Degradation of Acetylcholine Receptor in Diaphragms of Rats with Experimental Autoimmune Myasthenia Gravis*

(Received for publication, December 22, 1978)

John Paul Merlie,‡ Stephen Heinemann,¶ Brett Einarbon, and Jon M. Lindstrom‡

From the ‡Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and the Salk Institute for Biological Studies, San Diego, California 92112

The degradation of acetylcholine receptor observed in denervated and innervated normal rat diaphragms in organ culture is stimulated by exogenous antireceptor serum. In this paper we demonstrate that diaphragms from rats with experimental autoimmune myasthenia gravis contain reduced amounts of acetylcholine receptor. Acetylcholine receptor from myasthenic, but not from normal, rats has antibody bound to it and is degraded at an accelerated rate. We conclude that in the chronic phase of experimental autoimmune myasthenia gravis increased acetylcholine receptor degradation can be accounted for by a mechanism involving antigenic modulation, and that such a process can contribute to the clinical symptoms of impaired neuromuscular transmission.

The human neuromuscular disease myasthenia gravis is manifested by muscle weakness and fatiguability (see Ref. 1 for a review). An autoimmune etiology for myasthenia has been proposed on the basis of clinical observations (2, 3). In 1973 it was reported that immunization of rabbits with purified ACh receptor from Electrophorus electricus resulted in an immune response with animals exhibiting clinical features of myasthenia gravis (4). Once this clue to the identity of the autoantigen was obtained, the demonstration of ACh receptor antibodies in the sera of myasthenic humans progressed rapidly (5-11). A sensitive radioimmune assay (12) employing solubilized ACh receptor radiolabeled with 125I-BT was used to demonstrate antireceptor activity in sera from 90% of myasthenic patients (5).

There are many parallels between the human disease and the experimental animal model. Autoradiography of 125I-BT-labeled muscle fibers from biopsies of myasthenic patients showed that the number of available 125I-BT binding sites in myasthenic fibers was reduced (13). The amount of detergent-extractable 125I-BT binding sites in myasthenic patients (14) and immunized animals (15) is also reduced. Loss of ACh receptor in both myasthenic patients and animals with experimental autoimmune myasthenia gravis has also been documented using horseradish peroxidase-conjugated toxin to stain ACh receptor in electron microscopic studies (16-18). Reduction in receptor number correlates well with loss of synaptic function (14, 15). Muscle fibers from myasthenic patients (14, 19, 20) and immunized rats (21, 22) have smaller miniature endplate potentials and are less sensitive to iontophoretically applied ACh (21).

In both the human disease (16) and the animal model (23, 17), IgG and the C3 component of complement have been detected at endplates, and a large fraction of ACh receptor has antibody bound to it (15, 14). In both, the structure of the postsynaptic membrane is greatly simplified from its normally complex architecture (18). Fragments of membrane staining positively for ACh receptor and C3 are observed in the synaptic cleft (24, 16).

There is experimental evidence for a number of mechanisms by which antibodies to ACh receptor can impair neuromuscular transmission. These mechanisms fall into two categories: mechanisms which impair ACh receptor function, and mechanisms which cause loss of ACh receptor protein from the membrane. Mechanisms by which antibodies can impair ACh receptor function are: 1) reducing single channel conductance and mean channel opening time (25), and 2) sterically blocking binding of ACh to the receptor site (10-12). Mechanisms which can cause loss of ACh receptor are: 1) complement-dependent processes which may be cell-mediated during the acute phase of experimental autoimmune myasthenia and during passive transfer of the disease (26, 27, 28, 39), 2) complement-dependent mechanisms which appear to be independent of phagocytic cells (29, 17, 24), and 3) stimulation of ACh receptor degradation by antibody by a complement-independent mechanism (antigenic modulation) (25, 30-32).

Our previous experiments have shown that ACh receptor antibodies in the sera of myasthenic patients and immunized animals will bind to and stimulate the intracellular degradation or surface ACh receptor on normal rodent and human skeletal muscle cells in tissue culture (25, 30). ACh receptor antisera stimulate receptor degradation in denervated and innervated normal adult rat diaphragms in organ culture (33, 34, 35). Here we report experiments designed to evaluate the importance of accelerated ACh receptor degradation to receptor loss in the diaphragms of rats suffering from experimental autoimmune myasthenia gravis.

**EXPERIMENTAL PROCEDURES**

125I-a-Bungarotoxin—a-Bungarotoxin was purified from the venom of Bungarous multicinctus as previously described (36, 37) and was labeled with Na125I by the chloramine-T method (36, 38, 39). From analysis of the degradation products of receptor-bound 125I-BT in organ cultures of rat hemidiaphragms (see below), and from pronase digestion in vitro, it was determined that 125I-BT obtained by the above procedures was primarily the monoiodotyrosine derivative (38, 25, 35).

High titer anti-ACh receptor serum was prepared by multiple...
immunization of female Lewis rats with 10 μg of purified ACh receptor from *Torpedo californica* in Freund's complete adjuvant (35, 36). Such serum contained concentrations of anti-*T. californica* ACh receptor of 1 to 5 × 10⁻⁷ M and anti-rat ACh receptor of 1 to 3 × 10⁻⁷ M as measured by radioimmunoassay (see below). Anti-rat IgG serum was prepared in goats by immunization with a rat IgG fraction purified by ammonium sulfate fractionation and ultragel ACA44 chromatography (40).

**Immunized and Control Rats**—Control rats and rats with experimental autoimmune myasthenia were prepared by injection of 8-week-old female Lewis rats with a single dose of Freund's complete adjuvant or adjuvant containing 15 to 30 μg of purified ACh receptor from the electric organs of either *E. electricus* or *T. californica* (40, 36). Rats immunized without Bordetella pertussis as a supplementary adjuvant did not exhibit clinical symptoms of an acute phase (see Ref. 41 and "Discussion").

**Denervation, Labeling, and Dissection of Rat Diaphragms**—In order to examine the degradation of extrajunctional receptor normally found in denervated muscle, the left hemidiaphragm of immunized rats was denervated under mild ether anesthesia by phrenic nerve section (35, 36, 42, 43). Denervation was performed 4 to 8 weeks after immunization. Diaphragms were removed for ACh receptor quantitation and determination of the ACh receptor degradation rate 10 to 15 days after denervation (35).

**Assay of ACh Receptor and Antibody-Receptor Complexes**—ACh receptor and ACh receptor-IgG complexes were extracted from muscle and measured by radioimmunoassay as previously described (12, 15). ACh receptor is defined as ¹²⁵I-BT binding which is immunoprecipitated by anti-ACh receptor serum and inhibited by the specific cholinergic antagonist benzoquinonium (a gift from Sterling Winthrop Research Institute). ACh receptor-IgG complex is measured as ¹²⁵I-BT binding which is inhibited by benzoquinonium and is immunoprecipitated by goat anti-rat IgG alone (15).

**ACh Receptor Degradation in Organ Culture**—Degradation of ACh receptor in rat hemidiaphragms was determined by the method of Berg and Hall (42, 43) with some modifications (33, 35). Labeling of ACh receptor was done *in vivo* by intrathoracic injection of ¹²⁵I-BT 12 to 18 h before surgical removal of the diaphragm for organ culture. The quantity of toxin used for labeling (2 × 10⁻⁶ mol/100 g of body weight) was calculated from the data of Berg and Hall (43) to be well below the lethal dose for a normal rat. We have found that this dose labels only 8 to 90% of the available receptors in normal muscles, whereas in myasthenic rats, where the amount of ACh receptor is reduced, this dose was occasionally lethal. For this reason our results may be skewed by the possibility that the healthiest animals survived the toxin labeling and were available for study.

All aliquots of the culture medium were removed at intervals of several hours and stored frozen until all samples were collected. Samples were counted by y-scintillation counting, and the total radioactivity released into the medium at the sampling times was calculated.

**RESULTS AND DISCUSSION**

The reduction in total extractable ACh receptor in the carcasses of rats with experimental autoimmune myasthenia gravis has been previously documented (14, 15). Since the clinical manifestations of myasthenia may vary in different muscles (22, 44), and since experiments on ACh receptor sensitivity (21) and ACh receptor degradation have been done with the diaphragm muscle (33–35), we have measured the reduction of ACh receptor in the diaphragms of several groups of independently immunized age- and sex-matched rats. Table I contains data on ACh receptor levels for both innervated and denervated hemidiaphragms. These preparations differ in that they contain primarily junctional and extrajunctional ACh receptor, respectively. The rate of turnover of these two types of ACh receptor is markedly different (43, 46, 47, 33–35). Table I shows that both junctional and extrajunctional receptors are reduced in the diaphragms of rats with experimental autoimmune myasthenia gravis. Furthermore, a significant fraction of receptors from immunized animals have antibody molecules bound to them. Extrajunctional receptors in denervated diaphragms of myasthenic rats are not as severely reduced in number as are junctional receptors. The fraction of extrajunctional receptors which have antibody bound in myasthenic rats is smaller than the fraction of junctional receptors with antibody. These findings may be explained by the possibility that a large fraction of extrajunctional receptors are internal. ACh receptor determinations in these muscles have been done by extracting tissue homogenates with Triton X-100 and labeling all available toxin-binding sites with ¹²⁵I-BT. ACh receptor *in situ* can be labeled with ¹²⁵I-BT or by circulating antibodies only if it is exposed to the outer surface of the cell. Embryonic muscle cells in tissue culture contain a large fraction of ACh receptor which is not accessible to extracellular toxin labeling (39, 47). This may also be true for ACh receptor in adult denervated muscle fibers. We have found that the ratio (total ¹²⁵I-BT binding sites/¹²⁵I-BT binding *in situ*) is larger for denervated as compared with innervated muscle. Therefore, the 23 to 29% of total solubilized ACh receptor which is bound with antibody in the experiment described in Table I may actually represent 50 to 60% of the extrajunctional ACh receptor exposed at the surface of the plasma membrane.

The effect of experimental autoimmune myasthenia on the rate of degradation of extrajunctional ACh receptor is shown in Fig. 1. The maximum stimulation of degradation is approximately 2-fold. Some variability in values of rate constants occurs from one experiment to another, even in the case of normal, control rat diaphragms. The variability observed within an experiment, Fig. 18, is attributed to variability in disease intensity or antibody titer (15), or both, in different animals. Since the sickest animals may have been killed (see "Experimental Procedures") by the toxin labeling procedure, our calculations represent a minimum estimate of the effect of experimental autoimmune myasthenia on ACh receptor turnover.

The rate of degradation of ACh receptors in normal innervated fibers has recently been measured (33–35). Antireceptor sera added to such diaphragms in organ culture have been shown to stimulate the rate of receptor degradation by approximately 2-fold (33–35). In Fig. 2A and Table II we show that the innervated diaphragms of rats with myasthenia degrade ACh receptor approximately twice as fast as normal innervated diaphragms. Calculations which correct for the slow release of toxin from the muscle (25, 39) will increase this difference only slightly. Chromatographic analysis of radioactivity released from ¹²⁵I-BT-labeled myasthenic rat diaphragms in organ culture has shown that the radioactive end
6330 Acetylcholine Receptor Degradation in Myasthenia Gravis

B.

FIG. 1. ACh receptor degradation in denervated diaphragms of control and ACh receptor-immunized rats. Rats were immunized at age 8 weeks with 15 μg of purified T. californica ACh receptor. Twenty-five days later, the left hemidiaphragm was denervated as described in Table I. After 10 to 12 days, the diaphragms were labeled with 125I-BT by intrathoracic injection, removed, and placed in organ culture as described under "Experimental Procedures." ACh receptor degradation was determined by measuring the appearance of 125I radioactivity in the culture medium. The results are expressed as the per cent of the total amount of 125I-BT bound at time zero which remains as a function of time. The amount bound at zero time was calculated as the sum of all released radioactivity plus that remaining in the tissue at the end of the experiment. Since denervated diaphragms contain a significant amount of junctional receptor (which has a much slower turnover, see Fig. 2), the data were corrected for the slow release of radioactivity from the paired innervated hemidiaphragm in each case. The per cent of the initially bound radioactivity which remained at any given time was calculated as follows:

\[
100 - \frac{(\text{cpm released, denervated}) - (\text{cpm released, innervated})}{(\text{cpm bound, denervated}) - (\text{cpm bound, innervated})} \times 100.
\]

Panels A and B represent separate but similar experiments. Filled circles (●) indicate extrajunctional receptor degradation in four separate control rats. Open circles (○) indicate receptor degradation in three separate ACh receptor-immunized rats. Values in parentheses are half-lives of degradation.

Fig. 2. ACh receptor degradation in innervated diaphragms of control and ACh receptor-immunized rats. Rate were immunized as described in Fig. 1. Twenty-five days after immunization, diaphragms were labeled and established in organ culture as described for denervated diaphragms. The per cent of the initially bound radioactivity which remained at any given time was calculated as follows:

\[
\frac{(\text{cpm bound}) - (\text{cpm released})}{(\text{cpm bound})} \times 100.
\]

A. Control, adjuvant-immunized (●); ACh receptor-immunized (○); ACh receptor-immunized and anti-ACh receptor serum added to culture (△). B. Control plus antireceptor serum added to culture (○); immunized and treated in culture with the protease inhibitors leupeptin (9.3 × 10^{-5} M) and pepstatin (4.4 × 10^{-5} M) (●); immunized and treated in culture with the metabolic poisons, dinitrophenol (5 × 10^{-3} M) and NaF (2 × 10^{-2} M) (△). Values in parentheses are half-lives of degradation.

products (3-monoiodotyrosine and its breakdown product) and the time course of their production are identical to those observed when normal diaphragms are treated with exogenous anti-ACh receptor serum (data not shown) (35); i.e. degradation products comprise 70% or greater of the released radioactivity.

The accelerated rate of ACh receptor degradation in myasthenic rat diaphragms is inhibited by leupeptin and pepstatin (Fig. 2B). When used in combination, the protease inhibitors leupeptin (9.3 × 10^{-5} M) and pepstatin (4.4 × 10^{-5} M) inhibit ACh receptor degradation in muscle cells in tissue culture and in muscle tissue in organ culture (35). Leupeptin is known to inhibit plasmin, trypsin, papain, and cathepsin B; pepstatin inhibits pepsin, renin, cathepsin D, and other acid proteases (48). Both leupeptin and pepstatin are known to have very low toxicity (48). Devreotes and Fambrough (39) have reported that ACh receptor degradation in muscle cells in tissue culture was resistant to a number of protease inhibitors: phenylmethanesulfonyl fluoride, N-a-tosyl-L-arginine-methyl ester, soybean trypsin inhibitor, and pepstatin alone (10^{-5} M). Trypan blue, which is thought to be a nonspecific inhibitor of lysosomal proteases, was found to inhibit significantly ACh receptor degradation (39). All of these findings are consistent with the idea that ACh receptor is normally degraded by a

\[\text{J. P. Merlie and M. J. Anderson, unpublished observation.}\]
Acetylcholine Receptor Degradation in Myasthenia Gravis

Effect of experimental autoimmune myasthenia gravis on ACh receptor degradation in innervated rat diaphragm

<table>
<thead>
<tr>
<th></th>
<th>Control ( t_{1/2} )</th>
<th>EAMG ( t_{1/2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>152</td>
<td>116</td>
</tr>
<tr>
<td>2</td>
<td>128</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>190</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>150</td>
<td>68</td>
</tr>
</tbody>
</table>

TABLE II

Half-lives were determined for four control and four ACh receptor-immunized rat diaphragms as described in Fig. 2. EAMG, experimental autoimmune myasthenia gravis.

The rate of receptor degradation in myasthenia is faster than normal. The effects of exogenous anti-ACh receptor serum and myasthenia on the degradation of receptor are not additive is consistent with the interpretation without effect on the rate of receptor degradation in the myasthenic and normal diaphragms, respectively. As previously reported (33-35), the effect on normal diaphragms is to increase the rate of receptor degradation by a maximum of 2-fold. However, addition of exogenous antireceptor serum is without effect on the rate of receptor degradation in the myasthenic diaphragm. That the effects of exogenous anti-ACh receptor serum and myasthenia on the degradation of receptor are not additive is consistent with the interpretation that the rate of receptor degradation in myasthenia is faster than normal due to the stimulation of degradation by bound antibodies (see Table I).

The evidence presented here shows that ACh receptor in the diaphragms of rats with experimental autoimmune myasthenia is degraded at a rate approximately twice that of ACh receptor in normal innervated rat diaphragms. The identity of the end product of degradation, presence of antibody bound to ACh receptor, energy dependence, protease inhibitor sensitivity, and lack of synergism by exogenous anti-ACh receptor serum added in organ culture are consistent with the conclusion that the accelerated rate of ACh receptor degradation observed is due to a mechanism similar or identical to that observed for ACh receptor in muscle cells in tissue culture (25).

The mechanism of stimulation of ACh receptor degradation by antibody binding is unknown, but in many of its characteristics it resembles antigenic modulation described in other systems (49-52): 1) stimulation of degradation is a result of binding of antireceptor antibodies to ACh receptor at the cell surface (25,35), 2) treatment of muscle with anti-ACh receptor sera results in a loss of functional ACh receptor (21,25,31), 3) ACh receptor degradation is an active cellular process which can be inhibited by energy poisons and low temperature, and 4) degradation of ACh receptor is a proteolytic process with free \(^{125}\)I-tyrosine (from labeled a-bungarotoxin) as one of its products. Proteolysis can be inhibited by the specific protease inhibitors leupeptin and pepstatin.

Antigenic modulation of surface IgG from normal B lymphocytes has been explained in terms of patching and capping (51). These phenomena were observed with Ig anti-IgG but not with univalent Fab anti-IgG. (However, others have reported modulation of lymphocyte TL antigens with Fab fragments (53).) Recent experiments have shown that monovalent Fab fragments prepared from myasthenic human serum IgG (54) or rat anti-ACh receptor IgG (55) do not stimulate ACh receptor degradation in muscle cell cultures. F(ab\(^{-}\)) or bound Fab treated with anti-IgG serum were just as active as IgG in stimulating degradation. These experiments have been interpreted in terms of a mechanism by which antibody cross-links two or more receptor molecules at the plasmalemma and thereby stimulates its selection for degradation. Whether the cross-linked receptor-antibody complex is internalized specifically or as part of a larger unit of membrane structure is not yet known, but it is presumed to proceed through lysosomes. Intracellular localizations of toxin-labeled receptor have been observed following degradation of surface labeled receptors (56). The specificity of inhibition of ACh receptor degradation by protease inhibitors is also suggestive of lysosomal involvement.

Although antigenic modulation has been defined as being independent of complement (49-53), the complement pathway has been implicated in the disease process. Passive transfer of myasthenia from humans to mice using an IgG fraction from patient serum was attenuated in recipient mice depleted of C3 but not in those lacking in C5 (28). Depletion of the C3 component in recipient rats resulted in inhibition of development of the clinical symptoms of the acute phase of experimental autoimmune myasthenia and in the passive transfer of the disease between rats (26). The stimulation of ACh receptor degradation by anti-ACh receptor antibody in cell (25) and organ culture (33-35) is probably independent of complement. since either the sera used in those experiments were heat-inactivated or a purified IgG fraction was used. It is possible that the cells or tissues in these experiments produce complement components, but this seems unlikely. In diseased humans and rats, C3 has been detected bound to the muscle membrane (17, 24). It therefore remains a possibility that complement is playing some role in the faster-than-normal degradation of ACh receptor in the experiments reported here. However, the enhanced degradation observed in these experiments is probably due to antigenic modulation, since a C3-dependent mechanism would not be expected to result in release of \(^{125}\)I-tyrosine and would not be expected to be inhibited by leupeptin and pepstatin.

Two further aspects of the interpretation of our results must be considered. First, since neuromuscular transmission is severely impaired in experimental autoimmune myasthenia, and since the structure of the postsynaptic membrane is altered, it is possible that increased ACh receptor degradation is a direct result of these altered properties of the muscle. This possibility cannot be evaluated at present. Second, the in vitro rate of degradation of ACh receptor in innervated diaphragms may not accurately reflect the in vivo rate. We regard this as unlikely, since only receptors prelabeled in vivo under normal conditions are labeled by the method used, and the observed rates extrapolate back to 100% at time zero, only 2 h after the diaphragm was removed from the animal. Furthermore, we find good agreement between rates of degradation of both junctional and extrajunctional receptors measured in organ culture and those measured in vivo by Huang and Chang (46). That receptors observed in innervated diaphragms are truly junctional is supported by the fact that a single first order rate of degradation for up to 50% of the labeled receptors is observed. The observed rate constant (approximately 0.008 h\(^{-}\)) is too low to be that found for rapidly turning over extrajunctional receptors (approximately 0.08 h\(^{-}\)) (33-35).

Due to the great difference in rate constants (10-fold), a mixed population of junctional and extrajunctional ACh receptor containing as little as 20% extrajunctional could have been distinguished by these experiments.

In summary, we have shown that ACh receptor in the diaphragms from rats with autoimmune myasthenia is degraded at twice the normal rate. On the basis of similarity to antigenic modulation of ACh receptor in cell (25) and organ culture (33-35), we conclude that antigenic modulation does occur in myasthenic muscle fibers. The degree of ACh receptor loss which can be predicted from the stimulation of ACh receptor degradation observed here could contribute to some
of the clinical symptoms of the chronic phase of experimental autoimmune myasthenia gravis.

REFERENCES

Degradation of acetylcholine receptor in diaphragms of rats with experimental autoimmune myasthenia gravis.
J P Merlie, S Heinemann, B Einarson and J M Lindstrom


Access the most updated version of this article at http://www.jbc.org/content/254/14/6328

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/14/6328.full.html#ref-list-1