused into myotubes as previously described (32).

Monoclonal antibodies and Fab fragments

All mAbs used in this study were derived from rats immunized with intact AChR isolated from human muscles (mAb192), Torpedo (mAb155 and mAb164) or *Electrophorus electricus* organs (mAb35). The mAbs were obtained from cloned hybridoma supernatants and were prepared as previously described (33). mAb35 cross-reacts with muscle AChRs from all tested species (mammalian, Torpedo, and chick) and binds to the MIR on the AChR α -subunit (34). mAb192 cross-reacts strongly with human and calf muscle AChRs. The mAb192 and mAb35 epitope includes the segment α 67–76 located in the extrasynaptic region of the α -subunit (35–36). mAb155 and mAb164 cross-reacts with Torpedo and mammalian intact AChR (37). Their epitope includes the segment α 373–380 located in the cytoplasmic part of the α -subunit (38). Because all these mAbs bind to the α -subunit that is identical between embryonic and adult receptor, they bind to both.

Fab35 fragments were obtained by papain treatment of mAb35 as previously described (33).

Evaluation of surface ¹²⁵I- α -Bgt-binding sites

To assess the effect of mAb (mAb155, mAb164, and mAb35) on the number of ¹²⁵I- α -Bgt-binding sites, 4 d after plating, TE671 cell culture medium was replaced by 1 ml of fresh culture medium containing an optimal concentration of mAb (usually 1/1,000) with or without 40 μ g/ml cycloheximide. Similarly, after a 5-d culture of fused normal myotubes, mAb and FAb35 fragments (1/400) were added to the fusion medium. Each condition was tested in triplicate. After 1, 3, 6, 9, 12, and 24 h of incubation at 37°C with the mAb, the medium was replaced by 1 ml of fresh medium containing 10 nM of ¹²⁵I- α -Bgt, and cultures were maintained for another 20 min at room temperature. Subsequently, the cells were washed four to five times with medium, and proteins were extracted in 1 ml of 0.1% Triton X-100 for 30 min. The protein extracts were counted in a gamma liquid scintillation counter (LKB-Wallac, Turku, Finland). Background radioactivity was estimated by incubating cells with 13 μ M unlabeled α -Bgt for 1 h before adding 10 nM ¹²⁵I- α -Bgt. Data from experiments in which non-specific binding was > 20% were discarded. Bound ¹²⁵I- α -Bgt was considered to represent surface nAChR.

Table II. Characteristics of the Oligonucleotides Used in the Study

Application	Name	Strand	Sequence	Hybridization temperature °C	Expected size bp
RT-PCR of β -subunit mRNA	β 1	–	5' GTGTCAGGGTCAGCGTTGGT 3'	63	578 and 484*
	β 2	+	TCCGGCGGATGATGAGGTAG	63	
RT-PCR of δ -subunit mRNA	δ 1	–	GCCCTCACACTCTCCAACCT	62	468 and 387*
	δ 2	+	TCTCCCACTCCCGTTCTCT	62	
RT-PCR of ϵ -subunit mRNA	ϵ 1	–	CTACTCGCTCATCATCCGC	58	357 and 310*
	ϵ 2	+	ACGTTCTCCTGGAGCTGCTG	58	
RT-PCR of γ -subunit mRNA	γ 1	–	CAGCACCGACCAGCCAAGAT	56	513
	γ 2	+	CCGAGGAGCCATCTGTAGC	56	
Probe used in γ Southern blot	PBGAM	–	CATCAGCAAGTACCTGACCT	42	—
RT-PCR of GAPDH mRNA	G1	–	ATCACCATCTTCCAGGAGCG	62	573
	G2	+	CCTGCTTCAACCCTTCTTG	62	

*Expected size of specific target and internal standard amplification products, respectively.

TE671 cells were incubated in 35-mm Petri dishes with mAb for 1, 3, 6, 9, 12, and 24 h, whereas normal human embryonic myotubes were incubated in 50-mm Petri dishes. Cells were washed in PBS, and total RNAs were extracted using the RNA+ kit protocol (BIO-PROBE, Paris, France). Total muscle RNAs were extracted from human biopsy samples with or without endplates as previously described (27).

The quality of the preparation was checked by electrophoresis of 5 μ g of total RNA on an alkaline denaturant agarose gel. A 28S/18S band intensity ratio of around two demonstrated the quality of the RNA.

Oligonucleotides used for amplification

Based on the known cDNA sequence of the nAChR β -, δ -, γ -, and ϵ -subunit genes (39), we designed a set of primers (sense and antisense) with the help of Oligo software (Med Probe, Oslo, Norway), a computer program used to optimize the annealing temperature and sequence specificity, and to limit self-complementarity. The oligonucleotide primers were purchased from Genset (Paris, France). The oligonucleotide primers and the expected size of the amplified products are presented in Table II, which also shows the oligonucleotide primer used as probe in nAChR γ -subunit Southern blotting. Primers G1 and G2 are used to amplify a 573-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

Quantitative RT-PCR

Because of tube-to-tube variability in amplification efficiency, an internal control was coamplified in the same tube. We used PCR-mutagenesis to construct an internal standard for each subunit, differing from the β , δ , and ϵ -subunit mRNA only by deletions of 94, 81, and 47 bp, respectively. This modification does not affect amplification efficiency, and allows the amplified product of the competitor to be distinguished from the transcripts of the nAChR subunit in electrophoresis gel. In addition, as we used the same primers to coamplify the nAChR subunit cDNA and the internal standard, the ratio of products remained constant throughout the exponential phase of amplification. The introduction of a known quantity of internal standard RNA in the RT assay serves first as an internal control mRNA for the RT reaction and, second, to generate a standard curve for quantifying the specific target of each nAChR subunit mRNA from experimental samples.

Construction of the β -, δ -, and ϵ -internal standard subunit. The mutant fragments of β -, δ -, and ϵ -subunit nAChR cDNA used as internal controls were constructed by using a mutagenesis assay similar to that published by Higuchi et al. (40), and based on oligonucleotide overlap extension and amplification by PCR from plasmids containing fragments or full-length sequences of nAChR β -, δ -, and ϵ -subunits cDNA. The human nAChR δ -subunit cDNA is ligated into the EcoRI site of plasmid pSP19; the human nAChR β -subunit cDNA is ligated into plasmid pGem4Z with EcoRI linkers; and the human nAChR ϵ -subunit cDNA fragment of \sim 450 bp is located between base numbers 570 and 1,028, and ligated with the SmaI site of plasmid pGem4Z (39).

The mutagenesis technique allowed us to delete nAChR β -, δ -, and ϵ -subunit cDNA fragments of 94, 81, and 47 bp, respectively. After construction, the human mutant β -, δ -, and ϵ -subunit nAChR gene fragments (respectively 515, 418, and 341 bp) were subcloned into the pCR-Script SK(+) cloning vector linearized at the SrfI site according to the Stratagene cloning kit protocol (Stratagene Inc., La Jolla, CA) under the control of the T3 RNA polymerase promoter.

Mutated RNAs were transcribed in vitro from 2 μ g of EcoRV-cut DNA plasmid, used as the template for transcription by T3 RNA polymerase (20 U; Promega, Madison, WI) in a 50- μ l reaction mixture containing 10 μ l of 5 \times transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM MgCl₂, 10 mM spermidine, and 50 mM NaCl), 10 mM DTT, 50 U of RNasin ribonuclease inhibitor (Promega), and 500 mM each rNTP. The resulting complementary RNA product was separated from the DNA template by phenol-chloroform extraction and

ethanol precipitation after 1 h of incubation at 37°C with 10 U of RNase-free DNase (Promega). The cRNA concentration was determined by measuring absorbance at 260 nm on a Gene QuantII spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and the samples were stored at -80°C . The size and homogeneity of the products were checked by agarose gel electrophoresis.

Quantitative analysis. Total muscle and TE671 RNAs were reverse transcribed in a total volume of 50 μ l, containing 2 μ g of total RNA, known amounts of internal standard, 5 μ l of 10 \times RT buffer (500 mM Tris-HCl, pH 8.3, 60 mM MgCl₂, 400 mM KCl, 40 mM dithiothreitol), 1.5 mM dNTPs, 39 U of RNasin (ribonuclease inhibitor; Promega), 50 pmoles of nAChR subunit-specific antisense primer (β 2, δ 2, or ϵ 2; Table II), and 2–5 U of avian myeloblastosis virus reverse transcriptase, incubated at 42°C for 60 min, and then quickly chilled on ice.

PCR was carried out in a total volume of 100 μ l, containing 10 μ l of RT reaction mix, 10 μ l of 10 \times PCR buffer (670 mM Tris HCl, pH 8.8, 160 mM (NH₄)₂ SO₄, 0.1% Tween 20), 200 μ M each dNTP, 50 pmoles of both nAChR subunit-specific sense and antisense primers (Table II), 1.5 mM MgCl₂, and 2.5 U of Taq polymerase (EURO-BIO). A trace amount of ³²P-labeled 5' primer was added (2–3 \times 10⁶ cpm/tube). The mixture was overlaid with mineral oil, and then amplified in a PHC3 thermal cycler (Techne, Cambridge, UK) for 36 cycles (39 cycles for the ϵ -subunit). The amplification profile involved denaturation at 94°C for 1 min, primer annealing at 56–63°C (depending on the primer set) for 1 min, and extension at 72°C for 2 min. The final elongation step lasted 10 min.

RT was performed as described above. To ensure that quantification took place in the exponential phase of PCR, RT products (i.e., known amount of nAChR subunit-specific internal control and muscle cDNAs) were amplified for 36 cycles (39 cycles for the ϵ -subunit) and aliquots of 7.5 μ l were taken at various times (22–39 cycles). PCR products were electrophoresed on 1.5% agarose gel, containing ethidium bromide, in Tris borate EDTA buffer. Gels were visualized under ultraviolet light and photographed. The appropriate bands were cut out of the gel and radioactivity was determined by scintillation counting. The amount of radioactivity (cpm) recovered from the excised gel bands was plotted against the number of PCR cycles. In all experiments, contaminants were checked for by amplification of a control sample, in which RT was omitted from the reaction mixture.

Northern blotting

Northern blot analysis was performed as previously described (41) using 35 μ g of total muscle RNA per lane. Briefly, total RNA was denatured in formaldehyde at 65°C and subjected to electrophoresis on 1% agarose gel in 16% formaldehyde. It was then transferred onto nylon membranes (Hybond N+; Amersham, Buckinghamshire, England). Prehybridization was performed at 65°C for 1 h and hybridization at 65°C for 2 h, using a rapid hybridization buffer kit (Amersham). Prehybridization, hybridization, washing, and dehybridization of the blots were carried out according to standard procedures. The myogenic-specific hybridization probes used for the myogenin and MRF4 factors were derived from human full-length cDNA clones ligated into the EcoRI site of pEMSV-scribe vector. These plasmids were a generous gift from Hans Henning Arnold (Institute of Biochemistry and Biotechnology, Technical University of Braunschweig, Braunschweig, Germany). All probes were labeled with α -³²P-labeled dCTP by using a random priming labeling kit (Rediprime; Amersham). The specific activity of the probes was 3–4 \times 10⁹ dpm/ μ g of DNA. Blots were autoradiographed with intensifying screens (Appligene, Illkirch-Graffenstaden, France) at -80°C for up to 48 h. Autoradiograms of the Northern blots were scanned with an IMSTAR computing densitometer and Image Quant software to obtain volume integration (total OD in the area of the signal). The results are averages of two blots. To normalize for RNA actually present on the filters, we used a 20-mer oligonucleotide complementary to part of the sequence of rat 18S ribosomal RNA (a generous gift from M. Heimburger, Centre National de la Recherche Scientifique ERS-566, Le

Plessis-Robinson, France) labeled with γ - ^{32}P -labeled dATP by T4 polynucleotide kinase (Promega).

Statistical analysis

Results are expressed as mean \pm SEM. The nonparametric Mann-Whitney test was used throughout.

Results

Expression of adult nAChR in MG muscle

We have previously demonstrated that nAChR α -subunit mRNA is increased in MG patients with severe disease but not in moderately affected patients, both compared to controls (27). To determine whether this upregulation also concerns the other nAChR subunits, and to determine whether the adult (ϵ -subunit) or fetal (γ -subunit) form of nAChR is affected, we performed RT-PCR with primers specific for the γ -subunit ($\gamma 1$ and $\gamma 2$) and ϵ -subunit ($\epsilon 1$ and $\epsilon 2$) on total muscle RNA from six MG patients (three severely and three moderately affected) and three controls. Total RNA extracted from the TE671 muscular cell line (mainly expressing the fetal form of nAChR; 42) was used as a positive control for γ -subunit expression. The γ -subunit was not amplified by PCR in MG or control muscle, contrary to the TE671 muscular cell line. Southern blotting of PCR amplification products obtained using the γ -specific primer labeled with γ - ^{32}P -labeled dATP as probe showed a faint band in a few samples (Fig. 1), indicating that the fetal subunit is hardly expressed in the human adult sternocleidomastoid muscle explored in this study. This obser-

vation is probably muscle type-specific, as extraocular muscle was shown to express the nAChR γ -subunit gene (43). The signals observed after amplification of the GAPDH housekeeping gene (573 bp) were nearly similar for all the muscular samples. RT-PCR experiments with primers specific for the nAChR ϵ -subunit yielded a 357-bp fragment amplified in all muscle samples (three normal samples; three severely and three moderately affected MG samples). These results demonstrated that sternocleidomastoid muscle from both MG patients and controls mainly expresses the adult nAChR genes.

β -, δ -, and ϵ -subunit transcript levels are increased in muscle from severely affected MG patients

To determine the level of nAChR mRNA subunit expression, we used quantitative RT-PCR as described in Methods. The analysis of β -, δ -, and ϵ -subunit mRNA from representative muscle samples is presented in Figs. 2, A, C, and E, respectively. As regards the β -subunit, two distinct amplification products of expected size were visible: the first (578 bp) corresponds to muscle cDNA of the nAChR β -subunit, and the second (484 bp) to internal standard cDNA. The amplification rate was exponential between 26 and 30 cycles and then declined drastically and approached a plateau (Fig. 2 A). With the δ -subunit, the first amplification product (468 bp) corresponds to muscle cDNA of the δ -subunit, and the second (387 bp) to internal standard cDNA. The amplification was exponential between 24 and 28 cycles (Fig. 2 C). With the ϵ -subunit, the first amplification product (357 bp) corresponds to muscle cDNA of the ϵ -subunit, and the second (310 bp) to internal

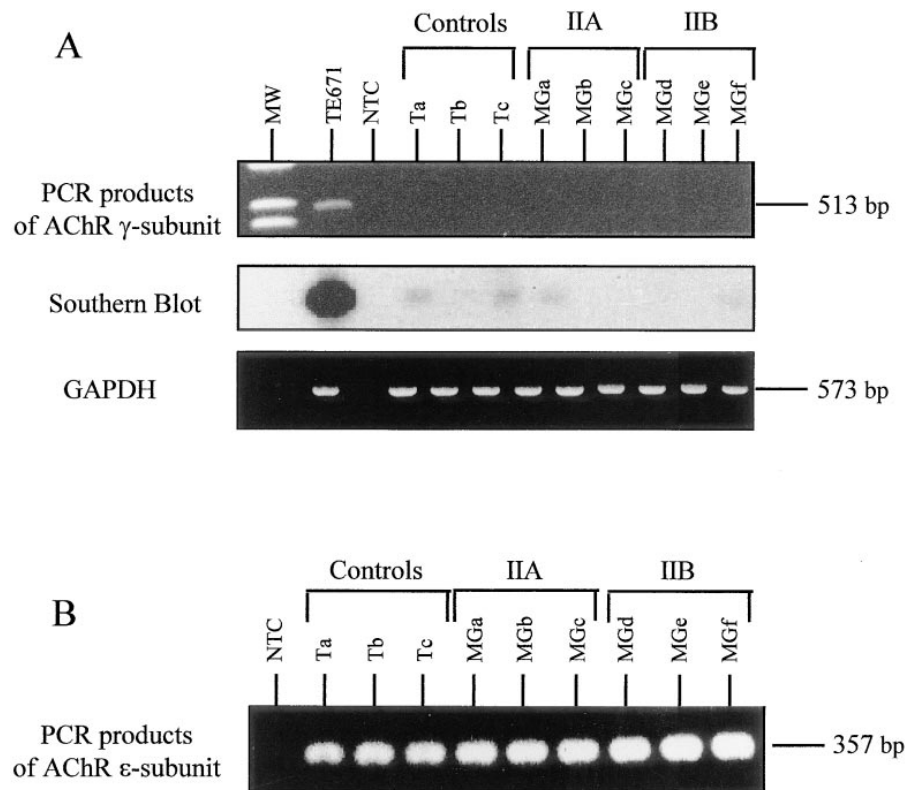


Figure 1. Expression of nAChR ϵ - and γ -subunits in human MG muscle. (A) Total RNA (2 μg) extracted from MG and control muscles was subjected to RT-PCR with primers $\gamma 1$ and $\gamma 2$ covering a 513-bp fragment of the γ -subunit after analysis of amplification products on 1.5% agarose electrophoresis gel. Lane 1 shows the molecular weight marker ($\phi\text{X}174$ HaeIII/ λ HindIII). Lane 2 corresponds to RT-PCR amplification of total RNA extracted from the TE671 cell line. Lane 3 is the nontemplate PCR control. Lanes 4–6 correspond to RT-PCR amplification products of total RNA extracted from healthy muscle (Ta, Tb, and Tc). Lanes 7–12 correspond to RT-PCR amplification products of total RNA extracted from muscle of moderately (MGa, MGb, and MGc, lanes 7–9) and severely affected MG patients (MGd, MGe, and MGf, lanes 10–12) (For patient clinical characteristics see Table I). These PCR products were submitted to Southern blot hybridization using the γ -specific primer labeled with γ - ^{32}P -labeled dATP. TE671 cells clearly express the γ -subunit, whereas muscle samples from controls and MG patients present a faint band after Southern hybridization. Amplification products of GAPDH housekeeping gene were also analyzed for all total RNA samples. A 573-bp

fragment was obtained in all the muscular samples studied. (B) Total RNA (2 μg) extracted from MG and control muscles was subjected to RT-PCR with primers $\epsilon 1$ and $\epsilon 2$ covering a 357-bp fragment of the ϵ -subunit after analysis of amplification products on 1.5% agarose gel. An amplified product is observed in all muscle samples.

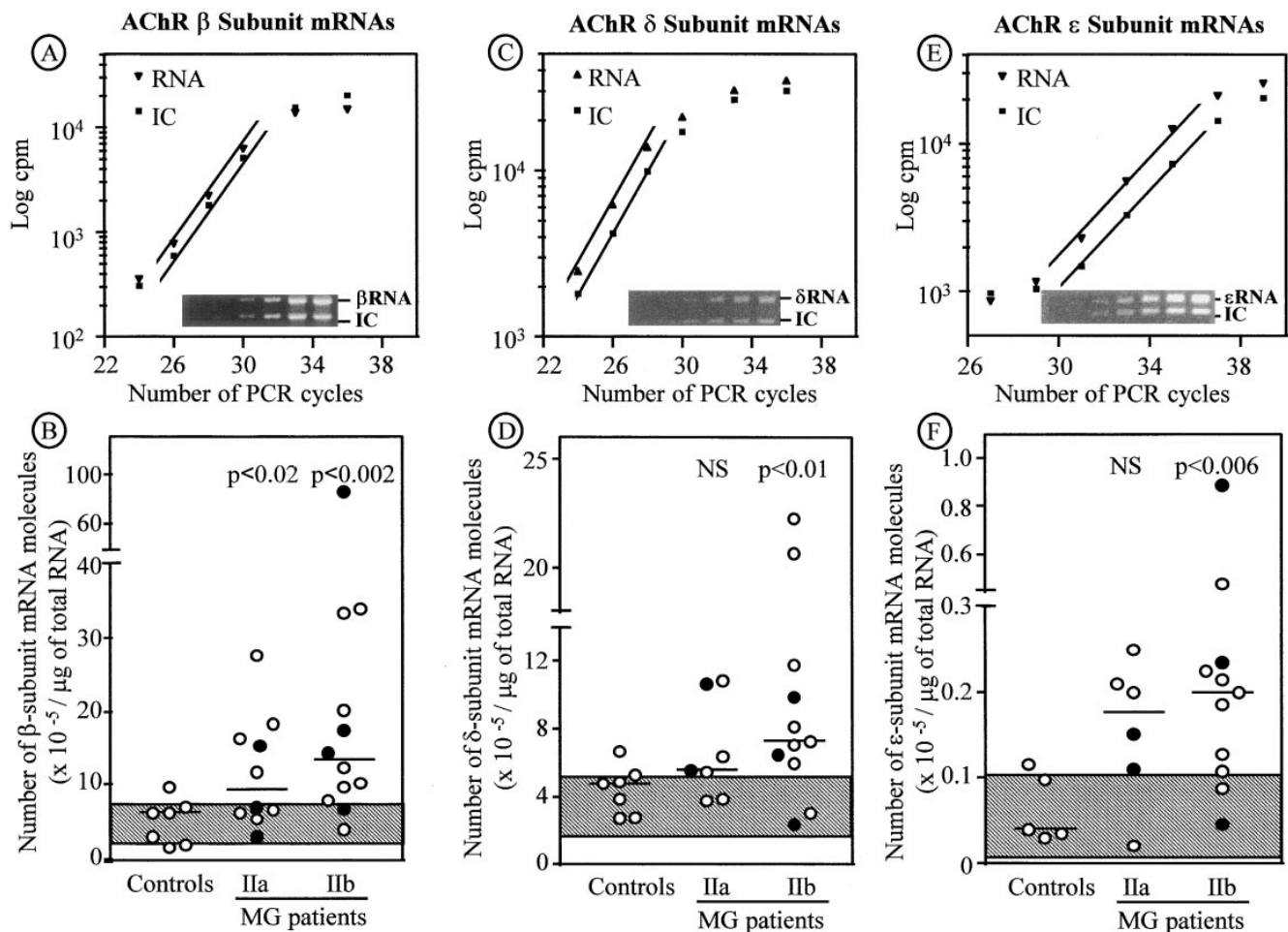


Figure 2. Expression of β -, δ -, and ϵ -subunit RNA in human MG muscle samples. (A, C, and E) Examples of quantitative PCR for the β -, δ -, and ϵ -subunit, respectively; 2 μg of total RNA and an appropriate amount of internal control (IC) were reverse transcribed and the resulting cDNA was amplified for various numbers of cycles in the presence of the two primers, one of which was labeled with γ - ^{32}P -labeled dATP. At various stages of PCR, aliquots were taken and loaded on 1.5% agarose electrophoresis gel. The two expected bands were excised from the gel and radioactivity was determined by scintillation counting. The number of cycles was then plotted against cpm (logarithmic scale). The linear part of the curve indicates that amplification was exponential and similar for the two products. As the amount of internal standard added to the reaction mix is known, we extrapolated from the linear part of the curve to determine the number of RNA molecules in the muscle sample. (B, D, and F) β -, δ -, and ϵ -subunit mRNA expression in 22 MG patients and 7 controls, in 18 MG patients and 7 controls, and in 17 MG patients and 5 controls, respectively. Results are expressed as the number of molecules coding for the β -, δ -, or ϵ -subunit $\times 10^5/\mu\text{g}$ of total RNA. Each point represents an individual value. The bar represents the median value. The striped area represents the control 95% confidence interval. Anti-nAChR seronegative and seropositive patients are represented by closed (●) and open circles (○), respectively. Regarding nAChR β -subunit mRNA expression, note the significant increase in both severely affected patients ($P < 0.002$) and moderately affected patients ($P < 0.02$) relative to controls. There was a significant increase in expression of nAChR δ - and nAChR ϵ -subunit mRNA in severely affected patients ($P < 0.01$ and $P < 0.006$, respectively) but not in moderately affected patients. Seronegative patients (●) showed a similar increase in β -, δ -, and ϵ -subunit mRNA levels, as in seropositive patients (○).

standard cDNA. The amplification was exponential between 30 and 36 cycles (Fig. 2 E). In all samples, the two curves were parallel within the exponential phase, indicating that the amplification efficiency was the same for the coamplified targets (Figs. 2, A, C, and E), and that the amount of nAChR (β , δ , or ϵ) subunit mRNA could be calculated during this exponential phase by extrapolation from the amount of internal standard added to the reaction mix.

This technique was applied to muscle biopsy samples from 22 MG patients (10 with a mild form and 12 with a severe form) and 7 controls for the β -subunit, from 18 MG patients (7 moderately affected and 11 severely affected) and 7 controls

for the δ -subunit, from 17 MG patients (6 moderately and 11 severely affected) and 5 controls for the ϵ -subunit.

The results of nAChR β -, δ -, and ϵ -subunit mRNA quantification, expressed as the total number of molecules of nAChR subunit mRNA per μg of total RNA, are presented in Figs. 2, B, D, and F, respectively. The mean number of nAChR β -subunit molecules was $5.3 \pm 1.1 \times 10^5/\mu\text{g}$ of total RNA in control samples, and $12 \pm 2.4 \times 10^5$ ($P < 0.02$ versus controls) and $21.5 \pm 6.4 \times 10^5/\mu\text{g}$ of total RNA ($P < 0.002$ versus controls) in MG patients with moderate and severe forms, respectively. 16 out of 22 patients (73%) had a significant increase in β -subunit mRNA levels compared to the control 95% confidence in-

terval (Fig. 2 B). The results of nAChR δ -subunit mRNA quantification are shown in Fig. 2 D. The mean number of nAChR δ -subunit molecules was $4.4 \pm 0.5 \times 10^5/\mu\text{g}$ of total RNA in control samples and $6.7 \pm 1.1 \times 10^5$ (not significantly different from controls) and $9.5 \pm 1.9 \times 10^5$ ($P < 0.01$ versus controls) in patients with moderate and severe forms, respectively. As with β -subunit transcripts, 12 out of 18 patients (67%) had a significant increase in δ -subunit mRNA levels compared to the control 95% confidence interval (Fig. 2 D). The results of nAChR ϵ -subunit mRNA quantification are presented in Fig. 2 F. The mean number of nAChR ϵ -subunit molecules was $0.06 \pm 0.01 \times 10^5/\mu\text{g}$ of total RNA in control samples and $0.15 \pm 0.03 \times 10^5$ (not significantly different from controls) and $0.25 \pm 0.07 \times 10^5$ ($P < 0.006$ versus controls) in patients with moderate and severe forms, respectively. 14 out of 17 patients (82%) had a significant increase in ϵ -subunit mRNA levels, compared to the control 95% confidence interval. Interestingly, muscle from seronegative patients showed an increase in β - as well as δ - or ϵ -subunit mRNA similar to that in seropositive patients. These data demonstrate that the increase in nAChR subunit mRNA observed in muscle from most MG patients does not correlate with the anti-nAChR autoantibody titer. The wide-ranging values obtained with MG muscles suggest the existence of factors that control nAChR mRNA level with variable efficiency among individuals.

Comparative mRNA expression of the different nAChR subunits: ϵ -subunit mRNA is low expressed compared to the other subunits

Comparative mRNA values obtained for each subunit by quantitative RT-PCR in MG patients and controls are presented in Fig. 3. α -Subunit mRNA levels presented here are partial results obtained in our previous study (27). α P3A+ and α P3A- isoforms correspond to the alternate nAChR α -RNA splicing of a 75-bp exon (P3A) (44). All the data are expressed as the mean number of mRNA molecules of each subunit per μg of total muscle RNA. In controls, the mean number of β -

and δ -subunit mRNA molecules was similar ($5.3 \pm 1.1 \times 10^5$ and $4.4 \pm 0.5 \times 10^5$ molecules/ μg of total RNA, respectively), whereas the mean expression of α -subunit mRNA was higher (29.9×10^5 molecules/ μg of total RNA corresponding to 14.4×10^5 and 15.4×10^5 molecules/ μg of total RNA for the P3A+ and P3A- isoforms, respectively). Interestingly, the mean ϵ -subunit mRNA level was much lower than that of the other subunits ($0.06 \pm 0.01 \times 10^5$ molecules/ μg of total RNA), raising the possibility that ϵ -subunit mRNA expression is limiting in control muscle (Fig. 3 A). The ratios relative to the ϵ -subunit were 497, 88, and 73 for α (P3A+ and P3A- isoforms)-, β -, and δ -subunit mRNA, respectively. The same pattern of relative expression between the ϵ -subunit and the other subunit mRNA levels was observed in muscle from MG patients with moderate disease and severe disease (Figs. 3, B and C). Finally, in both normal and MG muscles, ϵ -subunit mRNA is more weakly expressed compared to the other subunits.

To determine which nAChR subunit is most sensitive to regulation, the increase in mRNA expression for each subunit was calculated for all MG muscle samples as the ratio of the mRNA molecule number to the mean mRNA molecule number in control muscle (Table III). Median values indicated that the increase in ϵ -subunit mRNA was greater than that in the other subunit mRNAs, both in MG patients with a severe form of the disease (3.1-fold for ϵ -subunit versus 2.5-fold for β -subunit, 2.1-fold for α -subunit, and 1.7-fold for δ -subunit mRNA) and those with a moderate form (2.8-fold for ϵ -subunit versus 1.8-fold for β -subunit, 1.3-fold for δ -subunit, and 0.6-fold for α -subunit mRNA). The δ -subunit appeared to be less sensitive to regulation in both moderately and severely affected MG patients (Table III). In addition, the maximum increase in transcripts coding for each nAChR subunit was about 18-, 16-, and 13.9-fold for α -, β -, and ϵ -subunit mRNA, whereas the maximum increase in δ -subunit mRNA was only about fivefold. The δ -subunit thus appears to be the least sensitive to regulation.

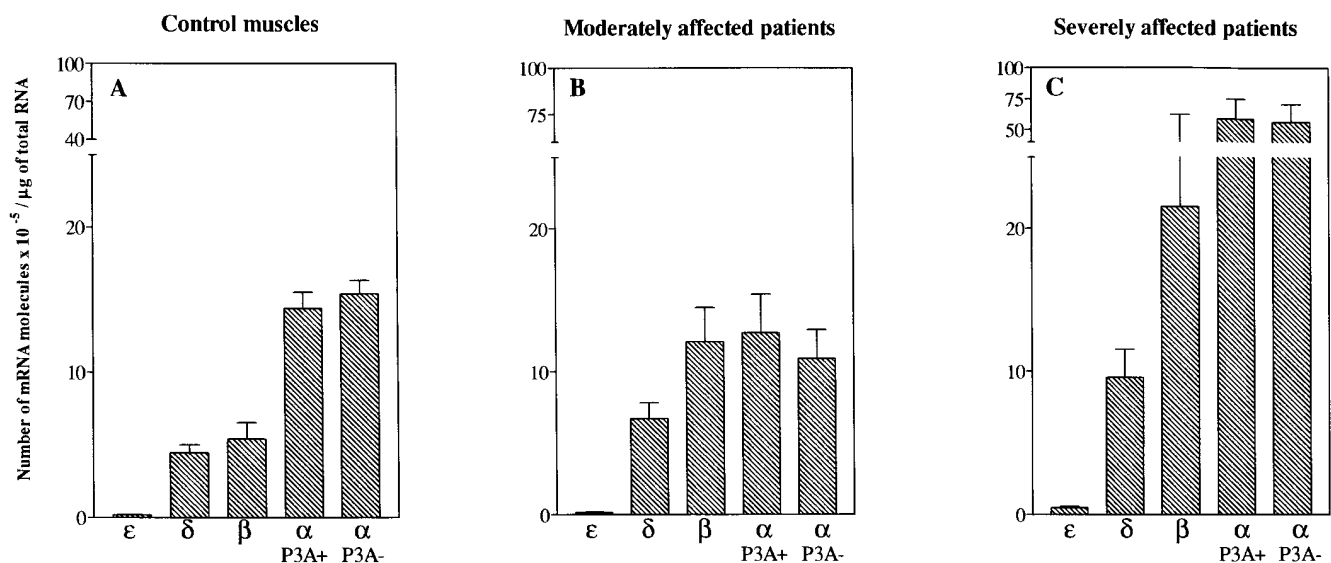


Figure 3. Comparative expression of nAChR α -, β -, δ - and ϵ -subunits in muscle from moderately (IIa) and severely (IIb) affected MG patients and controls. Results are expressed as the mean \pm SEM of the number of molecules coding for each subunit $\times 10^5/\mu\text{g}$ of total RNA. ϵ -Subunit mRNA expression was limiting in muscle from controls and MG patients.

Table III. Increase Factor for Each AChR Subunit mRNA in Muscle of MG Patients

	α -Subunit	β -Subunit	δ -Subunit	ϵ -Subunit
Patients with a mild form (IIA)	0.8 (0.6)*	2.3 (1.8)*	1.5 (1.3)*	2.4 (2.8)*
Patients with a severe form (IIB)	3.8 (2.1)*	4.0 (2.5)*	2.2 (1.7)*	4.0 (3.1)
Maximum increase	18.00	16.00	5.00	13.9
Patients with a significant increase**	20 out of 26	15 out of 22	12 out of 18	13 out of 17

The increase factors were calculated as the ratio of the mRNA molecule number obtained for the MG muscle sample to the mean mRNA molecule number in controls. *Ratios are expressed as the mean value; the numbers in brackets correspond to the median. **Higher than the control 95% confidence interval.

Expression of nAChR subunit mRNA in junctional enriched regions compared to nonjunctional regions

In situ hybridization studies with ϵ -subunit-specific antisense complementary RNAs suggested that ϵ -subunit mRNA is confined to the motor endplate in adult fibers (45). Muscle biopsy samples were thus routinely checked for the presence of motor endplates by using Koelle and Friedenwald's modified method of acetylcholinesterase staining after dissection of the muscle fibers and microscopic examination. In two patients, we studied in parallel the expression level of nAChR messengers in samples with and without endplates. The two samples containing endplates were divided into two parts (one containing the junctional compartment) and total RNA was extracted from

both for quantitative PCR. The results, expressed as the number of molecules coding for nAChR β -, δ -, and ϵ -subunit per μg of total RNA, are presented in Fig. 4. A difference of only about twofold was observed between muscle fiber regions enriched or not in motor endplates, ruling out the possibility that the absence of motor endplates influenced our observations. Thus, the large nAChR mRNA increase observed in muscle samples of MG patients (up to 16-fold for the β -subunit) could not be due to the distance of the sample from an endplate region.

These results are surprising at first sight, as nAChR is mainly expressed at the endplate, but could be explained by the fact that the endplate represents a very small surface area

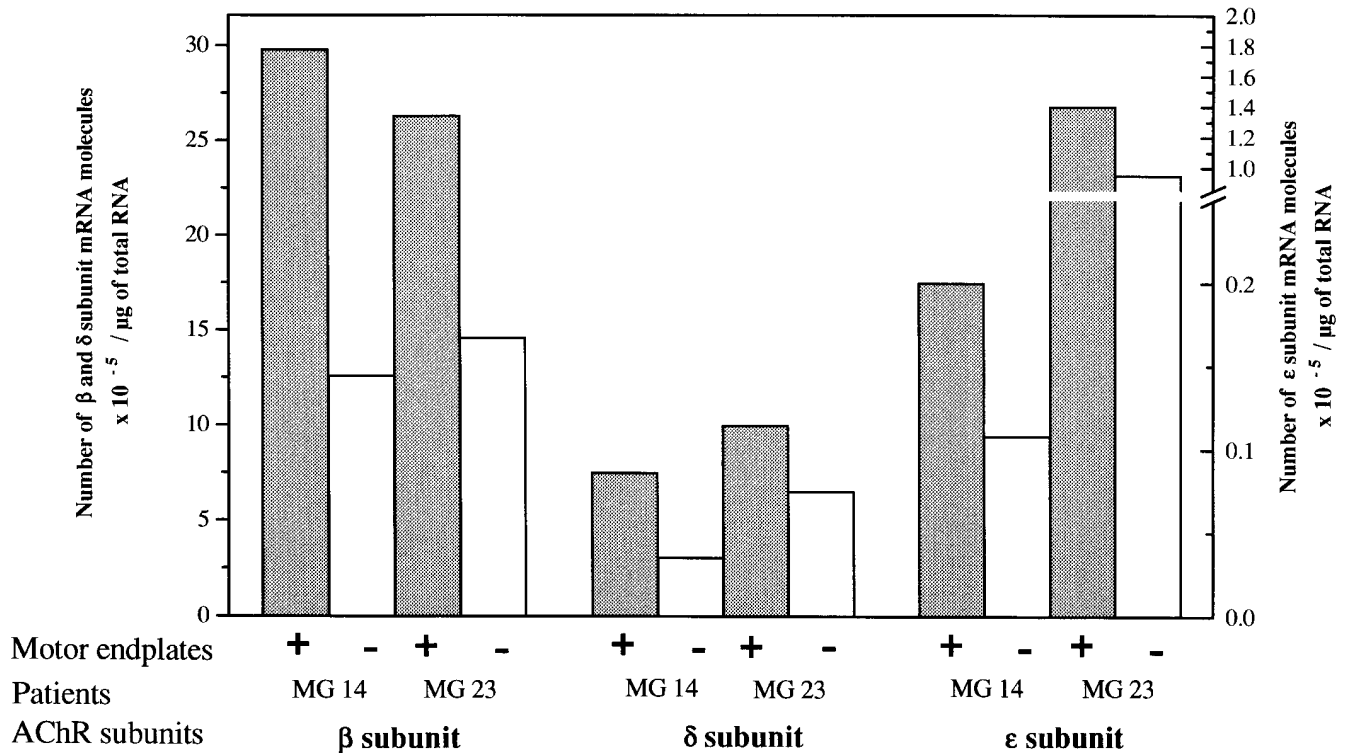


Figure 4. Differential expression of nAChR subunit mRNA in junctional and extrajunctional regions of human MG muscle. Two muscle samples obtained from a seronegative patient (case 23) and a seropositive patient (case 14) were shown to contain motor endplate regions by using the modified Koelle and Friedenwald method of acetylcholinesterase staining. These samples were divided into two parts, one including the motor endplates. Quantitative RT-PCR was performed on total RNA extracted from the two fragments of these MG muscle samples. For the three subunits analysed (β , δ , and ϵ), the number of mRNA molecules $\times 10^5 / \mu\text{g}$ of total RNA was moderately higher in junctional enriched regions compared to nonjunctional regions (about twofold).

of the muscle sample, meaning that synaptic mRNA is diluted in nonsynaptic mRNA. In addition, these results demonstrate that muscle samples without endplates are relevant materials to compare nAChR mRNA expression between MG and healthy subjects.

Expression of myogenic factors in MG muscle biopsies compared to controls

To analyze the level of myogenic factor transcripts in muscle biopsies, Northern blot of total muscle RNA from eight MG patients (patient number 5, 10, 2, 9, 11, 23, 19, and 12) and four control muscle samples were hybridized to ³²P-labeled cDNA Myf probes. In these experiments (Fig. 5), a 1.8-Kb myogenin transcript and a 1.5-Kb MRF4 transcript were detected in all muscle samples, whereas MyoD1 and Myf5 transcripts were not detected in any of the samples tested (data not shown). The rhabdomyosarcoma TE671 cell line, used as control, contained only the 1.8-Kb myogenin transcript, suggesting differential expression of myogenic factor genes in human muscle and rhabdomyosarcomas (46). A semi-quantitative analysis was then performed by using densitometry with the 18S oligonucleotide probe as a control for the total amount of RNA loaded on the filter. The mean ratio of myogenin and MRF4 to 18S was 28.1 ± 7.7 and 22.9 ± 2.0 , respectively, in the controls and 19.2 ± 3.3 and 15.6 ± 2.9 , respectively, in MG patients, not significantly different from controls. These data suggest that the myogenic factors myogenin and MRF4 are expressed in human muscle with similar mRNA levels in MG patients and controls.

A compensatory mechanism is induced after nAChR loss triggered by anti-MIR monoclonal antibody in TE671 muscle cells and in human myotube cultures

To explore whether the increase in nAChR messengers was due to anti-nAChR antibodies, we analyzed the effect of an anti-AChR monoclonal antibody (mAb35) on both surface nAChR expression (by ¹²⁵I- α -Bgt-binding studies) and nAChR α -, δ -, and β -subunit mRNA levels (by quantitative PCR assays) in the TE671 muscle cell line, which expresses nAChR (42). We used mAb35, directed against the extracellular MIR

region of nAChR, to induce surface nAChR internalization by antigenic modulation in muscle cells (33). The expression of surface nAChR results from difference between the AChR internalization rate and the insertion rate of new nAChRs in the plasma membrane.

Kinetic studies. The normal nAChR internalization rate was determined by analysis of nAChR expression in TE671 cells treated with cycloheximide, which blocks protein synthesis (Fig. 6 A). The half-life of the nAChRs was ~ 10 h, which is similar to that observed for nAChRs in cultured normal human embryonic myotubes (47). No modification of the nAChR half-life was observed in TE671 cells incubated with mAbs directed against the cytoplasmic nAChR epitope (mAb164). To check that the cycloheximide concentration (40 μ g/ml) was sufficient to block protein synthesis, we performed ³⁵S-labeled methionine protein studies (Fig. 6 B). After 1 h, $> 70\%$ of protein synthesis was abolished in TE671 cells. At 24 h, the total remaining protein synthesis in cycloheximide-treated cells was $> 20\%$. These data confirmed that the cycloheximide concentration used in this study was sufficient to block protein synthesis.

The kinetics of toxin binding and mRNA levels in TE671 cells are presented in Figs. 6, C and D, respectively. A clear reduction in the nAChR half-life was observed in mAb35-treated TE671 cells (3 h; Fig. 6 C) compared to untreated or mAb164-treated cells (10 h; Fig. 6 A). Between 1 and 6 h, the nAChRs loss in cycloheximide-treated TE671 cells incubated with mAb35 was similar to that measured in cycloheximide-untreated TE671 cells incubated with mAb35 (Fig. 6 C), suggesting the absence of early compensatory nAChRs synthesis. Interestingly, between 1 and 3 h of mAb35 incubation without cycloheximide (Fig. 6 D), β -subunit messenger levels fell by $\sim 35\%$ relative to controls, suggesting that a regulatory mechanism compensated for the loss of nAChR by using the messenger pool present in the cells.

Between 6 and 9 h, in the absence of cycloheximide, the number of nAChR ¹²⁵I- α -Bgt-binding sites increased and reached a plateau of $\sim 50\%$ of control until 12 h, whereas in the presence of cycloheximide it continued to decrease, sug-

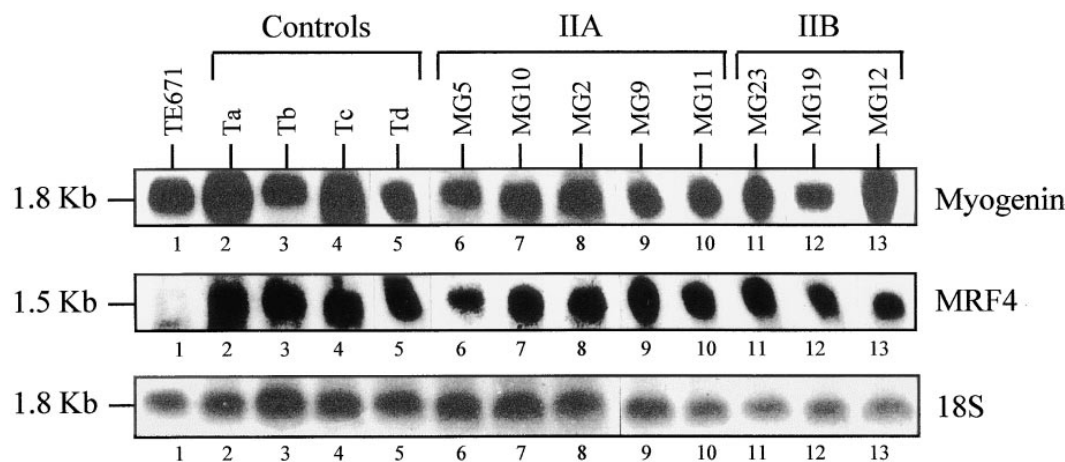


Figure 5. Expression of members of the myogenic gene family in MG and control muscle biopsies and in the TE671 rhabdomyosarcoma cell line. Northern blots of both muscle RNA (35 μ g per lane) and TE671 RNA (20 μ g per lane) were hybridized to myogenin and MRF4 probes in conditions which prevent non-specific cross-hybridization. The same blot was reused in the order indicated after removal of the hybridization signals of

the previous probe. Myogenin and MRF4 transcripts were clearly expressed in both healthy muscles (*Ta*, *Tb*, *Tc*, and *Td*) and MG muscle samples (*MG5*, *MG10*, *MG2*, *MG9*, *MG11*, *MG23*, *MG19*, and *MG12*) (see Table I for clinical characteristics of the patients). Only myogenin transcripts were expressed in TE671 cell line. Loading of RNA samples was assessed by staining RNA gels with ethidium bromide and by hybridization of Northern blots to an 18S oligonucleotide.

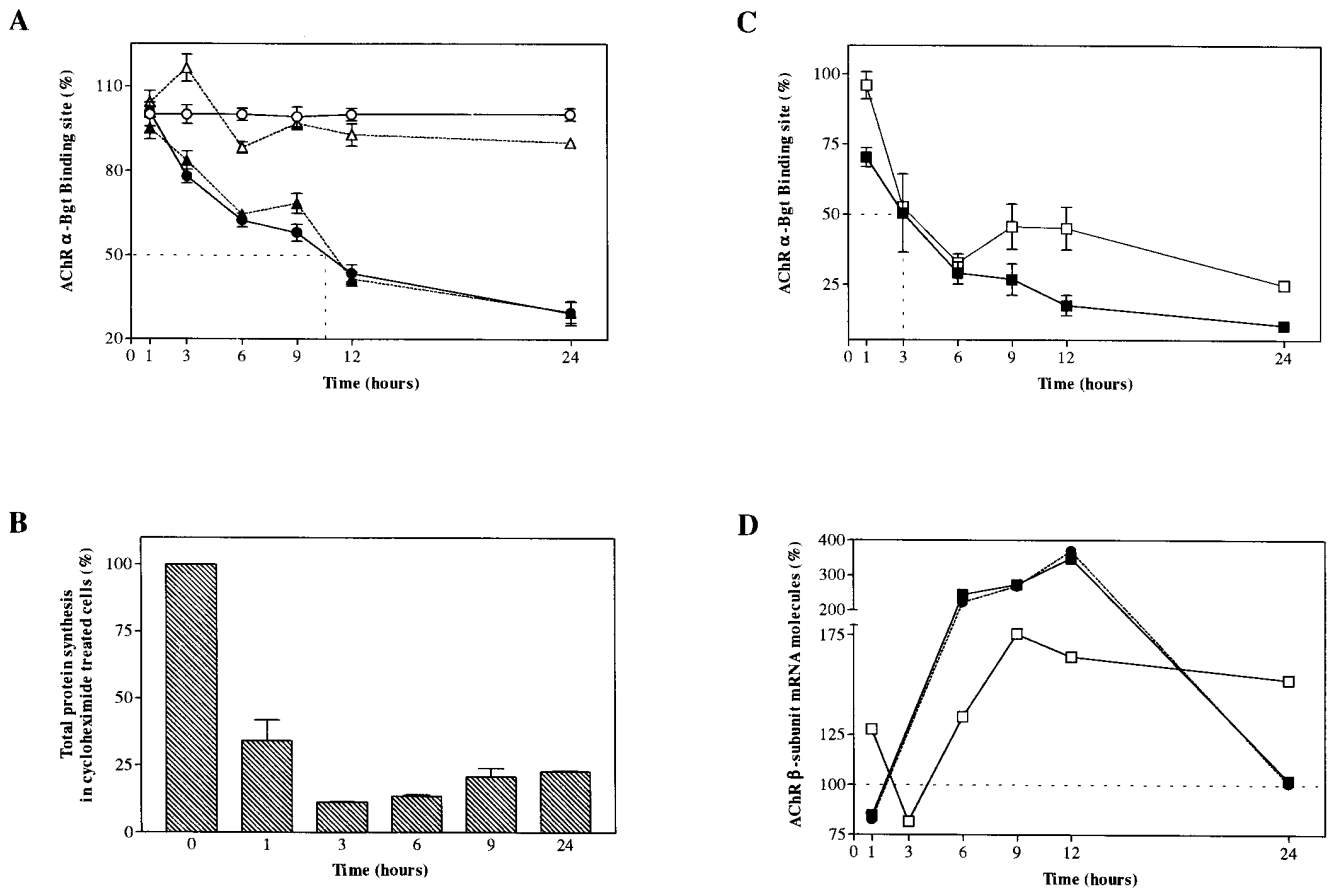


Figure 6. Effect of mAb35 exposure on nAChR β -subunit mRNA levels and nAChR expression in TE671 cells. (A) The number of nAChR α -Bgt-binding sites was determined in TE671 cells treated with (●) or without (○) 40 μ g/ml cycloheximide. The half-life of the nAChR is around 10 h. No modification of the nAChR half-life was observed in mAb164-incubated TE671 cells treated with (σ) or without cycloheximide (δ). The number of nAChR α -Bgt-binding sites corresponds to the mean percentage \pm SEM of triplicate values compared to values obtained in untreated cells. (B) Control for total protein synthesis blockade by 40 μ g/ml cycloheximide. (C) The number of nAChR α -Bgt-binding sites was determined in TE671 cells treated with mAb35 in the presence (■) or absence of cycloheximide (□). The number of nAChR α -Bgt-binding sites corresponds to the mean percentage \pm SEM of triplicate values compared to values obtained in untreated cells. (D) The number of β -subunit mRNA molecules was determined in TE671 cells treated with mAb35 in the presence (■) or absence of cycloheximide (□) and in control cells (without mAb35) treated with cycloheximide (λ). The number of β -subunit mRNA molecules is expressed as a percentage of nAChR β -subunit mRNA molecules in untreated control cells. Similar results were obtained in three independent experiments.

gesting that the increase in nAChR 125 I- α -Bgt-binding sites corresponded to nAChR neosynthesis rather than membrane expression of the presynthesized receptor pool. A clear increase in nAChR β -subunit mRNA was observed between 3 and 9 h, suggesting that the increase in nAChR 125 I- α -Bgt-binding sites was due to nAChR neosynthesis and demonstrating that messenger upregulation is triggered after \sim 55% loss of nAChR.

Upregulation of AChR chains is associated with internalization. To investigate the mechanism of AChR subunit upregulation, we compared the effect of anti-AChR antibodies to that of α -Bgt (1 μ g/ml). Incubation of TE671 cells for 16 h with mAb35 increased β - and δ -subunit messenger levels, whereas α -Bgt had no effect (Fig. 7 A), suggesting the involvement of nAChR internalization in mRNA expression. The specificity of the antibody effect on nAChR expression was checked by incubating TE671 cells for 16 h with two monoclonal antibodies (mAb164 and mAb155) directed against the intracellular

part of nAChR (Fig. 7 A). Interestingly, in the same conditions, no modification of α -subunit messenger levels was observed (Fig. 7 A), possibly due to the strong expression of a partially mature form of the nAChR α -subunit found in the cytoplasm of TE671 cells (48).

To check that the antibody-mediated nAChR mRNA upregulation was not specific to TE671 cell line, similar experiments were performed on cultured normal human embryonic myotubes (Fig. 7 B). We observed a similar increase in nAChR β -subunit mRNA expression with mAb192 and mAb35, two mAbs directed against the extracytoplasmic part of the nAChR, whereas mAb155 and mAb164 (directed to the cytoplasmic domains of nAChR) had no effect. A clear reduction in nAChR 125 I- α -Bgt-binding sites was observed in human myotubes incubated with mAb35 and mAb192, as found in TE671 cells. Interestingly, both α -Bgt and FAb35 fragments, which do not induce antigenic modulation of nAChR (33), had no effect. Taken together, these observations strengthen the

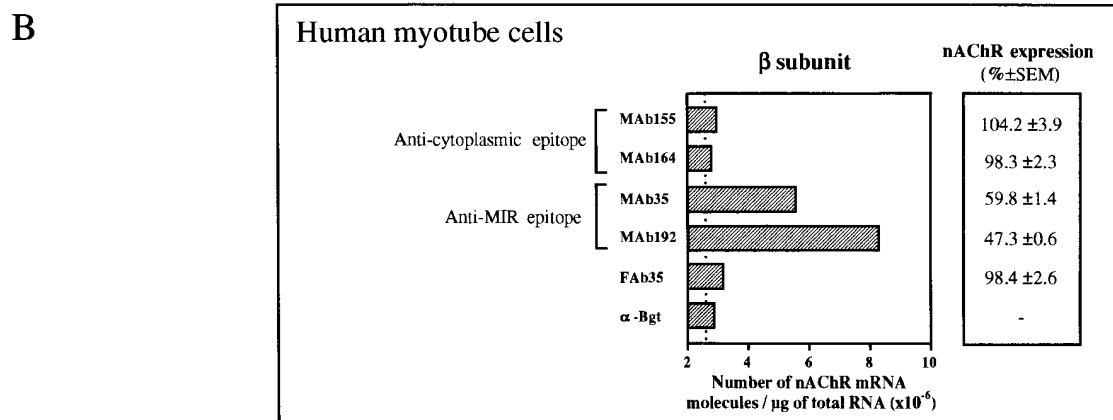
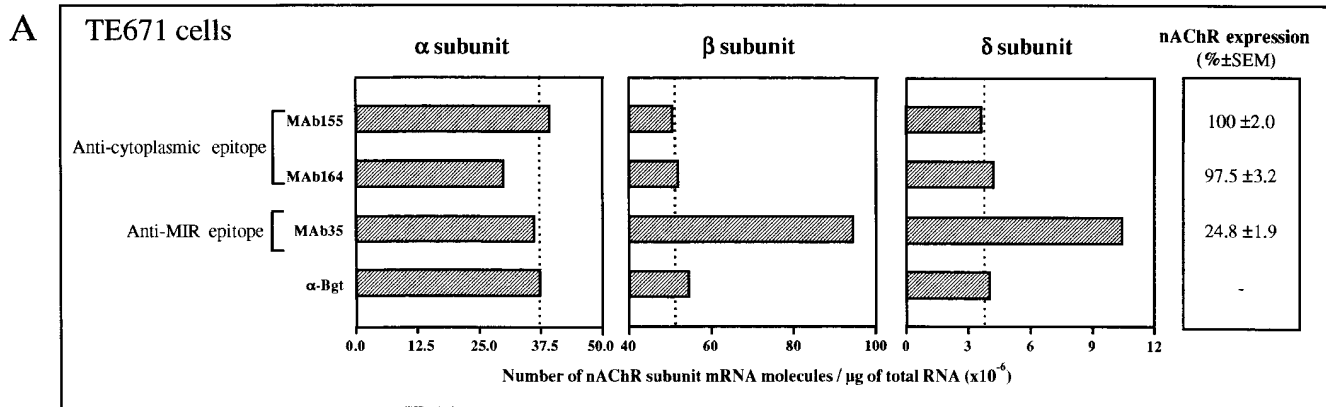


Figure 7. Effect of monoclonal anti-nAChR antibodies and α -Bgt on nAChR mRNA levels. (A) Analysis of TE671 cells. Results are expressed as the number of AChR mRNA molecules/ μ g total RNA $\times 10^6$ for the α -, β -, and δ - subunits. RNA was extracted from α -Bgt, mAb164/mAb155 (anti-cytoplasmic epitopes), and mAb35 (anti-MIR)-treated cells. A clear increase in β - and δ - subunit mRNA levels was observed in mAb35-treated cells, whereas α -Bgt and mAb164/mAb155 had no effect on messenger levels. The loss of nAChR is indicated in each set of conditions. (B) Analysis of human myotube cultures. Results are expressed as the number of AChR β -subunit mRNA molecules/ μ g total RNA $\times 10^6$. RNA was extracted from α -Bgt, FAb35, mAb164/mAb155 (anti-cytoplasmic epitopes) and mAb35/mAb192 (anti-MIR)-treated cells. A clear increase in β -subunit mRNA levels was observed in mAb35 and mAb192-treated cells, whereas α -Bgt, FAb35, and mAb164/mAb155 had no effect on messenger levels. The loss of nAChR is indicated for each set of conditions.

hypothesis that internalization of nAChR triggers nAChR mRNA upregulation.

Discussion

We investigated, in patients with MG, whether changes in the level of the affected autoantigen (nAChR) alter the regulation of nAChR subunit gene transcription. (a) We found an increase in nAChR β -, δ -, and ϵ -subunit transcripts, similar to that in the α -subunit that we had previously described (27), in sternocleidomastoid muscle from MG patients, which correlated with the severity of the disease; γ -subunit transcripts were almost undetectable. (b) We identified a compensatory mechanism triggered by nAChR internalization after a certain threshold loss of receptor by analyzing the effects of monoclonal anti-AChR antibodies in the TE671 muscle cell line. (c) We show that the α -, β -, and δ -subunits are expressed to the same order ($\sim 10^5$ molecules/ μ g of total RNA), whereas expression of the ϵ -subunit was much lower ($\sim 10^3$ molecules/ μ g of total RNA) in both healthy and MG muscles. (d) Finally, we

detected no difference in the expression of myogenic factor (myogenin and MRF4) mRNA between MG patients and controls.

Evidence of ϵ but not γ expression in human sternocleidomastoid muscles. The absence of γ -subunit mRNA expression in human MG is in agreement with results obtained on leg muscle of the experimental autoimmune myasthenia gravis (EAMG) rat, mouse, and rabbit models, which show no γ -subunit expression throughout the muscle fiber (49, 50). However, RNase protection assays on other human muscles (innervated intercostal and gastrocnemius normal muscles) showed γ -subunit messenger expression (51). In addition, nAChR γ -subunit gene expression has also been reported in extraocular muscle (43). These conflicting findings are probably due to differences in muscle type. Indeed, it is well established that the expression of the muscle gene repertoire is different in terms of ultrastructural proteins and regulatory factors between muscular fibers or muscle types (52). Thus, it is possible that γ -subunit messengers are expressed at very low levels in sternocleidomastoid muscle (faint bands were observed by Southern blot

of PCR products) compared to intercostal and gastrocnemius muscles. The γ -subunit mRNA expression could be incompletely shut-off and may depend on the muscle origin.

In situ hybridization studies with ϵ -subunit-specific antisense-complementary RNAs have suggested that ϵ -subunit mRNA is confined to the motor endplate in adult fibers (45). In this study, we observed low but significant expression of ϵ -subunit mRNA in extrasynaptic regions of muscle fibers. This was probably due to the high sensitivity of the PCR-based technique that we used, relative to in situ hybridization. This low expression of ϵ -subunit mRNA in the extrasynaptic region could be explained by the involvement of diffusible neurotrophic factors such as acetylcholine receptor-inducing activity (ARIA), neuregulin, which promotes an increase in ϵ -subunit mRNA levels (53) on binding to ErbB2/ErbB3 or ErbB2/ErbB4 receptors (54). ErbB2 and, to a lesser extent, ErbB4 receptors are expressed throughout the muscle fiber (55) and may be involved in extrasynaptic ϵ -subunit expression. The upregulation of ϵ -subunit expression observed in MG patients could be due to an increase in ARIA release from the basal lamina (56) and derived from proteolytic processing after postsynaptic destruction by autoantibodies. Nevertheless, the expression of ϵ -subunit mRNA was very low compared to the other subunits (100-fold) and it remains to be determined whether new adult nAChR synthesis occurs in the extrajunctional area. Finally, we cannot exclude the possibility that ϵ -subunit messengers are more efficiently translated than those of the other subunits. In addition, it is possible that nAChR mRNA levels exceed those required to account for the observed receptor synthesis rates. It is thus conceivable that the excess of ϵ -subunit mRNA is smaller than that of the other subunits.

Regulation of nAChR genes in human MG muscle: comparison with the denervation and EAMG animal models. Our data showing an upregulation of nAChR β - and δ -subunit transcripts in MG compared to normal muscles confirm the increase in α -subunit mRNA previously described in human MG muscles (27), and are also in accordance with data obtained in denervation models and EAMG muscles, where a clear increase in nAChR messengers was observed by in situ in all the muscle fibers (45, 50). However, several arguments indicate that the mechanism involved in the regulation of nAChR expression in human MG differs from that underlying the denervation process: (a) MG is not generally associated with denervation or morphological modifications of motoneuron endings (57) and acetylcholine release at muscle endplates from MG patients is increased (58). (b) We observed no increase in myogenic transcript levels in MG muscles compared to healthy muscles, whereas myogenin, MyoD, and MRF4 mRNA levels are upregulated in denervated models (50, 59). (c) The expression of γ -subunit mRNA was almost undetectable in both healthy and MG muscles, whereas a strong increase in γ -subunit mRNA levels is observed in denervation models (60). Similarly, we observed ϵ -subunit mRNA upregulation in MG muscles, whereas minor modifications of ϵ -subunit mRNA levels are observed in denervation models (61). Although a decrease in miniature endplate potential amplitude is generally observed in MG muscles, as in denervation models, our observation is compatible with data showing that blockade of electrical activity does not necessarily modify γ -subunit gene expression. Indeed, γ -subunit mRNA expression is virtually unmodified in tetrodotoxin and α -Bgt-blocked motoneuron

models (61). Alternatively, the observed changes in myasthenic muscle could be explained by adaptation to long-term muscle inactivity. Indeed, Adams et al. found an adaptive response in long-term denervated rat muscles, in which nAChR γ -subunit mRNA levels are decreased, whereas nAChR ϵ -subunit mRNA levels are increased (62). However, this is unlikely, as no expression of the fetal subunit was observed in EAMG rodent muscles after early exploration (50).

nAChR messenger upregulation in MG muscles: evidence for a transcriptional compensatory mechanism. The increase in nAChR messengers observed in MG muscles correlated with the severity of the disease. As the loss of nAChR was greater in the severely affected group of patients (63, 64), the most likely explanation is the existence of a compensatory mechanism for the loss of surface nAChR due to autoimmune attack. This increase in nAChR subunit mRNA could result from an enhanced transcription rate of the subunit gene or from alterations in mRNA processing or degradation. Evidence that regulation occurs at least in part at the level of gene transcription was obtained in our previous investigations, in which we found an increase in α -subunit mRNA precursor levels by means of PCR with intronic probes (27). Nevertheless, our data obtained with TE671 cells incubated with monoclonal antibodies (mAb35) suggest that the mechanism of regulation triggered by surface nAChR loss also occurs at the level of gene translation or nAChR assembly. Indeed, mAb35 induced a 225% increase in β -subunit mRNA levels, but only a 30% increase in nAChR content. These observations indicate a post-translational regulatory mechanism and are in accordance with the observation that tetrodotoxin treatment of cultured chick myotubes induced a 13-fold increase in α -subunit mRNA levels, but only a twofold increase in nAChR content (65).

To explore the putative impact of anti-nAChR antibodies on the triggering of the transcriptional compensatory mechanism in human seropositive MG muscles, we developed a kinetic study based on muscle cell culture and incubation with an anti-MIR anti-nAChR monoclonal antibody (mAb35) that was effective in antigenic modulation experiments with TE671 cells (33). We found an upregulation of β - and δ -subunit messengers, which was maximum after 6 h (~ 2.5 -fold) for β -subunit mRNA, when nAChR loss reached $\sim 55\%$; this increase in mRNA levels was followed by a 30% increase in nAChR expression. These findings suggest that anti-nAChR antibodies can induce a transcriptional compensatory mechanism for the loss of receptor, but only above a certain threshold loss of nAChR. Analysis of cycloheximide effects in these experiments also suggested that the increase in surface receptor expression was due to the use of neosynthesized RNA messengers and not a presynthesized nAChR pool. This "safety margin" is consistent with animals studies that showed a large reduction in nAChR content associated with a large increase in nAChR mRNA levels, whereas a small reduction in nAChR content was not compensated for by an increase in nAChR mRNA (41, 49). In addition, the safety margin value we observed in TE671 cells ($\sim 55\%$) is similar to that observed in muscle of MG patients. The loss of nAChR in mild disease is $\sim 40\%$ compared to $\sim 60\%$ loss in severe forms (63, 64).

This compensatory mechanism is very effective in muscle cells, as the loss of nAChR was $\sim 65\%$ at 6 h, and this was reduced to only 30% at 12 h.

Putative mechanisms triggering nAChR messenger upregulation in MG muscles. The increase in nAChR subunit mes-

senger levels we observed in MG muscles was similar in patients with and without detectable anti-nAChR antibodies. Thus, a nonanti-nAChR antibody-dependent regulatory mechanism seems to be involved in seronegative patients. It has been established that altered nAChR function can modulate the electrical activity of muscle cells and could in turn increase nAChR gene expression via activation of second messengers such as Ca^{2+} (66). As the impairment of electrical activity is clear in seronegative patients (67), the most likely hypothesis is that a muscle activity pathway is involved in the compensatory mechanism induced in seronegative MG muscles. However, we found that the monoclonal antibody directed against the MIR region part of the nAChR induced a loss of nAChR expression at the TE671 cell surface and human myotube membrane, which was then compensated for by an upregulation of nAChR messengers. Similarly, sera from MG patients containing anti-nAChR antibodies induce both a loss of nAChR expression and an upregulation of nAChR messengers (our manuscript in preparation). As there is no spontaneous electrical activity in our culture system, these findings suggest an original anti-nAChR antibody-dependent regulatory mechanism in addition to the muscular activity pathway that could be involved in seropositive MG muscles. It is not clear, however, how nAChR loss at the cell surface triggers such a compensatory mechanism, as nAChR is not directly coupled to second messenger generation.

We showed that blockade of nAChR function by α -Bgt, which does not induce nAChR internalization in TE671 cells, did not induce an increase in the nAChR β - and δ -subunit mRNA level. Similarly, incubation of human myotube with Fab35 fragments that do not induce antigenic modulation, did not induce an increase in nAChR messengers. The loss of surface nAChR after anti-nAChR antibody exposure is essentially due to antigenic modulation requiring antibody-dependent cross-linking of membrane-bound receptors (33), resulting in nAChR internalization and in an increased degradation rate as well as to complement mediated membrane lysis (68). Thus, our findings suggest the involvement of nAChR internalization and/or membrane lysis in the upregulation of mRNA expression. As regards the regulatory signaling pathway triggered by nAChR antibody mediated loss, there are two main possibilities:

(a) The synaptic junction forms a superstructure characterized by an extraordinarily dense clustering of receptors for nerve-derived signals and colocalized with nAChR. Some receptors, such as ErbB3/ErbB2 for heregulin (ARIA), which display intrinsic tyrosine kinase activity, have been studied. These receptors accumulate densely at the neuromuscular junction and are involved in nAChR expression (55). Heregulin stimulate nAChR gene expression in muscle via an independent electrical activity-signaling pathway (69). Thus, in the case of seropositive MG patients, internalization of nAChR at the muscle endplate and/or postsynaptic membrane lysis could locally modify the structure of the postsynaptic membrane and the ultrastructural conformation of these receptors, which could then activate transducer signals and a tyrosine kinase-signaling pathway, which could in turn trigger transcription of nAChR gene subunits.

(b) Disruption of actin networks by cytochalasin has been reported to upregulate nAChR expression in TE671 cells, suggesting the involvement of the cytoskeleton in membrane receptor regulation (70). Some physical interactions between

nAChR and other cytoskeletal proteins have been reported, namely interactions between nAChR β -subunit and rapsyn involved in phosphorylation of the receptor and its clustering (71). Recently, an association between nAChR, src, and fyn (a member of the tyrosine kinase src family) was also observed (72). Evidence for an interaction between the src SH3 domain and an actin-associated protein of 110 KD has been described (73). Thus, cointernalization of nAChR and the attached protein and/or postsynaptic membrane lysis could modulate the stability of the neighboring proteins and/or modulate the activity of tyrosine kinase-coupled proteins, which could in turn trigger upregulation of nAChR messengers.

Taken together, our data show that the mechanism regulating the expression of nAChR genes in muscle from patients with MG differs from that of denervation models. We demonstrate that blockade of electrical activity is not the only regulatory pathway involved in nAChR mRNA regulation, as monoclonal anti-nAChR antibodies also upregulated nAChR mRNA expression. The compensatory mechanism triggered by nAChR loss demonstrates that the target muscle is not passively attacked by the autoantibodies; this mechanism of compensation could be one factor contributing to the mildness of the disease.

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