The Effects of a Myasthenic Serum on the Acetylcholine Receptors of C2 Myotubes

I. Immunological Distinction between the Two Toxin-binding Sites of the Receptor¹

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Abstract

We have examined the effect of a serum from a patient with myasthenia gravis on the binding of α -bungarotoxin (α -BuTx) to the acetylcholine receptors (AChRs) of a mouse muscle cell line, C2. After a 2-hr incubation, antibodies in the serum reduced toxin binding to C2 myotubes to a maximal extent of approximately 50%. The degradation of surface AChRs could account for the loss of only 5% of sites during the incubation; the remainder, therefore, must have been lost by blockage of binding. To investigate whether the antibodies blocked specifically one of the two toxin-binding sites that each AChR possesses, we used an analysis based on that of Sine, S. W., and P. Taylor, ((1981) *J. Biol. Chem.* 255: 10144–10156). Although the two sites could not be distinguished by their rates of binding of α -BuTx, *d*-tubocurarine (dTC) inhibition of the initial rate of toxin binding revealed that the sites had affinities for dTC that differed by approximately 30-fold. Incubation with the myasthenic antibodies reduced the number of high affinity dTC sites, without affecting those of low affinity. We conclude that the two toxin-binding sites of the AChR are immunologically distinct.

The antibodies to the acetylcholine receptor (AChR) that are found in the sera of patients with myasthenia gravis are thought to play a crucial role in the pathogenesis of the disease (Vincent, 1980; Drachman, 1981; Lindstrom and Engel, 1981). Thus, muscle weakness and other symptoms of myasthenia can be produced in experimental animals by active immunization against the purified AChR (Patrick and Lindstrom, 1973) or by passive immunization with immunoglobulin from myasthenic patients (Toyka et al., 1975).

The antibodies to the AChR in myasthenic sera are heterogeneous, and the poor correlation between their titer and the severity of the disease (Lindstrom et al., 1976; Vincent and Newsom-Davis, 1980; Roses et al., 1981) suggests that only certain classes of them are deleterious. One class in which the concentration correlates with the clinical state of the disease is that of the antibodies that inhibit the binding of α -bungarotoxin (α -BuTx) to the AChR (Drachman et al., 1982).

In addition to their importance in the pathophysiology of the disease, myasthenic antibodies can also serve as useful tools for investigating the structure and regulation of the AChR. Sera from most patients, for example, have antibodies that distinguish the AChRs in the extrajunctional membranes of developing or denervated mammalian muscle from those at mature endplates (Almon et al., 1974; Bender et al., 1975; Weinberg and Hall, 1979; Dwyer et al., 1981; Lefvert, 1982). Although the AChR is extremely well characterized biochemically, the specificities of myasthenic antibodies have been defined at a molecular level only in a few instances (Tzartos et al., 1982), and their use as structural probes of the AChR has been limited.

We have recently described a myasthenic serum with an unusually high degree of specificity for AChRs in the extrajunctional membranes of developing and denervated muscle. The antibodies in this serum also bind AChRs at developing endplates in rat muscle until the third postnatal week (Hall et al., 1985). The specificity of these antibodies is even more striking in that they appear to bind almost exclusively at or near the toxin-binding sites on the receptor (Hall et al., 1985). The AChR has two toxin-binding sites, which are associated with each of the two α -subunits of the AChR (Karlin, 1980; Haggerty and Froehner, 1981; Conti-Tronconi and Raftery, 1982; Oblas et al., 1983; Gershoni et al., 1983.) In muscle cells, these two sites can be distinguished by agonists and antagonists of the AChR, although not by the toxin itself (Sine and Taylor, 1979, 1980, 1981). We report here experiments in which we have used a mouse muscle cell line, C2, to investigate the specificity of the myasthenic serum for the two toxin-binding sites of the AChR *in situ*. In the following paper (Maricq et al., 1985) we examine the effects of the serum on the functional response of C2 AChRs to agonists.

Some of the experiments described here have been reported in a preliminary communication (Hall et al., 1983).

Materials and Methods

Myasthenic antibodies. The antibodies have been described previously (Hall et al., 1983, 1985). They are derived from a serum taken at plasmapheresis by Dr. Peter Dau at Children's Hospital in San Francisco in February 1978. A 40% ammonium sulfate precipitate, after storage for 3 years at Children's Hospital, was dialyzed against 0.02 M ammonium bicarbonate, lyophilized, and stored at -20°C . In all experiments except the immunofluo-

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rescence experiments, the source of antibodies was the lyophilized protein, taken up just before use in Buffer A.

Other reagents. Monoiodinated ^{125}I - α -BuTx was prepared and purified as previously described (Inestrosa et al., 1983). Rhodamine-conjugated α -BuTx was prepared as described by Ravdin and Axelrod (1977). *d*-Tubocurarine (dTC) was from Calbiochem. An antiserum to the myasthenic antibodies was raised in rabbits using a DEAE fraction of the myasthenic antibody preparation prepared as described by Hall et al. (1985).

Tissue culture. The C2 mouse cell line, originally isolated by Yaffe and Saxel (1977), was maintained as described by Inestrosa et al. (1983). Myoblasts were grown in a medium consisting of Delbecco's modified Eagle's medium with 1 gm/liter of glucose (DME H-16) (GIBCO), supplemented with 20% fetal calf serum (GIBCO) and 0.5% chick embryo extract (GIBCO; growth medium). To stimulate cell differentiation, growth medium was replaced by DME H-16 supplemented with 10% horse serum (GIBCO; fusion medium). Within several days, large multinucleated myotubes appeared with AChRs on their surfaces (Inestrosa et al., 1983).

Except where noted, myoblasts were plated in 24-well plastic tissue culture plates (Falcon) at a density of about 3000 cells/cm². For measurement of AChR turnover, the wells were precoated with collagen to increase the attachment of cells to the dish. For immunohistochemical staining, the myoblasts were plated on plastic coverslips in 24-well plates. In all cases the growth medium was replaced by fusion medium after 48 hr, and the cultures were used for experiments within the next 4 days.

Kinetics of α -toxin binding in normal and antibody-treated cells. The general procedures for studying α -BuTx binding to C2 cells was similar to that described by Sine and Taylor (1979) for BC3H-1, a non-fusing mouse muscle cell line. Fusion medium was removed from C2 myotubes, and the cells were equilibrated for 20 min at room temperature in Buffer A consisting of 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.7 mM MgSO₄, 1.0 mM Na₂HPO₄, 5.5 mM glucose, 25 mM HEPES, and 60 μ g/ml of bovine serum albumin, pH 7.4. The binding reaction was initiated by transferring the cells to the same medium containing 10 nM ^{125}I - α -BuTx. The reaction was terminated at various intervals by aspiration, followed by three 1-ml washes with Buffer A. Cells were then extracted twice in 0.5 ml of 0.1 N NaOH, and the combined extracts were counted in a Beckman II gamma counter. The total number of α -BuTx-binding sites was determined by incubating the cells with ^{125}I - α -BuTx for 3 hr under the same conditions. The association rate constant, k_t , was calculated using the integrated form of the second-order rate equation,

$$\frac{1}{T_0 - R_0} \ln \frac{R_0 T}{T_0 R} = k_t t$$

where T_0 is the initial concentration of ^{125}I - α -BuTx, R_0 is the initial concentration of receptor, expressed in α -BuTx-binding sites, and R and T are the concentrations of AChR and ^{125}I - α -BuTx at time t . R and T were determined by subtracting the amount of toxin-receptor complex present at time t from the initial concentrations, R_0 and T_0 , respectively. For experiments with antibody, cells were incubated with 4 mg/ml of antibody in Buffer A for 2 hr at 37°C before measuring the kinetics of toxin binding. In all experiments, ^{125}I - α -BuTx binding in the presence of an excess amount of unlabeled α -BuTx was subtracted from the total binding.

Effect of dTC on α -BuTx binding rate. The effect of dTC on the initial rate

of α -BuTx binding was monitored with or without pretreatment of the cells with the antibody. After incubation with 4 mg/ml of antibody or buffer for 2 hr at 37°C, cells were rinsed as above and preincubated with various concentrations of dTC or buffer for 20 min at room temperature. The medium was then replaced by the same medium containing 10 nM ^{125}I - α -BuTx. After 10 min the binding reaction was stopped, the cells were washed, and the amount of bound toxin was determined as described above. Control experiments established that the formation of toxin-receptor complex was linear with time during this period (see Fig. 6). The inhibition data were fit to a two-site model for antagonist binding as described by Sine and Taylor (1981):

$$\frac{k_{\text{obs}}}{k_t} = N_A \frac{K_A}{K_A + L} + N_B \frac{K_B}{K_B + L}$$

where k_{obs} and k_t are the association rate constants for α -BuTx binding to the AChR in the presence and absence, respectively, of dTC at concentration L , and N_A and N_B are the relative fractions of binding sites for dTC with dissociation constants of K_A and K_B , respectively. In the absence of antibody, N_A and N_B were both assumed to be equal to 0.5, and the values for K_A and K_B were obtained by linear regression, treating the two extreme portions of the curve separately. Because the difference between K_A and K_B was so large, the equation could be simplified to linear equations at the two extremes of the curve. In experiments involving antibody treatment, data were analyzed by assuming that K_A and K_B were unchanged. The values for N_A and N_B were calculated from the data for total toxin binding inhibition according to different models for the binding of antibody (see "Results").

AChR degradation. C2 myotubes grown in collagen-coated 24-well plates were rinsed and incubated with buffer or antibody (4 to 6 mg/ml). The receptor was then labeled with ^{125}I - α -BuTx by incubation with 10 nM toxin for 2 hr. After extensive washing to remove free toxin, the myotubes were incubated in 0.5 ml of fusion medium at 37°C. At intervals of 1 to 3 hr the culture medium was replaced by 0.5 ml of fresh medium, counted, and frozen for later analysis. At the end of the experiment, the cells were extracted twice with 0.5 ml of 0.1 N NaOH and the combined extracts were counted. The radioactivity released into the medium at each time point was analyzed by P-2 column chromatography to separate degradation products from intact toxin that had been released by dissociation of toxin-receptor complex. The data for degradation and dissociation were then plotted separately on a semilog plot as percentage of total toxin bound. Because both degradation and dissociation affect the same population of toxin-receptor complex, this method of graphical analysis is not rigorously correct; however, it does allow a comparison between cells incubated with and without antibody that is sufficiently accurate for the purposes of this paper.

Immunofluorescence. Because of the high background found with the crude antibody preparation used in other experiments, immunofluorescence experiments were performed with an immunoglobulin fraction purified by passage over a DEAE column and, in some cases, by further fractionation on a Sephacryl S-200 column. Details of the preparations are given in Hall et al. (1985) and in Silberstein et al. (1983). Similar results were obtained with both preparations of antibody. Myotubes grown on coverslips were incubated with 2 mg/ml of antibody in a total volume of 200 μ l for 60 min followed by fluorescein-conjugated goat anti-human IgG F_{ab} fragment (Cappel Laboratories) and rhodamine-conjugated α -BuTx. In some experiments, the rhoda-

Figure 1. Binding of the antibodies to AChRs in C2 myotubes *in situ*. C2 myotubes grown on coverslips were incubated with an S-200 fraction of the myasthenic serum prepared as described under "Materials and Methods," followed by fluorescein-conjugated second antibody and rhodamine-labeled α -BuTx. Cells were then photographed with fluorescein (B and D) or rhodamine (A and C) optics. In C and D the cells were incubated with rhodamine-labeled α -BuTx before the antibody.

Figure 2. Myasthenic antibodies inhibit toxin binding to AChRs in C2 myotubes *in situ*. C2 myotubes were grown in 24-well plates and were incubated for 2 h at 37°C in 0.2 ml of the myasthenic antibodies at the indicated concentrations. Binding of 125 I- α -BuTx was then measured as described under "Materials and Methods." Determinations were made in duplicate. In this experiment, the 100% value was 14 fmol/well. Ab, antibody.

Figure 3. Effect of the antibodies on C2 AChR turnover. C2 myotubes grown in collagen-coated 24-well plates were incubated with 4 mg/ml of antibody or buffer for 2 hr at 37°C. The receptors were then labeled with 125 I- α -BuTx. The radioactivity released into the culture medium was monitored as described under "Materials and Methods." Determinations were made in duplicate. In this experiment, the 100% value in the absence of antibody ($-Ab$) was 89.1 ± 4.0 fmol/well ($n = 4$), and the 100% value in the presence of antibody ($+Ab$) was 51.6 ± 4.5 fmol/well ($n = 6$). The half-lives ($t_{1/2}$) of the AChR in the presence and absence of antibody were 10.4 and 13 hr, respectively.

mine-conjugated α -BuTx was added before the myasthenic antibodies.

Results

Binding of myasthenic antibodies to C2 myotubes. Binding of the myasthenic antibodies to C2 myotubes was initially tested by immunofluorescence. Incubation of the myotubes with the myasthenic antibodies, followed by fluorescein-conjugated second antibody and rhodamine-conjugated α -BuTx revealed clusters of AChRs on the myotube surface (Fig. 1, A and B). As with the AChRs at developing endplates (Hall et al., 1985), preincubation of the myotubes with rhodamine-toxin before incubation with the antibodies completely blocked antibody binding (Fig. 1, C and D). These results suggested that the antibodies bind at or near the α -BuTx-binding site of the C2 AChR.

This interpretation was confirmed by examining the effects of antibody on the binding of 125 I- α -BuTx to the AChRs in C2 myotubes. Preincubation with increasing concentrations of antibody progressively reduced the binding of 125 I- α -BuTx to the myotubes (Fig. 2). The maximal extent of inhibition seen in several experiments was approximately 50%. This result is similar to those seen earlier with solubilized AChRs from denervated rat muscle or from *Torpedo* electric organ (Hall et al., 1983, 1985).

Effect of antibodies on AChR degradation. Because these experiments were carried out on AChRs in intact cells, a possible explanation for the decrease in toxin binding caused by the antibodies is that they increased the rate of internalization and degradation of the surface AChRs. To test this possibility, we incubated the myotubes with the myasthenic antibodies, followed by 125 I- α -BuTx to label the

Figure 4. Kinetics of ^{125}I - α -BuTx binding to C2 AChRs *in situ*. C2 myotubes in 24-well plates were incubated with 10 nM ^{125}I - α -BuTx for various times at room temperature. The binding kinetics were then analyzed using a bimolecular rate equation as described under "Materials and Methods." Linear regression of these data reveals a slope, k_r , of $2.6 \times 10^4 \text{ M}^{-1}\text{S}^{-1}$ ($r = 0.992$) (inset). In this experiment, the total receptor concentration $R_0 = 79.4 \text{ fmol/well}$. All points were the average of three determinations.

Figure 5. Effect of the antibodies on the association rate of ^{125}I - α -BuTx and C2 AChRs *in situ*. The experiment was carried out as described in the legend to Figure 4, except that the cells were incubated with a 4 mg/ml of antibody or buffer for 2 hr at 37°C prior to the initiation of ^{125}I - α -BuTx binding. In the experiment shown, $R_0 = 71.2 \text{ fmol/well}$ in the absence of antibody (-Ab) and 43.4 in the presence of antibody (+Ab). Each point is the average of two determinations. In the presence and absence of antibody, the association rate constants were 1.5×10^4 and $2.5 \times 10^4 \text{ M}^{-1}\text{S}^{-1}$, respectively.

AChRs, and then followed the rate of receptor degradation by the appearance of radioactivity in the medium. P-2 column chromatography was used to separate degradation products from intact toxin released by dissociation. The results indicated (Fig. 3) that, although the antibodies had little or no effect on dissociation of toxin-receptor complex, the rate of degradation of the complex was slightly increased. Thus, the half-time of degradation of surface AChRs measured in the absence of antibody was 13 hr, in agreement with

previous results on C2 myotubes (Miller, 1984). Preincubation with the myasthenic antibodies resulted in a decrease in the half-time of degradation to 10.4 hr (Fig. 3). No further increase in the degradation rate of myasthenic antibody-treated myotubes was seen on addition of a second antibody. In some experiments toxin was added before the myasthenic antibodies. In these cases, the antibodies also caused an increase in degradation that was similar to that seen when antibody was added before toxin (data not shown).

Figure 6. ^{125}I - α -BuTx-AChR complex formation in the presence and absence of dTC. C2 cells were rinsed and preincubated with different concentrations of dTC or buffer for 20 min at room temperature. The solution was then replaced by the same solution containing 10 nM ^{125}I - α -BuTx. ^{125}I - α -BuTx-AChR complex formation was monitored at different time points. This experiment demonstrated that the ^{125}I - α -BuTx-AChR complex formation was linear for at least 10 min under the conditions used.

Figure 7. dTC inhibits the initial rate of α -BuTx binding to AChRs on C2 myotubes. C2 myotubes in 24-well plates were preincubated at room temperature for 20 min with the indicated concentrations of dTC, followed by the same solution containing 10 nM ^{125}I - α -BuTx. The amount of bound toxin in 10 min was then determined as described under "Materials and Methods." The data shown were taken from five independent experiments. All individual values were duplicates and each point represents the mean $\pm$ SE. The solid line is a theoretical curve expressing inhibition by two independent sites of equal concentration, of which the dissociation constants for dTC are 5.6×10^{-8} and 1.6×10^{-6} M, respectively.

Since, in our experiments on toxin binding, cells were incubated for 2 hr or less after antibody treatment, the small increase in AChR degradation could contribute only slightly to the reduction in toxin binding seen in antibody-treated cells. Thus, the increased degradation caused by the antibody in a 2-hr period in the experiment shown in Figure 3 resulted in a loss of approximately 5% of the total toxin-binding sites, whereas in this same experiment there was a reduction of 42% in total toxin binding during this period. We conclude that the decrease in number of active toxin-binding sites is largely due to inhibition of toxin binding and not to loss of AChRs from the myotube surface.

The antibodies bind to one of the two toxin-binding sites. Each AChR has two toxin-binding sites associated with each of the two α -subunits that the receptor possesses. One possible explanation for the partial inhibition of toxin binding by the myasthenic antibodies

is that the antibodies bind to only one of the two toxin-binding sites on each AChR. This could occur either because the antibodies recognize specifically one of the two sites, or because nonselective binding of antibody to either of the two sites blocks binding to the other. Sine and Taylor (1979, 1980, 1981) have carried out an extensive study of the toxin-binding sites of the AChRs of BC3H-1, a non-fusing mouse muscle cell line. We have used an analysis similar to theirs to investigate the specificity of binding of the myasthenic antibodies to the AChR of C2 myotubes.

When the binding of α -BuTx to C2 myotubes was measured, kinetic analysis showed a single association rate constant of $2.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 4). Thus, as in BC3H-1 cells, the two toxin-binding sites have identical, or closely similar, association rate constants for toxin binding. We also examined the kinetics of toxin binding after incubation with antibody (Fig. 5). The sites remaining after antibody

Figure 8. Effect of partial block of α -BuTx-binding sites on N_A/N_B ratio. C2 myotubes were incubated with 2.5 nM unlabeled α -BuTx for 1.5 to 2 hr at 37°C. After extensive washing, the cells were incubated with dTC and then with 125 I- α -BuTx as described in the legend to Figure 7. The data were from two independent experiments in which the number of active sites was reduced by unlabeled α -BuTx to 52% and 25%. The solid curve is the same curve that is shown in Figure 7.

treatment showed an association rate constant of $1.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, a value that is about 40% lower than that seen in control myotubes. The decrease could result from reduced accessibility of the remaining binding sites to toxin or to a change in conformation of the AChR induced by antibody binding.

The initial rate of binding of 125 I- α -BuTx to the AChR was also decreased by dTC (Fig. 6). The inhibition occurred over a broad range of dTC concentrations, extending from 10^{-8} to 10^{-5} M (Fig. 7). Analysis of the inhibition curve showed that the data were well fit

by assuming that there are two independent sites, equal in number, with dissociation constants of 5.6×10^{-8} M and 1.6×10^{-6} M. The data for dTC inhibition could also be explained by negatively cooperative interactions between two kinetically identical sites. According to such a model, the two sites would initially have indistinguishable affinities for dTC, but binding to one site would reduce the affinity of the other for dTC. This possibility was examined by blocking a fraction of the binding sites with unlabeled α -BuTx; dTC inhibition of 125 I- α -BuTx binding to the remaining sites was then determined. In two experiments in which 75% and 48% of the sites were blocked, the inhibition curves that were obtained (Fig. 8) were similar to that seen in the absence of unlabeled toxin. These results are consistent with the interpretation that there are two independent sites.

We then examined the effect of antibody on the inhibition by dTC. In the experiment shown in Figure 9, the added antibody reduced the total number of toxin-binding sites by 40%. This decrease could have come about in three ways: (a) selective loss of high affinity dTC sites; (b) selective loss of the low affinity dTC sites; or (c) the loss of equal numbers of both sites. Theoretical curves for each of these possibilities were made, assuming that the antibody causes no change in either K_A or K_B , the dissociation constants for the two sites (Fig. 9). The data were best fit by the model in which the antibody caused selective loss of the high affinity site. We therefore conclude that the antibodies in the myasthenic serum selectively block toxin binding to one of the two toxin-binding sites on the AChR.

Discussion

The nicotinic AChR has two sites that bind agonists, antagonists, and elapid neurotoxins. Presumably these are structurally similar sites, since they are associated with the two α -subunits that the receptor contains (reviewed in Karlin, 1980; Conti-Tronconi and Raftery, 1982; Changeux et al., 1984). Experiments with affinity-alkylating agents (Damle and Karlin, 1978; Wolosin et al., 1980) and direct binding studies (Neubig and Cohen, 1979; Fels et al., 1982) with agonists and antagonists have established that, in *Torpedo* AChRs, the two sites are nonidentical. An extensive and elegant analysis of the two sites of the muscle AChR has been carried out

Figure 9. Incubation with myasthenic antibodies specifically blocked toxin binding to the site with high affinity for dTC. Experiments were carried out as described in the legend to Figure 7, except that cells were preincubated with 4 mg/ml of myasthenic antibody for 2 hr at 37°C. Each point represents the mean $\pm$ SE of four determinations. Incubation of cells with the antibody reduced total toxin binding, assayed under saturating conditions, to 60% of that seen without antibody. The solid curves were calculated by using the values for the dissociation constants obtained in Figure 7 and by assuming that the 40% sites blocked by the antibody were either all low affinity sites ($N_A = 0.83$, $N_B = 0.17$), all high affinity sites ($N_A = 0.17$, $N_B = 0.83$), or equally shared between the two sites ($N_A = 0.5$, $N_B = 0.5$).

by Sine and Taylor (1979, 1980, 1981), using BC3H-1, a non-fusing mouse muscle cell line. They found that the AChRs in BC3H-1 cells bind α -BuTx with a single rate constant, but that inhibition of toxin binding by antagonists reveals two populations of toxin-binding sites. The two populations of sites are of equal concentration and appear to be intrinsically different. The two sites must be associated with the same receptor, because occupation of both sites by agonists was required for activation of the ion channel.

We have used the studies of Sine and Taylor (1979, 1980, 1981) as a framework within which to investigate the effects of a myasthenic serum on the AChR of another mouse line, C2. This serum is of interest in part because of its biological specificity: it detects a developmental change in endplate AChRs that appears to be related to the channel properties of the receptor (Hall et al., 1985; Schuetze et al., 1985). In C2 cells, the antibodies in this serum reduce toxin binding to the myotubes by about 50%. Although the antibodies cause a slight increase in the AChR degradation rate, this effect is not large enough to account for the observed decrease in toxin binding, which therefore must occur by direct block. The observation that antibody binding to clustered AChRs, which can be visualized immunocytochemically, is largely blocked by preincubation with toxin (Fig. 1) is consistent with this interpretation. This result suggests that most of the antibodies in this serum are directed toward the toxin-binding sites; other antibodies must be present, however, because the serum increases the degradation of preformed toxin-receptor complex on the cell surface (data not shown). The exact proportions of antibodies directed against the toxin-binding site and those directed elsewhere are difficult to estimate from experiments such as these but could be determined by an assay that measured binding directly. We have been unable to successfully establish such an assay, presumably because of the low titer of the AChR-specific antibodies and the fact that they appear to be partially degraded (Silberstein et al., 1983).

Our experiments characterizing the toxin-binding sites on C2 myotubes confirm the earlier experiments on BC3H-1. Thus, there is a single binding constant for α -BuTx, but binding of dTC, measured by inhibition of toxin binding, occurred with two dissociation constants. Because the two dissociation constants are unchanged after partial block with toxin, we conclude that the two sites do not arise by negative cooperativity but are intrinsically different. We then used the differential sensitivity of the two sites to dTC to examine the specificity of the myasthenic serum. Our results showed clearly that incubation of the myotubes with the myasthenic antibodies selectively blocked binding of α -BuTx to the site with high affinity for dTC. Thus, the two sites are not only pharmacologically, but also immunologically, dissimilar. Evidence is presented in the following paper (Maricq et al., 1985) that both sites are associated with the same receptor.

There are several possible biochemical sources for the immunological difference between the two sites. Because the subunits in the AChR are arranged asymmetrically, the two α -chains have different neighbors that could differentially alter the conformation of each active site. Alternatively, the difference might be intrinsic to the α -subunits. Although current evidence indicates that there is only a single structural gene for the α -chain (Noda et al., 1983; Klarsfeld et al., 1984) and that the two α -subunits have identical amino acid sequences over the portions of the chain that have been analyzed (Rafferty et al., 1980; Conti-Tronconi et al., 1982, 1984), differential transcription or processing could give rise to small differences in the peptide backbone. Post-translational modifications that distinguish the two α -chains could also be the source of the different reactivity to the myasthenic antibodies. The cDNA sequence of the α -subunit indicates that there is a single site for glycosylation that is adjacent to the active site (Noda et al., 1982; Sumikawa et al., 1982; Devillers-Thiery et al., 1983), and recent biochemical experiments indicate that the two α -subunits differ in the extent of their glycosylation (Conti-Tronconi et al., 1984).

Antibodies in myasthenic sera that block toxin binding have been

described previously and appear to be relatively common (reviewed in Vincent, 1980). In several cases, inhibition to approximately 50% has been observed, suggesting a specificity for one of the two sites as seen here (Almon and Appel, 1975; Albuquerque et al., 1976; Lefvert and Bergstrom, 1977; Massa and Mittag, 1983). Further experiments will be required to determine whether other myasthenic antibodies are specific for the site with low affinity for dTC. Antibodies that block toxin binding are of particular clinical interest because their titer in myasthenic sera is correlated with the severity of the disease symptoms (Drachman et al., 1982). As binding to both agonist sites is required for full channel opening, block of one of these sites by antibody might be expected to have functional consequences. The effect of the antibodies described here on the physiological response to agonists of the AChRs in C2 myotubes is described in the following paper (Maricq et al., 1985).

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