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Monoclonal Antibodies Modify Acetylcholine-Induced Ionic Channel Properties in Cultured Chick Myoballs

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Summary. Monoclonal antibodies directed against the cholinergic binding site of the acetylcholine receptor were found to alter the ion channel properties in cultured chick "myoballs." Time and dose dependent reduction in acetylcholine sensitivity was observed. Noise analysis experiments indicated a decrease in the mean single channel conductance and an increase in the mean single channel open time.

Key Words acetylcholine receptor · monoclonal antibody · ionic channel · ACh noise

Introduction

Muscle weakness in myasthenia gravis is known to be associated with an autoimmune response to muscle acetylcholine receptor (AChR) (Drachman, 1978; Fuchs, 1979; Vincent, 1980). Studies based on the use of radioactively labeled alpha bungarotoxin have shown a reduction in the number of functional AChR's in myasthenia gravis (Fambrough, Drachman & Satyamurti, 1973; Green, Miledi, Perez de la Mora & Vincent, 1975). Electrophysiological studies have shown a reduction in the miniature end-plate potentials (mepp) in neuromuscular junctions obtained from myasthenic patients (Elmqvist, Hoffmann, Kugelberg & Quastel, 1964; Cull-Candy, Miledi & Trautmann, 1978; Ito, Miledi, Vincent & Newson-Davis, 1978), and in experimentally induced myasthenia (Lennon & Lambert, 1980; Pennefather & Quastel, 1980; Alema, Cull-Candy, Miledi & Trautmann, 1981; Hohlfeld et al., 1981). Sera obtained from myasthenic patients or immunized animals also reduced the acetylcholine (ACh) sensitivity of muscle cells in culture (Bevan, Kullberg & Rice, 1978; Heinemann et al., 1977). Single channel properties were unchanged in muscle obtained from myasthenic patients (Cull-Candy, Miledi & Trautmann, 1979; Cull-Candy, Miledi & Ochtel, 1980), or immunized rats (Alema et al., 1981), but a small decrease was found in single channel conductance (about 10%) in cultured rat muscle treated with sera from animals immunized with AChR (Heinemann et al., 1977). The reduction in mepp's amplitude and ACh sensitivity is attributed by most investigators to a reduced number of AChR in the post synaptic membrane (Alema et al., 1981; Hohlfeld et al., 1981; Heinemann et al., 1977). We have studied the effects of a monoclonal antibody (mcAb) directed against the cholinergic binding site of AChR in *acute* experiments, in which the antibody was applied to chick muscle cells during standard electrophysiological measurements *in vitro*. We found a time- and dose-dependent reduction in ACh sensitivity of the muscle cells. Moreover, the antibody produced marked changes in the ACh-induced single channel properties. No other anti-AChR monoclonal antibody exerted such an effect on ionic channel properties. Preliminary results of some of these data have already been reported (Goldberg, Mochly-Rosen, Fuchs & Lass, 1981).

Materials and Methods

ACh-induced transmembrane currents were recorded in voltage clamped large spherical muscle cells (Lass & Fischbach, 1976; Fischbach & Lass, 1978*a*, *b*). Such spheres or "myoballs" are more suitable than elongated cylindrical muscle fibers for voltage-clamp analysis. The interior of each myoball is isopotential and the membrane potential can be held constant, even in the face of large ACh response (up to 200 nA). Myoballs are "normal" in the sense that they are extremely sensitive to ACh and they contract. Control of membrane potential was achieved with two intracellular electrodes – one for measuring the membrane potential and the other for supplying the feedback current required to hold the membrane potential constant. The microelectrodes had a resistance of 5–10 MΩ when filled with 3 M KCl. A microelectrode filled with 2 M ACh was located near the cell, and ACh was ejected by a positive pulse. The Earle's balanced salt solution (EBSS) had the following composition (in mM): 116, NaCl; 5.3, KCl; 1, $NaH_2PO_4 \cdot H_2O$; 0.8, $MgSO_4 \cdot 7H_2O$; 5.5, glucose; 1.8, $CaCl_2$; 26, $NaHCO_3$ (or 20, HEPES). Temperature was controlled by perfusing ethanol between the glass bottom of the recording chamber and the objective of

the inverted microscope. In most experiments, the membrane potential was clamped at −50 mV. Feedback currents were stored on magnetic tape (frequency response 0−1, 250 Hz). The ACh noise was analyzed with a PDP 11/10 computer. The single channel conductance (γ) and the mean channel open time (τ) was estimated on line by curve fitting of a theoretical Lorentzian to the spectral density function, using γ and τ as free parameters.

Monoclonal antibodies with anti-AChR activity were formed as described previously (Mochly-Rosen, Fuchs & Eshhar, 1979), using the cell hybridization technique of Köhler and Milstein (1975). In principle, spleen cells of mice injected with AChR purified from *Torpedo californica* (Aharonov, Tarrab-Hazdai, Silman & Fuchs, 1977) were fused with P3-NSI/l-Ag4-1 (NSI) plasmacytoma cells in the presence of polyethyleneglycol. The hybrid lines having anti-AChR activity were cloned and propagated as ascitic fluids. The immunoglobulin (Ig) fraction of the ascitic fluid (0–45% ammonium sulfate fraction) was used for further assays. For the present study we have used an anti-AChR monoclonal antibody, designated mcAb 5.5.G.12, which was shown to be directed against the cholinergic binding site of the AChR and to cross react with muscle AChR (Mochly-Rosen & Fuchs, 1981). Another anti-AChR monoclonal antibody (5.34), that also cross reacts with muscle AChR (Souroujon, Mochly-Rosen, Gordon & Fuchs, 1983) was also tested in several experiments.

Antibodies were kept as a stock solution (4 mg/ml) in phosphate buffer saline (PBS) at −20 °C. Before the experiment, the mcAb was disolved in EBSS to appropriate concentration. The cells were continuously perfused with control EBSS, followed by the test solution. The rate of perfusion was 2 ml/min and the bath volume was 0.7 ml. Thus, wash-in of mcAb was rapid and cannot account for the reaction time shown in Fig. 2.

Results

In a standard experiment, the myoball was continuously perfused with EBSS and ACh was iontophoretically applied. A typical response to relatively short (10–15 msec) pulse of ACh is shown in Fig. 1. The ACh sensitivity was defined as the peak membrane ionic current (nA) divided by the charge ejected from the ACh pipette (nC). The control ACh sensitivity in 43 cells was 15.9±1.24 nA/nC. Subsequently we have tested the effect of anti-AChR mcAb 5.5.G.12 on ACh sensitivity. A marked reduction of the ACh sensitivity was observed after 16 min exposure to this specific monoclonal antibody (5.5.G.12 Ig fraction, 8 μg/ml). This anti-binding site mcAb has been previously shown to bind to chick, rat, and mouse muscle cells, to accelerate its turnover and to block sodium influx into chick muscle cells (Souroujon et al., 1983). No effect on the ACh response was observed when the ACh sensitvity was measured in the presence of normal mouse immunoglobulins (NMIg, 8 μg/ml).

The blocking action of mcAb 5.5.G.12 was time and dose dependent in the same cells as depicted in Fig. 2. Exposure of the cells to NMIg had no effect on the ACh response. Similar results were

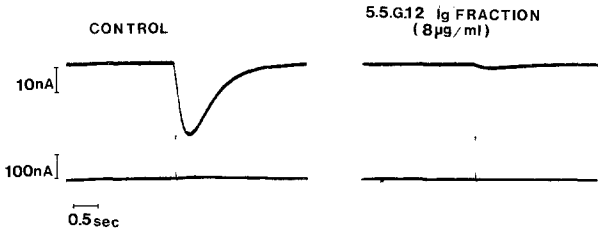

Fig. 1. Reduction of ACh sensitivity of one representative cell following acute application of a monoclonal antibody 5.5.G.12. The upper trace is the membrane ionic current, and the lower trace is the ionophoretic current. After 16 min exposure to high concentration of Ig fraction (8 μg/ml), the ACh sensitivity was reduced from 21.2 nA/nC to 1.5 nA/nC

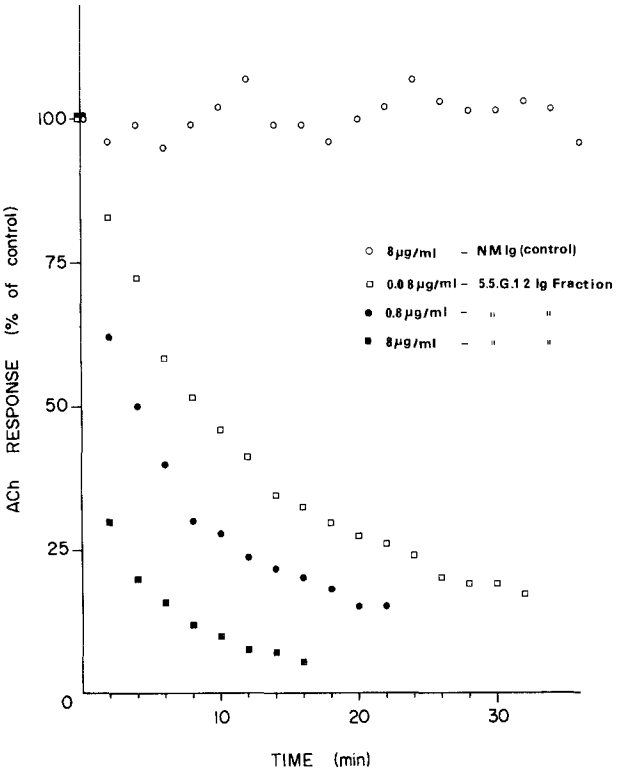

Fig. 2. Time- and dose-dependent reduction of ACh sensitivity induced by a monoclonal antibody 5.5.G.12. o – 8 μg/ml Normal Mouse Immunoglobuline-NMIg (control); ■ – 8 μg/ml 5.5.G.12 Ig fraction; ● – 0.8 μg/ml 5.5.G.12 Ig fraction; □ – 0.08 μg/ml 5.5.G.12 Ig fraction. Each graph represents ACh application in the same cell. NMIg had no effect on the ACh sensitivity, while 5.5.G.12 caused a marked reduction in the ACh sensitivity. Note a fast initial decay followed by a much slower decrease in ACh sensitivity. The cells were clamped to −50 mV and the temperature was 28 °C

observed in 21 cells. Figure 3 shows a semilogarithmic plot of the ACh sensitivity at 8 and 0.8 μg/ml Ig fraction (7 cells tested in each concentration). As shown in Figs. 2 and 3, a double exponential decay of the ACh sensitivity was observed. An initial fast decay was followed by a much slower re-

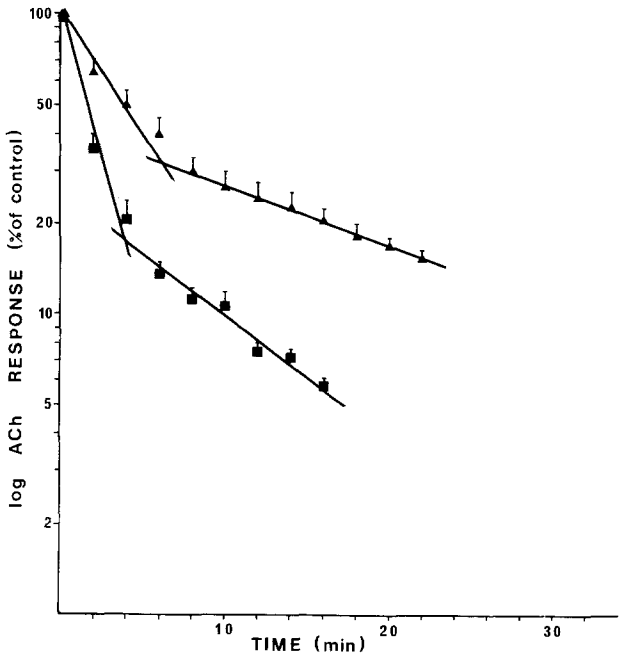

Fig. 3. Double exponential decay of ACh sensitivity in 14 cells, (7 cells tested in each concentration). ▲ – 0.8 μg/ml; ■ – 8 μg/ml 5.5.G.12 Ig fraction. The bar indicates 1 SEM

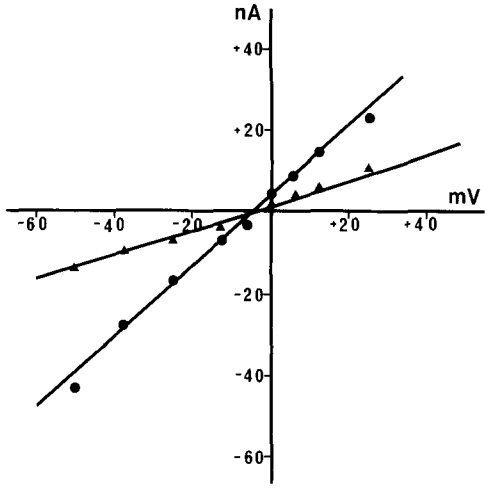

Fig. 4. Voltage current relationship of the ACh-induced membrane response. ● – 8 μg/ml NMIg (control); ▲ – 8 μg/ml 5.5.G.12 Ig fraction. Although the membrane response is depressed by the antibody, the ACh- reversal potential is unchanged

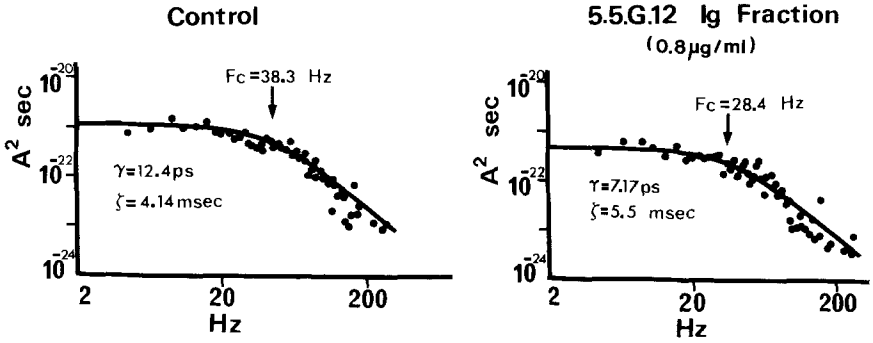

Fig. 5. ACh power spectra in control and after 15 min in 5.5.G.12 Ig fraction (0.8 μg/ml) in the same cell. The noise trace was recorded on magnetic tape and digitized with VR 14 and analyzed with PDP 11/10 computer. Spectrum points were calculated between 2–300 Hz after subtraction of background noise. In each graph the power spectral density (in A^2 sec, ordinate) is plotted against the spectral frequency (in Hz, abscissa). Not logarithmic coordinates. Points at frequency >200 Hz have been omitted because here the filter started reducing the signal amplitude. Sampling frequency was 500 Hz. The spectrum is the average of 23 spectra in the presence of ACh, after subtraction of the average of 5 spectra of background noise (in the absence of ACh). The arrow indicates the half power frequency (fc). From this spectrum the channel open time (τ) was computed using the equation $\tau = 1/2\pi fc$, and the average single channel conductance (γ) was calculated using equation:

$$\gamma = \frac{S_{(o)}\,\pi f_c}{2\mu_i(V - V_o)}.$$

The line is plotted according to equation:

$$S_{(f)} = \frac{S_{(o)}}{1 + (f/fc)^2}.$$

The holding voltage (V) was −50 mV, temperature 28 °C, and the ACh reversal potential (V_o) was −5 mV. The control mean ACh induced current (μ_i) was 60.5 nA; $\gamma = 12.4$ pS; $\tau = 4.14$ msec. In the presence of 5.5.G.12 (15 min) μ_i was 36.2 nA; $\gamma = 7.17$ pS; $\tau = 5.5$ msec

Table. Effects of 5.5.G.12 Ig fraction on single-channel parameters (γ, τ) calculated from "ACh noise" analysis[a]

Treatment	Control	5.5.G.12 Ig fraction (0.8 μg/ml)		
		5 min	10 min	15 min
Mean Single-channel conductance (γ) pS	14.5 ±1.17 $n=13$	7.2 ±0.86 $n=13$	6.5 ±1.05 $n=12$	6.2 ±1.09 $n=8$
Mean Single-channel open time (τ) msec	4.2 ±0.15 $n=13$	5.7 ±0.36 $n=13$	6.1 ±0.22 $n=12$	6.7 ±0.28 $n=8$

[a] The cells were clamped to −50 mV at 28 °C. The values of γ and τ were calculated in EBSS + NMIg (control) and following 5.5.G.12 application in the same cell. The values of γ (pS) and τ (msec) are given as mean ±1 SEM. γ was significantly reduced by the mcAb ($P<0.001$) and τ was prolonged ($P<0.001$).

duction in ACh sensitivity (*see* Discussion). Prolonged washing of the muscle cell (about 1 hr), after exposure to the monoclonal antibody was ineffective, and exploration of neighboring cells in the dish revealed almost total loss of ACh sensitivity even after washing of the bath for 2 hr. The blocking action of the monoclonal antibody was thus irreversible. The ACh reversal potential was measured in partially blocked cells at antibody concentration of 0.8 μg/ml. The antibody blocking action could not be explained by negative shift in the ACh reversal potential which remained fixed at −5 mV (Fig. 4).

The mcAb 5.5.G.12 effects on the ACh were further studied by ACh noise analysis which provides the single channel conductance (γ) and the mean open time (τ) (Katz & Miledi, 1972; Anderson & Stevens, 1973). Figure 5 shows power spectra of the ACh noise in the same cell, before and after the mcAb 5.5.G.12 application. In control γ was 12.4 pS and τ was 4.14 msec. Following 15 min exposure to mcAb 5.5.G.12 (0.8 μg/ml) in the same cell γ was reduced (7.17 pS) and τ was prolonged (5.59 msec). Another anti-AChR mcAb (5.34, 8 μg/ml) which binds to muscle AChR but is not directed against the receptor binding site (Souroujon et al., 1983) had no effect on the single channel properties. The Table summarizes the results obtained in 13 cells after application of mcAb 5.5.G.12 (0.8 μg/ml). γ was significantly reduced by the antibody and τ was slightly prolonged. No effect of NMIg on single channel parameters was observed.

The control single-channel conductance (14.56 ± 4.25 mean ± 1 SD) measured in this series of experiments was smaller than the value found in previous studies (Lass & Fischbach, 1976). The channel conductance values were quite variable (Steinbach, 1980), however, since γ values were determined before and after the antibody application to the *same* cell, the marked changes in channel properties were readily observed.

Discussion

Previous studies with muscle from myasthenic patients (Cull-Candy et al., 1978, 1979), or animals (Alema et al., 1981; Hohlfeld et al., 1981), and with polyclonal anti-AChR antibodies (Heinemann et al., 1977; Bevan et al., 1978), have demonstrated a reduction in ACh sensitivity. This effect was explained by a decreased number of active AChR in the postsynaptic membrane. No significant changes in single-channel parameters were reported. In the present report we have studied the effect of a particular monoclonal antibody – 5.5.G.12. This mcAb antibody was shown to be directed against the cholinergic binding site of the AChR (Mochly-Rosen et al., 1980; Mochly-Rosen & Fuchs, 1981), its binding to the receptor is inhibited by α-neurotoxins and by other cholinergic ligands, in accordance with their affinities to the nicotinic ACh (Mochly-Rosen & Fuchs, 1981). Moreover, this mcAb binds to muscle AChR of chick, rat, and mouse, and inhibits carbamylcholine-induced sodium influx into cultured chick muscle cells (Souroujon et al., 1983). We have demonstrated that a monoclonal antibody directed against the AChR binding site causes immediate reduction in ACh sensitivity, which is in parallel with the modification of single-channel parameters in the same muscle cell.

The fact that the activation of ACh-induced channels can take place in the presence of antibodies directed against the binding site deserves some explanation. There is evidence indicating that two ACh molecules are required for the activation of the ionic channel (Sheridan & Lester, 1977, 1982; Dionne, Steinbach & Stevens, 1978; Delegeane & McNamee, 1980; Trautmann & Feltz, 1980; Zingsheim et al., 1982), but that the binding of one molecule may be sufficient for a partial activation (Dionne et al., 1978). Moreover, recently it was shown that the mcAb 5.5.G.12 can accelerate AChR degradation only when subsaturating amounts of ^{125}I-α-Bungarotoxin (α-Butx) are used to label the cultures (Souroujon et al., 1983). These conditions enable the binding of mcAb 5.5.G.12 to one site per molecule of AChR, while the other

site is occupied by ^{125}I-α-BuTx, which serves as a marker for receptor degradation. At saturating amounts of ^{125}I-α-BuTx, both sites are occupied and 5.5.G.12 fails to accelerate receptor degradation.

It is possible that under the experimental conditions used in this study, the binding of one antibody molecule with a molecular weight of 150,000, to one ACh site, hampers, due to steric hindrance, the binding of the second antibody molecule. The much smaller ACh molecule can still bind to the free ACh site and partially activate the ionic channel. Thus, the antibody allows us to measure channel parameters under intermediate conditions where one site is occupied by a bulky antibody molecule and the other one by ACh. Partial activation of the receptor results in about 50% decrease in γ (*see* Table). After a prolonged exposure to the antibody, the two ACh binding sites may be occupied, resulting in a complete blocking effect as found with α-BuTx (Katz & Miledi, 1973, 1978), where the channel is completely blocked an $\gamma \simeq 0$. Therefore, although the ACh sensitivity is progressively decreased, γ is unchanged because a receptor site with $\gamma \simeq 0$ is "transparent" to noise analysis. The double exponential decay of the ACh sensitivity (Fig. 3) supports this hypothesis. The initial fast decay in ACh sensitivity may represent the relative rapid binding of mcAb to one site which results in a marked reduction in γ. The subsequent slow decay represents binding of mcAb to the second site, causing further decrease in ACh sensitivity with no change in ACh noise parameters as with α-BuTx (Katz & Miledi, 1973, 1978). Note that the slope of the slow decay in ACh sensitivity (Fig. 3) was dose dependent. This may represent the K_{on} of binding of the mcAb to the second binding site.

The modified channel properties (particularly the decreased γ values) brought about by the antibody may resemble the channel "substate" proposed recently by Hamill and Sakmann (1981). The antibody which behaves as a high molecular weight antagonist may increase the probability of the appearence of such a "substate." The increased channel open time observed in our experiments may result from the presence of a bulky antibody molecule bound to the receptor which slows down the conformational changes leading to channel closing.

To our knowledge, it is the first report on the modification of single channel properties of AChR or other ion channels by a specific monoclonal antibody. Further studies with monoclonal antibodies to AChR may provide useful information on the association between the ACh binding site and the ionic channel and their involvement in the mechanism underlying myasthenia gravis.

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