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

II. Functional Inactivation of the Receptor¹

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Abstract

We have investigated the effect of antibodies from a myasthenic serum on the physiological properties of acetylcholine receptors (AChRs) in myotubes of a mouse muscle cell line, C2. The antibodies in this serum blocked the binding of ¹²⁵I- α -bungarotoxin to the myotubes to an extent of about 50%. The antibodies also inhibited the increase in ²²Na influx caused by carbamylcholine (CARB). At a concentration of antibody that blocked about 50% of toxin binding, greater than 80% of the AChR-mediated ²²Na influx was blocked. The apparent K_D for CARB, estimated from the dose-response curve for ²²Na influx, was unaffected. The effect of the antibodies was further examined by patch-clamp recording. In greater than 30% of the patches from antibody-treated cells, no channel activity in response to acetylcholine was seen; in contrast, every patch from control cells showed activity. The channels that were seen after antibody treatment were indistinguishable from those seen in normal cells, both in their single-channel conductance and in the kinetic constants used to describe channel opening and closing. We conclude that the antibodies in this serum inhibit the functional response of AChRs in C2 myotubes to acetylcholine and do so by inactivating individual receptors.

Myasthenia gravis is an autoimmune disease in which patients are subject to sporadic weakness and fatigability. The physiological basis of these symptoms is a decreased number of functional acetylcholine receptors (AChRs) in the postsynaptic membrane. Thus, myasthenic endplates show reduced endplate potentials (Elmqvist et al., 1964; Albuquerque et al., 1976a, 1981; Ito et al., 1978; Cull-Candy et al., 1979), a diminished response to iontopho-

retically applied acetylcholine (ACh) (Albuquerque et al., 1976a; Cull-Candy et al., 1979), and a decreased number of binding sites for α -bungarotoxin (α -BuTx) (Fambrough et al., 1973; Green et al., 1975).

Antibodies to the AChR in the sera of myasthenic patients (Almon et al., 1974) are thought to be responsible for the loss of functional AChRs. Antibodies are bound to the postsynaptic membrane of the neuromuscular junctions in myasthenic patients (Engel et al., 1977), and injection of antibodies from myasthenic sera into mice induces symptoms of the disease, with reduced miniature endplate potentials (MEPPs) and a decreased number of toxin-binding sites (Toyka et al., 1975).

Antibodies to the AChR may exert their effect on the postsynaptic membrane by complement-mediated destruction of the membrane or by increasing the metabolic degradation of AChRs (reviewed by Drachman, 1981; Lindstrom and Engel, 1981). A third possibility is direct functional block of the AChR. Although many myasthenic sera have antibodies that block the binding of α -BuTx to the AChR (Almon et al., 1974; Bender et al., 1975; Lefvert and Bergstrom, 1977; Mittag et al., 1976; Lefvert et al., 1981; Drachman et al., 1982) and the presence of these antibodies is correlated with the severity of the disease (Drachman et al., 1982), myasthenic sera have rarely been found to inhibit the response of the receptor to ACh (Albuquerque et al., 1976b; Ito et al., 1978; Shibuya et al., 1978; Peper et al., 1981), and in no case has the inhibition been well characterized.

In the preceding paper (Gu et al., 1985), we have described a serum from a myasthenic patient that is highly specific when assayed against the AChRs from a mouse muscle cell line, C2. The antibodies in this serum recognize one of the two α -BuTx binding sites that each receptor possesses. Because this site is presumably close to, or part of, one of the binding sites for ACh, we wished to investigate the effect of these antibodies on the functional response of the C2 receptor to ACh. The antibodies in this serum are of additional interest because they recognize the AChRs at developing but not at adult rat endplates; recent experiments suggest that they bind specifically to AChRs with embryonic-type channels and not to those with adult-type channels (Schuetze et al., 1985). We report here that this antibody blocks the response of AChRs in C2 myotubes to agonists.

Materials and Methods

Tissue culture. The C2 mouse muscle cell line was maintained and grown as described previously (Inestrosa et al., 1983; Gu et al., 1985). For experiments with ²²Na, myoblasts were plated in 24-well plastic tissue culture plates (Falcon) precoated with collagen (Calbiochem), at a density of 6000 cells/well. Forty-eight hours later, the growth medium was replaced by fusion medium. Cells were used for experiments within 4 days after transfer to fusion medium. For patch-clamp experiments myoblasts were plated on ethanol-cleaned glass coverslips (13 mm in diameter) at a density of 2000

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cells/cover slip; after 3 days the medium was changed to fusion medium. Experiments were carried out 3 to 5 days later.

Measurement of ^{22}Na influx. The medium surrounding the cells was changed to a buffer consisting of 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl_2 , 1.7 mM MgSO_4 , 1.0 mM Na_2HPO_4 , 5.5 mM glucose, 25 mM HEPES, 60 $\mu\text{g}/\text{ml}$ of bovine serum albumin, pH 7.4 (assay buffer). After equilibration for 20 min at room temperature, the cells were further incubated at 4°C for 30 min. ^{22}Na influx measurements were initiated by replacing the buffer with the same medium chilled to 4°C and containing 5 $\mu\text{Ci}/\text{ml}$ of ^{22}Na (Amersham) and various concentrations of the cholinergic agonist, carbamylcholine (CARB) (Sigma). In a typical experiment, a final concentration of 100 μM CARB was used to activate the AChRs. After various periods of time, uptake measurements were terminated by aspiration of the medium, followed by three quick washes with ice-cold assay buffer containing 500 μM *d*-tubocurarine (Sigma) to inhibit the activation of channels. Cells in each well were extracted twice with 0.5 ml of 0.1 N NaOH.

The combined extracts were then counted in a Beckman II gamma counter optimized for ^{22}Na . In each experiment the rate of influx of ^{22}Na in cells pretreated with excess $\alpha\text{-BuTx}$ was determined and was subtracted from the rate of influx in cells not treated with $\alpha\text{-BuTx}$; the difference was considered to be the specific influx due to activation of the receptors.

A first-order kinetic analysis was made by plotting the integrated form of the rate equation, $\ln[(\text{Na})_{\text{in}}^\infty/((\text{Na})_{\text{in}}^\infty - (\text{Na})_{\text{in}})] = Kt$, where $(\text{Na})_{\text{in}}^\infty$ is the internal concentration of ^{22}Na at time t and K is a parameter that is proportional to permeability. If the permeability is constant, this plot yields a straight line.

For the determination of the effects of the myasthenia gravis serum on AChR function, the cells were preincubated with antibody for 2 hr at 37°C in a buffer similar to the assay buffer, but containing 140 mM NaCl and 5.4 mM KCl. After rinsing the cells once with assay buffer, ^{22}Na influx was measured as described above. The effect of antibody on toxin binding was monitored under the same conditions that were used for the ^{22}Na assay.

Electrical recording. Patch-clamp experiments were carried out at room temperature with the cells in a medium containing 135 mM NaCl, 2 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 1 mM KH_2PO_4 , 15 mM NaHCO_3 , 5 mM HEPES, 5 mM glucose, pH 7.25. The recording pipette was filled with the same solution. In all cases the solutions were filtered through a 0.22- μm filter (Millipore) immediately before use. To examine the effects of antibody on AChR function, the myotubes were incubated for 2 to 3 hr at 37°C with 3 mg/ml of antibody in the medium described above. After rinsing to remove excess antibody, the myotubes on coverslips were transferred to the recording chamber and used for 2 to 3 hr.

Pipettes for recording were pulled from acid-cleaned Hematocrit tubing (Yankee; Becton, Dickinson and Co.), with an inner diameter of 1.1 mm and an outer diameter of 1.5 mm. The pipettes were coated with Sylgard to within 50 μm of their tips and subsequently microforged to a final tip diameter of 0.5 to 1.0 μm . In the recording solution the DC resistance of the pipette was typically 4 to 8 megohms. The pipette was connected by an Ag-AgCl electrode to the amplifier head stage which was equipped with a 10-gigohm feedback resistor (Yale patch clamp amplifier). The bath electrode was 2% agarose in 1 M KCl and connected to ground via an Ag-AgCl electrode. Recordings from myotubes were made in the cell-attached patch-clamp configuration with various concentrations of ACh in the pipette. "Outside out" and "inside out" patch configurations were used less frequently (Hamill et al., 1981). The current signal was recorded on an FM tape recorder. The frequency response of the recording system was flat up to 4 kHz.

Data analysis. The electrical responses were low-pass filtered at 2 to 3 kHz by an eight-pole Bessel filter and digitized at 10 to 20 kHz. With a double-buffered data acquisition protocol, channel opening events were captured using a trigger value of one-half the unitary current amplitude. Data files consisting of open and closed durations and current amplitudes were organized into histograms with a flexible bin size such that each bin contained at least five events. Exponential or Gaussian functions were fitted to these histograms using the method of nonlinear least squares.

The reliability and accuracy of the methods of recording and analysis of the data were verified in the following manner. A computer simulation of a two-state (open-closed) channel was produced by applying command voltages of an exponentially distributed duration to the amplifier with a 1-gigohm resistor across the input. The voltage was adjusted so that the signal-to-noise ratio approximated that found in an experiment. The recording and data analysis applied to the simulated data were the same as those described above (filter set at 3 kHz). With these techniques, time constants greater than 0.06 msec could be estimated without systematic bias.

Antibodies. The antibodies used in this study have been described previously (Hall et al., 1983, 1985). They are derived from a serum taken at plasmapheresis by Dr. Pater Dau at Children's Hospital in San Francisco, in

February 1978. The 40% ammonium sulfate precipitate, after storage for 3 years at Children's Hospital, was dialyzed in our laboratory against 0.02 M ammonium bicarbonate, lyophilized, and stored at -20°C . For the ^{22}Na experiments, the lyophilized protein was taken up just before use in a buffer similar to the assay buffer but containing 140 mM NaCl and 5.4 mM KCl. For the electrophysiology experiments we used an IgG fraction prepared by DEAE chromatography as described previously (Hall et al., 1985).

Results

CARB-stimulated ^{22}Na influx. In our initial experiments we examined the effect of the myasthenic antibodies on CARB-stimulated ^{22}Na uptake into C2 myotubes as a measure of AChR function. Our assay was modeled on that described by Sine and Taylor (1979) for the non-fusing mouse muscle cell line, BC3H-1. Experiments were performed at 4°C to decrease the rate of receptor desensitization (Catterall, 1975), and the measurements were made in a buffer with a potassium concentration (140 mM) similar to the intracellular potassium concentration to eliminate the secondary effects of voltage changes on ^{22}Na influx. Under these conditions the membrane potential and the potassium equilibrium potential are close to zero, and the large increase in sodium and potassium permeabilities caused by the cholinergic agonist should have little effect on transmembrane voltage. In addition, the voltage-dependent sodium channels are inactivated.

When C2 myotubes were incubated with 100 μM CARB, there was an initial rapid influx of ^{22}Na followed by a slow, steady uptake (Fig. 1). The initial, rapid phase was completely blocked by $\alpha\text{-BuTx}$ and was therefore considered to be due to the AChR. ^{22}Na influx mediated by the AChR was complete within 2 min after the addition of CARB. A kinetic analysis (Fig. 1, inset) showed that the CARB-induced permeability was constant for only about 20 sec. The decrease after this time was presumably due to desensitization of the AChR. In all subsequent experiments, incubations were for 10 or 15 sec and the influx was measured for a single time point. Under these conditions, CARB-stimulated ^{22}Na influx was 6- to 10-fold higher in myotubes incubated without $\alpha\text{-BuTx}$ than in toxin-treated control cells.

Effects of the myasthenic serum. Incubation of C2 myotubes with

Figure 1. Time course of ^{22}Na uptake into C2 cells. The cell cultures were rinsed and transferred to an ice bath for about 30 min as described under "Materials and Methods." The buffer was then replaced with the same medium containing 5 $\mu\text{Ci}/\text{ml}$ of ^{22}Na and 100 μM CARB. The uptake was stopped at various time periods by aspiration and three quick washes. Cells were then extracted twice with 0.5 ml of 0.1 N NaOH and the combined extracts were counted in a gamma counter. In all experiments the uptake in cells pretreated with excess amounts of cold $\alpha\text{-BuTx}$ (▲) was subtracted from the total uptake (●) to yield the specific uptake (○). Each point is the average of three experimental determinations. Inset, The data are plotted in the integrated form of the first-order rate equation as described under "Materials and Methods" to show that, under our experimental conditions, the CARB-stimulated ^{22}Na permeability is constant for only about 20 sec.

Figure 2. Effects of a myasthenia gravis serum on the binding of α -BuTx to AChRs and on the ^{22}Na influx in C2 myotubes. **A.** The cells were preincubated with different concentrations of the antibody (Ab) for 2 hr at 37°C . ^{125}I -labeled α -BuTx was then added to a final concentration of 10 nM and the incubation was continued for another 2 hr. The medium was then aspirated, and the cells were rinsed three times, extracted twice in 0.5 ml of 0.1 N NaOH, and counted in a gamma counter. The binding in the presence of excess amounts of cold α -BuTx was considered to be nonspecific and was subtracted from the total binding. **B.** The cells were preincubated with various concentrations of the antibody for 2 hr, at 37°C , and then the ^{22}Na uptake assay was carried out as described under "Materials and Methods." The uptake of ^{22}Na in 15 sec was monitored and the percentage of uptake was plotted as a function of antibody concentration. The data are from two independent experiments, and each point is the average of the two determinations that are indicated by the error bars. **Inset.** Percentage of ^{22}Na uptake was plotted as a function of the percentage of α -BuTx-binding sites remaining after antibody treatment. The straight line represents the theoretical calculation based on a model in which the binding of antibody to one of the two binding sites completely blocks receptor function. It is assumed that the ^{22}Na uptake is directly proportional to the number of functioning receptors on the cell surface. The data shown in Figure 2A are reproduced from Gu et al. (1985) and are shown here for comparison.

the myasthenic serum for 2 hr at 37°C reduced the ^{22}Na influx induced by CARB. Increasing amounts of antibody gave a progressive inhibition of influx to a maximum of approximately 80% at 8 mg/ml of antibody (Fig. 2). When the inhibition by antibody of toxin binding and ^{22}Na influx was compared in the same experiment, the percentage of inhibition of influx was roughly 2 times the inhibition of toxin binding. Thus, for instance, an antibody concentration of 1.25 mg/ml gave 50% inhibition of ^{22}Na influx and 25% inhibition of toxin binding. This is the relation that one would expect if only one of the two toxin-binding sites associated with each receptor was susceptible to the antibody, and if block of this site inactivated the AChR. A plot of the extent of inhibition of toxin binding versus the extent of inhibition of ^{22}Na influx shows reasonable agreement with the curve predicted by this hypothesis (Fig. 2B, inset).

Effect on the apparent K_D for CARB. Because our assays are done at a constant CARB concentration, one interpretation of our results is that the antibody decreases the affinity of the AChR for CARB. In that case it should be possible to overcome the effect of

the antibody by increasing CARB concentration. To test this idea, we compared the concentration curves for CARB in the presence and absence of antibody. Myotubes were incubated in 4 mg/ml of antibody or in buffer for 2 hr at 37°C , and the ^{22}Na uptake induced by various concentrations of CARB was determined as described above. In the absence of antibody, CARB concentrations of 1 mM or higher were required to achieve the maximum effect, and 50% of the maximum was obtained at a CARB concentration of approximately 10^{-4} M.

In the presence of 4 mg/ml of antibody, although the maximal ^{22}Na influx was only 20 to 30% of that seen in untreated cells, the concentration curve was similar. Half-maximal influx occurred at about 10^{-4} M and maximal influx was seen at 1 mM. When normalized to the maximal influx as 100%, the curves from experiments with and without antibody were superimposable (Fig. 3). We conclude that the decrease in ^{22}Na influx caused by the antibody is not due to a decrease in the affinity of the AChR for agonist.

Single-channel analysis. The decrease in CARB-stimulated ^{22}Na

Figure 3. Determination of the apparent K_D of AChR for CARB in normal and antibody-treated C2 cells. Cells were incubated with 4 mg/ml of the antibody (□) or buffer (●, ▲) for 2 hr at 37°C. The cells were then rinsed and transferred onto ice. ^{22}Na influx was initiated by replacing the medium with the assay buffer containing 5 $\mu\text{Ci/ml}$ of ^{22}Na and various concentrations of CARB. After 10 sec, the uptake was stopped and the amount of ^{22}Na influx was determined. Each point is the average of two determinations. Different symbols represent independent experiments. The data were normalized and were expressed as a percentage of the maximal uptake in each experiment.

Figure 4. Single-channel records of ACh-induced current were recorded from patches of C2 myotubes in the cell-attached configuration. The applied membrane potential was -75 mV . The first and third traces are records from antibody-treated cells, and the second and fourth traces are from normal cells. The ACh concentration in the pipette was $1\text{ }\mu\text{M}$ in the first and second traces and $10\text{ }\mu\text{M}$ in the third and fourth traces.

influx caused by the antibodies could result from a reduction in the single-channel conductance of the AChR, a decrease in the mean channel open time, a reduced frequency of channel opening events due either to fewer functional channels or to a decreased probability of channel opening, or some combination of the above. To investigate whether these properties were affected by the antibodies we used patch-clamp techniques to carry out a single-channel analysis

Figure 5. Current-voltage relationship for the AChR channel. The lower symbols are values from an antibody-treated myotube. The slope conductance is 37.2 pS and the calculated resting potential (assuming a reversal potential of 0 mV) is -65 mV . The upper symbols are the control values. The slope conductance is 34.1 pS and the calculated resting potential is -54 mV .

of the AChR channel following incubation of C2 myotubes with antibody. The properties of the AChR channel in C2 myotubes and a kinetic analysis of their behavior have recently been described (S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication).

Channel conductance. Single-channel openings were seen both in control and antibody-treated myotubes. With either 1.0 or $10.0\text{ }\mu\text{M}$ ACh in the recording pipette no obvious effects of the antibodies were seen on channel amplitude or kinetics (Fig. 4). In most patches, as was true in control myotubes (S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication), the histograms of unitary current amplitude revealed only one peak. Occasionally a second minor peak was observed which corresponded to a larger current size. These latter events were not further analyzed. Plots of mean unitary current versus applied voltage for the major channel showed a linear relationship over a range of 0 to -125 mV (Fig. 5). The slope conductance for antibody-treated cells was $36.5 \pm 0.8\text{ pS}$, a value

ANTIBODY

CONTROL

Figure 6. Kinetic analysis of patch clamp current records. The average current size was 4.35 pA (SD, 0.8). **A**, The burst duration histogram was obtained from a patch incubated with 3.0 mg/ml of DEAE fraction of the myasthenic antibody and then exposed to 1.0 μ M ACh. Openings separated by a gap lasting less than 0.5 msec were considered to belong to the same burst. Two exponentials with the following time constants were required to fit the histogram: 0.20 msec with 24% power and 7.04 msec with 76% power. **B**, The closed time distribution obtained from the same patch as in **A**. Three exponentials were fit to these data with the following time constants: 0.08 msec with 9.5% power, 0.68 msec with 12.5% power, and 97.08 msec with 78% power. **C**, The burst duration histogram obtained from a control patch. The conditions were as in **A**, except there was no incubation with antibody. Two exponentials with the following time constants were required to fit the histogram: 0.28 msec with 15% power and 10.87 msec with 85% power. **D**, The closed time distribution obtained from the same control patch as in **C**. Three exponentials were fit to these data with the following time constants: 0.15 msec with 7% power, 0.98 msec with 5% power, and 125.0 msec with 88% power.

that was not significantly different from the control value of 35.0 ± 0.5 pS. Thus, under the ionic conditions used, the antibody does not impair the conductance of the AChR channel.

Channel open time. The channel, once open, conducts normally. Perhaps the decrease in ^{22}Na flux caused by the antibody occurs because the channel stays open for a shorter period of time. To investigate this possibility, we obtained records of channel opening events at a low ACh concentration (0.5 or 1.0 μ M). Under these conditions few channel events overlapped, and individual bursts of channel openings were well separated (a burst is defined here as a sequence of channel openings not interrupted by a gap greater than

0.5 msec (Colquhoun and Hawkes, 1981)). The burst duration histograms of channels from both antibody-treated and control cells were fit by two exponentials: a fast component and a dominant slow component (Fig. 6, **A** and **C**). These two components have been previously interpreted as corresponding to the channel closing rates from the singly and doubly liganded open states, respectively (S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication; see also Colquhoun and Sakmann, 1981). The time constants and the relative proportion of the two components are shown in Table I. No significant differences in time constants or relative proportions were found between the two groups except for the fast time

TABLE I
Channel properties of the AChR in antibody-treated and control C2 myotubes

	C2 Myotubes	
	Antibody-treated	Control
Conductance (pS)	36.5 $\pm$ 0.8 ^a	35.1 $\pm$ 0.5
Burst duration (msec)	0.34 $\pm$ 0.06 (15) ^b 7.4 $\pm$ 0.9 (85)	0.13 $\pm$ 0.02 (24) 7.2 $\pm$ 1.0 (76)
Closed time duration (msec)	0.1 $\pm$ 0.03 (15) 1.1 $\pm$ 0.2 (8)	0.11 $\pm$ 0.01 (15) 1.4 $\pm$ 0.2 (6)
	120.5 $\pm$ 24.7 (77)	81.3 $\pm$ 21.8 (79)

^a Values are the mean $\pm$ SEM ($N = 7$, antibody; $N = 6$, control). The ACh concentration was either 1.0 or 0.5 μ M.

^b Numbers in parentheses, percentages.

Figure 7. A histogram of the frequency of channel burst events in membrane patches from control and antibody-treated C2 myotubes. The frequency data from all patches were put into bins of 1 Hz. The *top panel* shows patches incubated with 3.0 mg/ml of DEAE fraction myasthenic antibody and exposed to 1.0 to 0.5 μ M ACh. The *bottom panel* shows patches from control sister cultures. All patches in which no channel activity was observed were included in the 0- to 1-Hz bin.

constant, which was approximately 2.5-fold longer in the antibody-treated cells.

Channel closed time. The interval between successive channel openings is referred to as the closed time. Analysis of the closed time can yield the binding constants for the agonist and the rate of the conformational change from the closed to the open state. A decrease in either the binding constants or the isomerization rate would result in a decrease in 22 Na flux. The closed time histograms, obtained from the same data as were the burst duration histograms, required three exponentials for a proper fit. This was true for both the control and antibody-treated channels (Fig. 6, B and D). As can be seen in Table I, the values for the time constants and the relative power contributions at a low ACh concentration did not significantly differ between the two groups. To test further whether the channel kinetic parameters were affected by the antibody, we examined the closed time histogram of channels exposed to a higher concentration of ACh. As the ACh concentration is raised, the closed time histogram exponentials collapse to a single component, the rate of which depends on the ACh concentration and which converges at high ACh concentrations to the rate of isomerization from closed to open channels. At 10 μ M ACh, the time constant of the major component

was 2.2 msec for channels in antibody-treated cells, a value that was not significantly different from the control value of 2.5 msec (S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication). The fact that the multi-exponential functions required to fit the closed time histogram under several different ACh concentrations were not different between antibody and control groups provides an additional assurance, independent of the particular kinetic model chosen, that the underlying kinetics of channel opening had not been changed by the antibody.

Frequency of channel bursts. The kinetic analysis of the AChR channels was performed on records which contained at least 500 channel opening events in several minutes and which did not contain so many events that channel overlap hindered the analysis. The burst frequencies obtained from antibody-treated channels were therefore not very different from those of controls. The patches selected for kinetic analysis, however, were a minority of the successful patches that we obtained in the case of antibody-treated cells. Although it was difficult to control for differences in the area of the membrane patch or the specific location of the patch on the cell, it was clear, in a series of experiments on antibody-treated cells and sister control cells, that many of the patches from antibody-treated cells showed a reduced number of bursts compared to the control patches (Fig. 7). In fact, approximately 30% of the patches from antibody-treated cells showed no channel activity over periods of 5 to 10 min, a phenomenon that was never observed in patches from control myotubes.

Discussion

Although virtually all myasthenic sera have antibodies directed against the AChR, and many sera have antibodies that block α -BuTx binding to the AChR, few sera have been found that affect the functional response of the receptor molecule to ACh (Nastuck et al., 1959; Namba and Grob, 1969; Albuquerque et al., 1976b, 1981; Harvey et al., 1978; Ito et al., 1978; Peper et al., 1981). There are a few incidental accounts of sera that have inhibitory effects on the response to ACh, but the action of these sera has invariably been found to be weak or partial (Ito et al., 1978; Shibuya et al., 1978; Peper et al., 1981; see also an unpublished experiment reported by Bevan et al., 1977). The most effective of these was a single example found by Ito et al. (1978) that reduced the amplitude of MEPPs in human muscle by 50% over a period of 5 hr.

The myasthenic antibodies that we have studied have a more dramatic effect. After 2 hr of incubation they reduced CARB-stimulated 22 Na influx in C2 myotubes by at least 80%. Because the antibodies used in our experiments increase the degradation rate of the AChR only slightly and have little effect on the number of AChRs (Gu et al., 1985), the inhibition of 22 Na uptake must result from a direct effect on AChR function. Antibodies in this serum also blocked toxin binding to the AChR to a maximal extent of about 50% (Fig. 2; Gu et al., 1985). The inhibition of 22 Na uptake occurs over the same range of concentration of antibody, and the relation between inhibition of toxin binding and of 22 Na uptake suggests that the same antibodies are responsible for both effects (Fig. 1, *inset*). C2 myotubes have two classes of toxin-binding sites, and the antibodies specifically block one of these (Gu et al., 1985). The fact that almost complete inhibition of 22 Na influx can be obtained when only 50% of the toxin sites are blocked is consistent with a model in which both sites are associated with each AChR channel and block of one is sufficient to diminish ion flow through the channel (Sine and Taylor, 1980).

To further characterize the interaction of the antibodies with the AChR, we examined, at the single-channel level, the residual current that remained after exposure to the antibody. The reduced 22 Na influx could arise from altered channel properties resulting in less current being carried per channel, or it could result from a reduction in the number of functional channels.

The single-channel current traces appeared to be indistinguishable in antibody-treated and control cells. Amplitude histograms revealed

only one unitary current peak under either condition, and the calculated slope conductance for the AChRs in antibody-treated cells did not significantly differ from that of AChRs in control myotubes. Thus, once open, the channel conducts normally.

Although conducting normally, the channel might stay open for a shorter period of time, which would be reflected in the distribution of burst durations, or the channel might stay closed for a longer period of time, which would be seen in the distribution of closed times. Virtually all of the time constants used to describe the kinetic behavior of the AChR channel were unaffected by prior incubation with the myasthenic antibody. Thus, both opening and closing rates, and the agonist binding rates, were unchanged. The only significant difference was a 2.5-fold decrease in the fast component of the burst duration. This decrease would have negligible effects on the macroscopic current measurements and is also in the wrong direction to explain the flux studies. Thus, the decrease in ^{22}Na flux observed as a consequence of incubation with myasthenic serum cannot be due to altered channel function and must result from a decrease in the number of functional channels.

After antibody treatment, we observed an overall decrease in the frequency of channel opening events which is consistent with there being fewer functional channels in the patch (Fig. 7). Approximately 30% of the patches from antibody-treated cells showed no channel activity; this never occurred with control cells. Because patch areas and conditions are variable, it is difficult to quantitate changes in the frequency of channel opening. It is possible, for instance, that the presence of antibody changed the physical properties of the membrane, causing it to seal over during recording. Another possible explanation for the difference is that the presence of the antibody caused the AChRs to aggregate, thus denuding most of the membrane of receptors. Immunocytochemical experiments, however, showed a distribution of AChR clusters that appeared similar to that seen in normal cells (Gu et al., 1985; L. Silberstein and Z. W. Hall, unpublished observations). Thus, the simplest explanation is that the antibody decreases ^{22}Na influx by impairing the ability of the channel to open in response to ACh. Recent experiments on the effect of these antibodies on the AChRs in developing rat muscle (Schuetze et al., 1985) are also consistent with this interpretation.

The mechanism by which the antibody impairs AChR function is not clear. The antibody blocks one of the toxin-binding sites and most likely blocks the associated agonist-binding site. If the second binding site is accessible to the agonist, one might predict an increase in the proportion of singly liganded, compared to doubly liganded, receptor channel openings (S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication). However, no such increase was seen. This suggests that the antibody either prevents agonist binding to receptor, prevents a conformational change required for channel opening, or blocks access of ions to the channel.

The question of the residual activity seen in the presence of the antibody remains to be addressed. An incomplete block has also been seen in experiments on developing rat muscle (Schuetze et al., 1985). One possibility is that not all AChRs have bound antibody. The low titer and limited amounts of antibody available make it difficult to achieve saturation. A second possibility is that there is a class of channels that is resistant to the antibody. This hypothesis is an attractive one because the antibodies used in our experiments do not recognize AChRs at rat endplates with adult-type channel properties (Hall et al., 1985; Schuetze et al., 1985), and AChRs with such properties have recently been reported in cultured cells (Hamill and Sakmann, 1981; Brehm et al., 1982). Recordings from C2 myotubes, however, revealed essentially a single-channel type (Figs. 4 to 6; S. Hestrin, J. I. Korenbrot, and A. V. Maricq, submitted for publication) the properties of which were unchanged by incubation with antibody. Thus, if resistant channels are present, they are not distinguishable from susceptible ones by any of the criteria that we have used, including inhibition of toxin binding.

A final possibility is that the antibodies are heterogeneous, and include those that block both toxin binding and function and those

that block toxin binding without affecting function. Antibodies in the latter class are not uncommon and, in some cases, have been reported to be specific for extrajunctional receptors (Albuquerque et al., 1976b). The heterogeneity could reflect a polyclonal population of antibodies in the original serum or could be the result of partial degradation that could have occurred during storage.

These observations thus provide a striking example of the ability of antibodies in a myasthenic serum to functionally inactivate the AChR. Further experiments will be required to determine the importance of antibodies of this type in the pathophysiology of the disease.

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