The conformation of the main immunogenic region on the α-subunit of muscle acetylcholine receptor is affected by neighboring receptor subunits

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Abstract Myasthenia gravis (MG) is caused by autoantibodies to the acetylcholine receptor (AChR). Experiments with fetal ($\alpha_2\beta\gamma\delta$) and adult ($\alpha_2\beta\varepsilon\delta$) AChR and with recombinant subunit dimers showed that some monoclonal antibodies (mAbs) against the main immunogenic region (MIR), located on the α-subunit of the AChR, bind better to fetal AChR and to αγ subunit dimer than to adult AChR and αε dimer and equally to both αβ and αδ. However, other anti-MIR mAbs prefer adult AChR and αε dimer, bind well to αβ but weakly to αδ. These results suggest that the MIR conformation is affected by the neighboring γ/ε- and δ-subunits and may contribute to understanding the antibody specificities in MG.

Key words: Acetylcholine receptor; Monoclonal antibody; Main immunogenic region; Epitope conformation

1. Introduction

The human muscle acetylcholine receptor (AChR) is a ligand-gated cation channel located on the postsynaptic membrane of the neuromuscular junction. Its role is to transmit the electric signal through the chemical synapse from the motor nerve terminal to the end-plate. It is a pentameric glycoprotein that exists in the form of two subtypes, fetal and adult. The fetal subtype (AChR-γ), with a stoichiometry $\alpha_2\beta\gamma\delta$, is found in fetal and denervated muscle, whereas the adult subtype (AChR-ε), with the composition $\alpha_2\beta\varepsilon\delta$, is present at the end-plate in innervated adult muscle [1,2].

In myasthenia gravis (MG) and its experimental animal model, autoantibodies target the muscle AChR [2–4]. Various studies have shown that the majority of anti-AChR antibodies in MG sera and experimental animals are directed against a specific region on each α-subunit of the AChR, termed the main immunogenic region (MIR) [5–7].

The MIR, a small extracellular conformationally dependent loop, contains a group of overlapping epitopes located at the crest of each α-subunit within an AChR pentamer [3,7,8]. Although the MIR's core epitope is believed to be α67-76 [9], there are other segments of the α-subunit, still unidentified, which possibly contribute to it [10,11]. The MIR is not involved directly in channel function but plays a crucial pathogenetic role, since the anti-MIR antibodies are highly pathogenic in experimental animals and responsible for most of the antigenic modulation activity in sera from MG patients [12–16].

In this study, we investigated whether the conformation of the MIR is affected by the subunits neighboring the two α-subunits. Such a possibility could have serious immunological implications since there are MG sera which differentiate between embryonic and adult AChRs and bind better to one or the other molecule [17–19]. The latter has been attributed to the presence of antibodies that bind selectively to the γ- or the ε-subunit of the AChR. The generation of γ-subunit specific phage display fragments antibody binding derived from myasthenic patients with serum antibodies specific for embryonic AChR suggests that anti-γ-subunit antibodies do indeed exist in these sera [20]. However, these findings do not exclude the possibility that differential binding results from conformational alterations imposed on epitopes on α-, β- or σ-subunits by the γ- to ε-subunit (fetal to adult) exchange. The latter might be important for understanding neonatal MG and arthrogryposis congenital syndrome passively transferred by MG mothers' antibodies [21,22].

2. Materials and methods

AChR-γ and AChR-ε were extracted from the TE671 and CN21 cell lines, respectively, as described earlier [11,19]. The subunit dimers (αβ, αγ, αδ and αε) as well as the lone α-subunit were generated by transient expression of the respective AChR subunit cDNAs in human embryonic kidney (HEK) fibroblast 293 cells. HEK293 cells were plated out at 5×10^5/well on 6-well tissue culture plates. cDNAs encoding each of the human AChR subunits were cloned into the expression vector pcDNA3 (Invitrogen). Each well was transfected with 4 μg of the respective AChR subunit cDNA combination using calcium phosphate precipitation, as described previously [23]. Cells were incubated for 48 h following transfection and then AChR subunit combinations were extracted in 25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1.25% Triton X-100, 1 mM PMSF. Subunit dimerization in the extracts was verified by the effective precipitation of [^{125}I]α-bungarotoxin-labeled αβ, αγ and αδ dimers using specific anti-β-(No. 73), anti-γ-(No. 67) and anti-δ-(No. 137) subunit monoclonal antibodies (mAbs), respectively. The mAbs used were derived earlier from rats immunized with intact AChR from several species; some of their characteristics are shown in Table 1.

Radioimmunoassays (RIAs) with AChR-γ and AChR-ε as well as with the subunit dimers αβ, αγ, αδ and αε were carried out in principle as described [24], with some modifications. Briefly, 200 μl of phosphate-buffered saline, pH 7.5 (PBS)–0.5% Triton X-100 containing [^{125}I]α-bungarotoxin-labeled AChR or recombinant subunit dimers, sufficient to give approximately 5000 and 1000 cpm, respectively, in the immunoprecipitates of the positive controls, were incubated with 10 μl of mAb diluted in PBS–0.2% bovine serum albumin containing 0.1 μl of normal rat serum, as carrier, for 2.5 h at 4°C.

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Abbreviations: AChR, acetylcholine receptor; mAb, monoclonal antibody; MG, myasthenia gravis; MIR, main immunogenic region; RIA, radioimmunoassay

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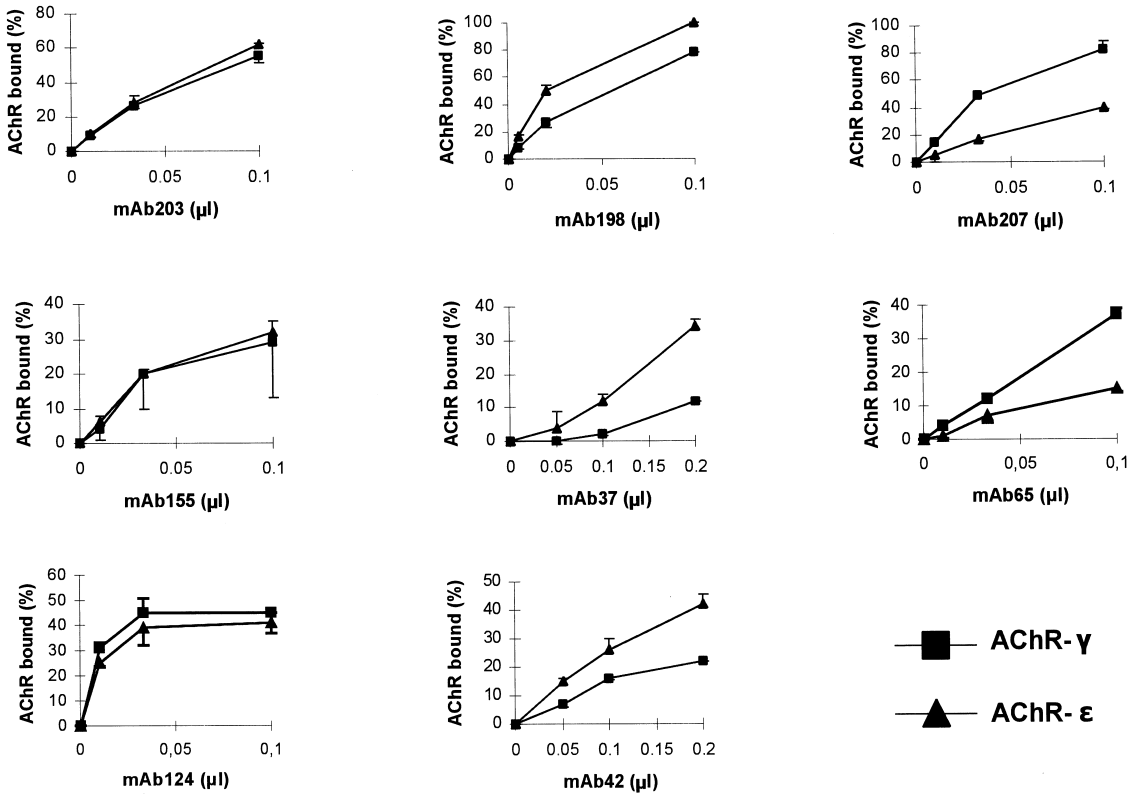

Fig. 1. Comparison of mAb binding to AChR-γ (squares) and AChR-ε (triangles) using RIAs. mAbs in the first column (No. 203, 124 and 155) show no discrimination between the two AChR subtypes. mAbs in the second column (No. 198, 42 and 37) bind better to AChR-ε (differences statistically significant, $P < 0.05$, except in mAb 37 for 0.05 μl), whereas mAbs in the last column (No. 65 and 207) bind better to AChR-γ (differences statistically significant, $P < 0.05$). Values shown are mean ± S.D., $n = 3$.

Immune complexes were quantitatively precipitated by 10 μl of rabbit anti-rat immunoglobulin serum. After 1.5 h at 4°C, the precipitates were washed three times with PBS–0.5% Triton X-100 and bound radioactivity was counted. Background radioactivity was determined in the presence of the non-binding mAb 25, while an excess of mAb 192 was used as a positive control.

Statistical analysis was performed by the use of Student's *t* test.

3. Results

The mAbs for this study were selected to have epitopes on subunits common to both AChR subtypes, i.e. MIR and non-MIR epitopes on the α-subunit and epitopes on the β-subunit. The binding of these mAbs to AChR-γ, AChR-ε as well as to αγ, αε, αβ and αδ subunit dimers was estimated based on the amount of [^{125}I]α-bungarotoxin-labeled subtype or subunit dimer combination immunoprecipitated (AChR bound in Fig. 1 and subunit dimer bound in Fig. 2). Similar experiments made using the α-subunit only showed that in the absence of other subunits, low cpm of the [^{125}I]α-bungarotoxin-labeled α-subunit could be precipitated (data not shown).

3.1. Binding of mAbs to fetal and adult AChRs

The left column in Fig. 1 shows that the mAbs 155 and 124 whose epitopes lie in the cytoplasmic domain of the α- and β-subunits, respectively, bind equally well to both AChR subtypes. Similar results are seen for the anti-MIR mAb 203. mAb 203 is representative of another six anti-MIR mAbs (No. 35, 190, 192, 195, 202 and 204), which also bound equally well to both AChR-γ and AChR-ε. In all cases, the difference in the binding of the above mAbs to the two AChRs did not exceed the 11% of the total binding. However, five anti-MIR mAbs (No. 198, 42, 37, 65 and 207) were found able to discriminate between the two forms of the AChR. Specifically, mAbs in the middle column of Fig. 1 (No. 198, 37 and 42) bound better to AChR-ε than AChR-γ, whereas

Table 1
Characteristics of anti-AChR mAbs used

mAb No.	Subunit, region and sequence specificity[a]	AChR and αε/αγ preference[b]
25	negative control	
35	α67-76, MIR	
190	α, MIR	
192	α, MIR	
195	α, MIR	
202	α, MIR	
203	α67-76, MIR	
204	α, MIR	
37	α67-76, MIR	AChR-ε/αε
42	α67-76, MIR	AChR-ε/NT[c]
198	α67-76, MIR	AChR-ε/αε
65	α, MIR	AChR-γ/αγ
207	α, MIR	AChR-γ/αγ
155	α371-378, cytoplasmic	
73	β, near MIR	
124	β354-359, cytoplasmic	
67	γ, near MIR	
137	δ374-391	

[a][9,11,25–27].
[b]From the present results.
[c]Not tested.

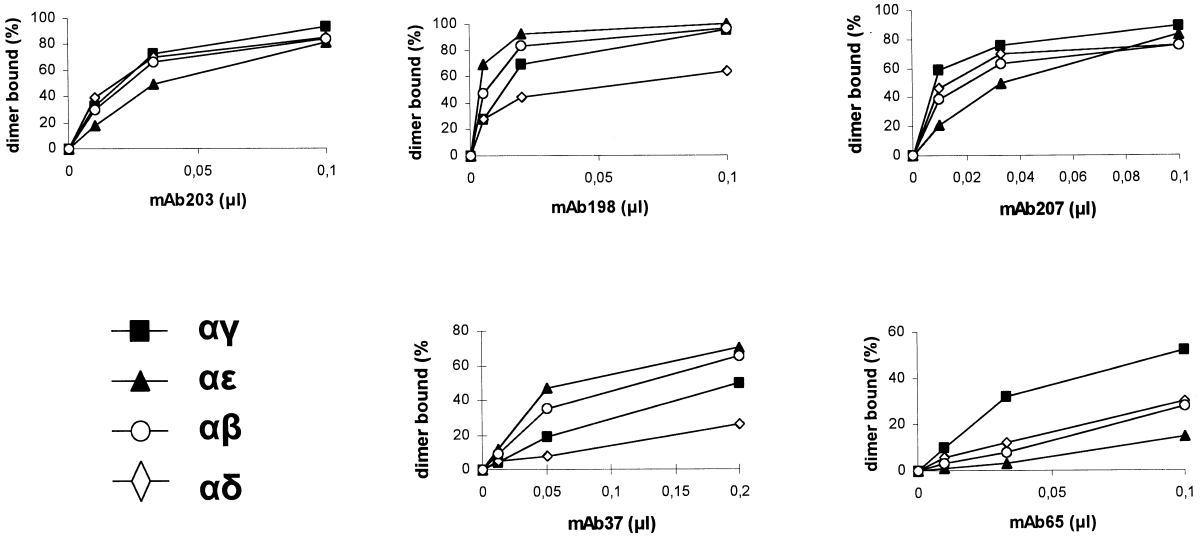

Fig. 2. Comparison of mAb binding to αγ (squares), αε (triangles), αβ (circles) and αδ (diamonds) subunit dimers using RIAs. mAbs in the second column (No. 198 and 37) bind better to αε dimer in comparison with αγ but although they bind well to αβ dimer, they bind more weakly to αδ. mAbs in the last column (No. 65 and 207) bind better to αγ than to αε and equally to αβ and αδ dimers. Values shown are mean, $n = 3$.

the converse was true for mAbs 65 and 207 (right column of Fig. 1).

3.2. Binding of mAbs to αγ, αε, αβ and αδ subunit dimers

To further confirm the observed differences and to study the possible effect of other AChR subunits besides the γ- or ε-subunits, the mAbs presented above were tested using recombinant αγ, αε, αβ and αδ subunit dimers. Fig. 2 shows that mAbs 198 and 37 bound better to αε subunit dimer than to αγ, whereas mAbs 65 and 207 bound better to αγ subunit dimer (differences statistically significant, $P < 0.05$), in agreement with their preference to AChR-ε and AChR-γ, respectively, as presented in Fig. 1. Although the differences in the mAb binding to αγ and αε subunit dimers were rather moderate at high antibody concentration, especially for mAbs 198 and 207, they were quite significant for small mAb concentrations.

As far as αβ and αδ subunit dimers are concerned, Fig. 2 also shows that mAb 203, which bound equally to both AChR subtypes, bound similarly to αβ and αδ subunit dimers. Likewise, mAbs 207 and 65, which preferred AChR-γ and αγ dimer, bound equally to both αβ and αδ subunit dimers, though less than to αγ. However, mAbs 198 and 37, which bound better to AChR-ε and αε dimer, bound considerably weakly to αδ subunit dimer although they bound well to αβ. The possibility that the good binding of all anti-MIR mAbs to αβ dimer was due to free α-subunit in the extract was excluded by the use of an anti-β-subunit mAb (No. 73) which could precipitate practically all (96%) of the [^{125}I]α-bungarotoxin-labeled α-subunit.

4. Discussion

The MIR of mammalian muscle and Torpedo electric organ AChRs is known to be located on the α-subunits of the AChR [3,9,11,25]. However, we present here anti-MIR mAbs that have differential binding and thus discriminate between the fetal and the adult AChR's MIR. This latter suggests either that the conformation of the MIR in the two subtypes is not identical or that the neighboring subunits participate in the MIR epitopes. Since the difference between fetal and adult AChR is the substitution of an ε-subunit for a γ, then it is these subunits that either contribute to the MIR epitope or are responsible for the altered MIR conformation. The recombinant subunit dimer experiments verified the above conclusion and further suggested that in addition to the γ/ε-subunits, the δ-subunit, too, affects the MIR conformation. On the other hand, although these experiments do not reveal any effect of the β-subunit on the conformation of the epitopes for the mAbs used in this study, a possible involvement of the β-subunit in determining the MIR conformation cannot be excluded.

The observation that the β-subunit does not affect the binding of the anti-MIR mAbs used in this study, yet binding to αβ dimer is relatively high, strongly suggests that non-α-subunits do not directly participate in the MIR and therefore the variations in mAb binding to the other dimers are due to conformational alterations imposed to the MIR by the γ-, ε- or δ-subunits.

Table 1 shows that the two groups of anti-MIR mAbs (AChR-ε/αε and AChR-γ/αγ preferring mAbs) differ in their specificity for the α67-76 synthetic peptide: the AChR-ε/αε preferring mAbs bind to the α67-76 peptide, whereas the AChR-γ/αγ preferring mAbs do not bind to any synthetic peptide. This differentiation may imply small differences in the actual epitopes of the two groups of mAbs. On the other hand, those mAbs which do not discriminate between the two AChR subtypes bind either to α67-76 or to unidentified epitopes.

4.1. Immunological implications

Several MG sera are found to bind preferentially to the fetal or the adult AChR subtype [17–19] and this differentiation seems to play an important role in the development of MG symptoms in the adults, where antibodies binding preferentially to adult AChR should be the most potent, as well as

in the development of neonatal MG and anti-AChR-mediated congenital arthrogryposis [21,22], in which antibodies binding preferentially to the embryonic form of the AChR should be most potent. The results we present imply that differences in the binding of MG patient's anti-AChR antibodies to fetal or adult AChR may not only be due to differential binding to the γ- or the ε-subunits but also to an altered conformation of the MIR (and possibly of other epitopes) on the two AChR subtypes.

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