Decrease in acetylcholine-receptor content of human myotube cultures mediated by monoclonal antibodies to α, β and γ subunits

Socrates J. Tzartos and Anna Starzinski-Powitz*

Laboratory of Biochemistry, Hellenic Pasteur Institute, 127, Ave. Vas. Sofias, Athens 115 21, Greece and **Institut für Genetik der Universität zu Köln, 5000 Köln 41, FRG*

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One of the two main causes of acetylcholine-receptor loss in myasthenia gravis is antigenic modulation, i.e. accelerated internalization and degradation rate by antibody-crosslinking. This phenomenon has been studied only in animal tissues. Therefore, we tested antigenic modulation of the acetylcholine receptor on human embryonic myotubes in culture. Several monoclonal antibodies to the α, β and γ subunits of the receptor reduced its concentration, in some cases down to one-third of the control. Some of these antibodies only form complexes of one antibody with two receptor molecules; consequently such small complexes are sufficient to accelerate internalization of the human acetylcholine receptor. This technique might be proved valuable for clinical screening of sera from myasthenic patients.

Acetylcholine receptor *Antigenic modulation* *Monoclonal antibody* *(Human myotube)* *Antibody specificity* *Receptor internalization*

1. INTRODUCTION

Nicotinic acetylcholine receptor is composed of 5 subunits (α_2, β, γ, δ) with a total molecular mass of ~250000. The 2 α subunits carry the acetylcholine binding sites which regulate the opening of the cation channel (reviewed in [1]). Myasthenia gravis is characterized by muscular weakness and fatigability largely due to autoantibody-mediated loss of AChR [2–5]. Direct blockage of AChR function by the antibodies probably also contributes to the disease [6–8]. Purified AChR or anti-AChR antibodies injected into animals cause experimental autoimmune myasthena gravis [2–5,9]. Two-thirds of the anti-AChR antibodies in immunized rats and myasthenic patients bind to the main immunogenic region which is located on the α subunit of the receptor [5,10–13]. mAbs to the MIR injected into rats cause experimental myasthenia ([5,10] and Tzartos and Lindstrom, in preparation).

Antibody-mediated loss of AChR is caused (i) by complement-mediated destruction of the postsynaptic membrane and (ii) by antigenic modulation, i.e. accelerated internalization and subsequently a degradation rate due to crosslinking of the receptors by the antibodies [3,4]. Myasthenic patients' sera [7,15], immune animal sera [14] and mAbs [5,9,16,17] have been shown to cause antigenic modulation of AChR in animal cell and organ cultures. Among the tested mAbs of known region specificity only the anti-MIR ones caused antigenic modulation of animal AChR [16,17]. Animal tissues used to evaluate the potencies of myasthenic patients' sera present the serious disadvantage of testing only the crossreactive with

Abbreviations: AChR, nicotinic acetylcholine receptor; MIR, main immunogenic region; mAb, monoclonal antibody; ^{125}I-toxin, ^{125}I-labeled α-bungarotoxin; fusion medium, Dulbecco's modified Eagles medium plus 4% heat-inactivated normal human serum

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the human AChR antibody fraction [18]. Therefore use of human muscle cell cultures was needed.

Here we report a net decrease in the overall AChR content after binding of mAbs to the MIR as well as to other regions using established embryonic muscle cell lines.

2. MATERIALS AND METHODS

2.1. *mAbs*

All mAbs were derived from rats immunized either with fish electric organ or mammalian muscle AChR or its SDS-denatured subunits [11,13,20]. mAb preparations were obtained from culture media concentrated and dialysed against fresh Dulbecco's modified Eagle's medium.

2.2. *Human muscle cells*

Embryonic human muscle cell cultures were established in principle according to [19]. Cells were cultured in Waymouth's medium supplemented with 20% fetal calf serum in 5% CO_2. At subconfluency, they were split 1:3 and/or to 96-well microtiter plates. All the experiments were performed in the microwells using cells of passage nos 10–17. When cells in microwells reached confluency their medium was substituted with 100 μl fusion medium. Surface AChR was evaluated by incubation with 40 μl fusion medium containing 10 nM ^{125}I-toxin of specific activity ~100 μCi/μg.

2.3. *Measurement of the effect of mAbs on the overall AChR content and on its internalization rate*

After ~48 h culture in fusion medium, this medium was replaced with 40 μl fresh fusion medium supplemented with varying amounts of mAbs. For each mAb concentration 4–6 wells were cultured at 37°C and 4–6 wells at 15°C. After 19 h, 10 μl fusion medium containing 50 nM ^{125}I-toxin (plus 5 μM unlabeled toxin for estimation of the background) were added to each microwell and cultures maintained for an additional hour at the same temperature. In some experiments 20 μg/ml cyclohexamide (Sigma) was added together with the mAbs; ^{125}I-toxin was added after 6 h and incubated for an additional hour. Subsequently, the wells were washed 5 times and the cell-bound radioactivity was released by 0.5% SDS and counted. Remaining surface AChRs were estimated by the equation:

$$\% \text{ remaining AChR} = 100 \times \frac{\text{fraction of detected toxin sites at } 37°C}{\text{fraction of detected toxin sites at } 15°C}$$

$$= 100 \times \frac{\Delta\text{cpm} \cdot 37(\text{mAb})/\Delta\text{cpm} \cdot 37}{\Delta\text{cpm} \cdot 15(\text{mAb})/\Delta\text{cpm} \cdot 15}$$

where Δcpm·37(mAb) and Δcpm·37 were the specific cell-bound radioactivity after incubation at 37°C with or without the mAb, respectively, whereas Δcpm·15(mAb) and Δcpm·15 were the corresponding values for incubation at 15°C.

3. RESULTS

3.1. *Human embryonic AChR on myotube cultures*

We first performed binding studies on the AChR of these cells and established conditions required in subsequent experiments. Significant amounts of ^{125}I-toxin binding sites appeared after ~24 h in fusion medium with the peak binding at day 3 (~3.5 fmol toxin binding sites per cm^2). When excess toxin was removed, about 80% of bound ^{125}I-toxin was already dissociated in the medium within 3 h of incubation. Therefore, myotubes were first incubated with the mAbs and subsequently with ^{125}I-toxin.

To evaluate the normal AChR internalization rate, in absence of mAb, the myotube cultures were incubated with cyclohexamide to block protein synthesis [21]. Fig.1 shows that concentrations of cyclohexamide in the range 5–100 μg/ml blocked AChR synthesis and apparently did not affect its internalization rate. Thus half-life of this AChR was ~8.5 h.

3.2. *mAbs directed against α and γ subunits affected the overall AChR content of the human myotube cell surface*

Properties of all mAbs tested are shown in table 1. They all crossreact with soluble human AChR (except of control mAb 26) and bind to various sites and subunits. Fig.2 shows the effect of mAbs on the overall AChR content after incubation for 20 h. Binding of anti-MIR mAb 35 to myotubes

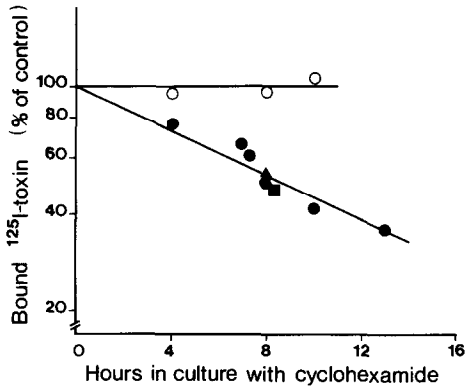

Fig.1. Cyclohexamide blocks synthesis of AChR of human embryonic myotubes. 3-day-old myotubes were cultured with 5 (●), 20 (■) or 100 μg/ml (▲) cyclohexamide at 37°C or with 20 μg/ml at 15°C (○). Remaining ^{125}I-toxin binding sites were measured as described in section 2 and divided by those in parallel cultures with no antibiotic.

decreased surface AChR content down to approximately one-third of the control. Interestingly, at high concentrations of mAb 35 most of its effect disappeared apparently because at high antibody excess it did not crosslink AChRs anymore, but formed only 2 mAb with 1 AChR molecule complexes [16]. We have observed this effect previously in animal cells, but to a much lesser degree [16,17]. The other 2 anti-MIR mAbs (192 and 203) also decreased the AChR content to a similar degree though their action was independent of input antibody concentration. Differences in the Ig subclass of these mAbs in their orientation on the AChR or in their affinity for the antigen might explain their different behaviour.

The effect of mAbs 74, 66 and 69 (fig.2) is especially interesting since it is the first time that mAbs to sites other than the MIR have been observed to cause antigenic modulation of AChR. mAb 66 only forms complexes of 1 mAb with 2 AChR molecules [20] thus precluding the formation of large complexes. This was expected since AChR has only one γ subunit but suggests that crosslinking of only 2 AChR molecules by a bivalent antibody is sufficient to accelerate their internalization. mAb 69 is unique in that it affected the AChR in 2 ways: blocking its toxin sites (as revealed by incubation at 15°C: fig.2, dashed line) and causing decrease in actual receptor number (fig.2, solid line). None of the other mAbs significantly blocked toxin binding at 15°C (not shown).

Several lines of evidence suggested that the effect of the mAbs was indeed on AChR internalization rate and not on the viability of the myotubes. First, their morphology was not affected and myotube formation continued for at least 5 days after mAb treatment. Second, high excess of some mAbs was at most as efficient if not less than lower

Table 1

Characteristics of anti-AChR mAbs used (from [11,13,20])

mAb	Subunit and region specificity	Ig class	Immunogen source	Crossreaction with soluble human AChR	Increase degradation of mouse AChRs
35	α, MIR	IgG1	Electroph.	+ + +	Yes
192	MIR	IgG2b	Human	+ + +	Yes
203	α, MIR	IgG2a	Human	+ + +	Yes
66	γ, near MIR	IgG2a	Calf	+ +	?
69	N.D.	IgG2a	Calf	+	?
73	β, near MIR	IgG1	Calf	+ +	Slightly
74	not near MIR	IgG1	Calf	+	?
64	α	IgG2a	Calf	+ +	No
124	β, cytoplasmic	IgG1	*Torpedo*	+ +	No
137	γ, cytoplasmic	IgM	*Torpedo*	+	?
148	β	N.D.	*Torpedo*	+ + +	?
26	near MIR	IgG2a	Electroph.	–	No
25		IgG2b	Electroph.	–	No

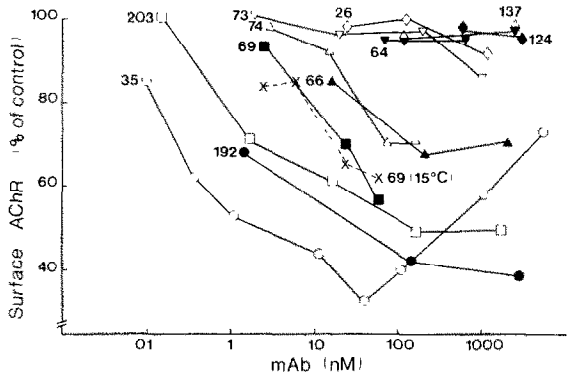

Fig.2. Effect of mAbs on the overall AChR content of human embryonic myotube surface. Incubation with the mAbs was at 37°C for 20 h. Inhibition of toxin binding to existing sites by steric hindrance was measured in parallel cultures at 15°C. This was ≤10% for all mAbs (not shown) except for mAb 69 (dashed line). Blocking of toxin binding was taken into account for calculation of the real AChR content at 37°C (see section 2). Most points are mean numbers of 2 or 3 independent expts with SD ≤ 12%. mAb concentrations were based on their apparent titer for the immunogen. Numbers denote mAb code numbers.

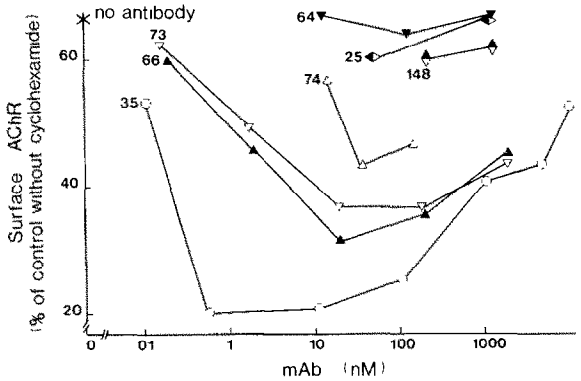

Fig.3. Effect of mAbs on the internalization rate of AChR by blocking synthesis with 20 μg/ml cyclohexamide. Incubation was for 7 h at 37°C. At 15°C mAbs exhibited ≤10% inhibition of toxin binding (not shown). Most points are mean numbers of 2 expts.

concentrations. Finally, in protein synthesis experiments with [^{35}S]methionine mAb-treated and untreated myotubes synthesized similar amounts of total protein (not shown).

3.3. *mAbs directed against α,β and γ subunits accelerated internalization of AChR*

We then tested separately the effect of mAbs on internalization of pre-existing surface AChRs; synthesis of new AChRs was prevented by blocking protein synthesis with cyclohexamide. Fig.3 shows the increased internalization of AChRs after incubation with different mAbs for 7 h. The effect of most mAbs could be correlated with their effect on overall AChR content demonstrated in fig.2. However, anti-β mAb 73, which slightly affected the net content of AChRs in the absence of cyclohexamide (fig.2) was found to increase the internalization rate of AChR very efficiently (fig.3). This mAb also caused very little antigenic modulation of mouse AChR despite sufficient binding [17]. These differences could be due to the absence of newly inserted AChRs in presence of cyclohexamide in fig.3. In cases where antibody-binding affinity is low, it is possible that transient crosslinking of 2 pre-existing AChR molecules might be more likely to allow their internalization than when 1 of the 2 receptors has been synthesized de novo. Furthermore, the decrease in the total number of AChRs, brought about by blocked synthesis, might facilitate antigenic modulation. In fact, when tested at low concentrations, most mAbs were more effective in causing loss of AChRs under conditions where protein synthesis was blocked (fig.3). Whatever the explanation, it is interesting that an antibody directed towards the β subunit is sufficient to cause antigenic modulation of AChR.

4. DISCUSSION

Human muscle cell cultures producing sufficient amounts of AChR were needed to investigate the role of human anti-AChR antibodies on AChR antigenic modulation. Such cultures were established and as a first step we identified their AChRs and studied the effect of binding of different mAbs to these receptors. mAbs against the α, β and γ subunits of AChR accelerated internalization of the receptor and reduced its overall surface content to an extent comparable to that observed in myasthenic patients and experimental animals [3,4]. A single antibody (mAb 69) exerted a dual function: it accelerated AChR internalization as well as partially blocked toxin binding to remaining receptors.

Until now it was not known whether the formation of large antibody-AChR complexes was required or simple crosslinking of 2 AChR molecules by a bivalent antibody sufficient to induce AChR internalization. Addition of single IgG mAbs directed to unique epitopes on the β or γ subunit should be unable to form complexes larger than 2 AChRs to 1 antibody molecule. Indeed sucrose-gradient-centrifugation experiments of mixtures containing anti-γ mAb 66 and solubilized bovine AChR showed that only such small complexes could be formed [20], whereas anti-MIR mAbs formed larger complexes as well [16,20]. Therefore, our observation that anti-γ mAb 66 and anti-β mAb 73 caused AChR loss suggests that such small complexes are also effective though with reduced efficiency.

Because toxin binding to the studied human AChR was reversible, toxin was added after incubation with the mAbs in order to measure the remaining surface AChRs. This technique gives greater experimental variation than prelabeling the AChRs with toxin but has the advantage of allowing the study of decrease in the overall surface AChR content of the myotubes as the result of both increased internalization and normal [3] if not increased [22] synthesis. Increased internalization was also tested independently of receptor synthesis by blocking its synthesis with cyclohexamide (fig.3). Using the established methods and cell cultures we are now testing sera from myasthenia gravis patients of different disease status, in an attempt to correlate their symptoms with the ability of their serum antibodies to mediate the loss of AChR.

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