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How Myasthenia Gravis Alters the Safety Factor for Neuromuscular Transmission

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Abstract

Myasthenia gravis (MG), the most common of autoimmune myasthenic syndromes, is characterized by antibodies directed against the skeletal muscle acetylcholine receptors (AChRs). Endplate Na⁺ channels ensure the efficiency of neuromuscular transmission by reducing the threshold depolarization needed to trigger an action potential. Postsynaptic AChRs and voltage-gated Na⁺ channels are both lost from the neuromuscular junction in MG. This study examined the impact of postsynaptic voltage-gated Na⁺ channel loss on the safety factor for neuromuscular transmission. In intercostal nerve-muscle preparations from MG patients, we found that endplate AChR loss decreases the size of the endplate potential, and endplate Na⁺ channel loss increases the threshold depolarization needed to produce a muscle action potential. To evaluate whether AChR-specific antibody impairs the function of Na⁺ channels, we tested omohyoid nerve-muscle preparations from rats injected with monoclonal myasthenogenic IgG (passive transfer model of MG [PTMG]). The AChR antibody that produces PTMG did not alter the function of Na⁺ channels. We conclude that loss of endplate Na⁺ channels in MG is due to complement-mediated loss of endplate membrane rather than a direct effect of myasthenogenic antibodies on endplate Na⁺ channels.

Introduction

Weakness in the autoimmune disease, myasthenia gravis (MG) is caused by antibodies directed against skeletal muscle acetylcholine receptors (AChR) on the muscle membrane portion of the endplate (Drachman, 1994; Vincent et al., 2003). These antibodies reduce the number of AChRs at the endplate (Drachman, 1994; Engel and Fumagalli, 1982; Engel et al., 1977; Fambrough et al., 1973; Kaminski and Ruff, 1999; Kao and Drachman, 1977) by a combination of complement-mediated membrane lysis (Engel and Fumagalli, 1982) and acceleration of AChR catabolism by receptor cross-linking (Drachman, 1994; Engel, 1994; Kao and Drachman, 1977; Vincent et al., 2003). The secondary synaptic folds are simplified due to loss of endplate membrane (Engel, 1994; Engel et al., 1977; Engel and Santa, 1971; Maselli et al., 1991; Santa et al., 1972). The serum level of AChR binding antibodies does not predict the severity of weakness (Drachman, 1994; Engel, 1994; Kaminski and Ruff, 1996), but the postsynaptic membrane area correlates with the size of the endplate potentials (EPP) miniature

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endplate potentials (MEPP) and with the patient's clinical signs of weakness (Engel et al., 1977).

MG is inducible in rats by immunization with foreign or self AChR (EAMG) or by passive transfer of myasthenogenic AChR-binding IgG (PTMG) (Drachman, 1994; Engel, 1994; Kaminski and Ruff, 1996; Lennon and Lambert, 1980; Lindstrom et al., 1976a; Lindstrom et al., 1976b). Weakness in PTMG begins about 12 hours after antibody injection and peaks at 48 hours (Lennon and Lambert, 1980; Lindstrom et al., 1976b). After an initial period of prominent macrophage invasion, electrophysiological and ultrastructural changes at the endplate are similar to those found in patients with acquired MG (Engel, 1994; Lennon and Lambert, 1980; Lindstrom et al., 1976b).

In addition to AChRs, the endplate membrane has a high density of voltage-gated Na⁺ channels (Caldwell et al., 1986; Milton et al., 1992; Ruff, 1992; Ruff, 1996c; Ruff and Whittlesey, 1992; Ruff and Whittlesey, 1993a; Ruff and Whittlesey, 1993b; Wood and Slater, 1995). AChRs are concentrated on the crests of primary membrane folds channels, but voltage-gated Na⁺ channels are concentrated in the depths of the secondary synaptic membrane folds (Angelides, 1986; Flucher and Daniels, 1989; Haimovich et al., 1987; Le Teut et al., 1990; Slater, 2007). The cation fluxes resulting from the opening of the AChRs on the crests of the primary synaptic folds initiates an endplate potential. Current arising from this localized depolarization is directed through the secondary synaptic folds to the voltage-gated Na⁺ channels (Wood and Slater, 1997). For muscle contraction to occur the endplate potential must trigger two action potentials (APs), which are depolarizing waves that propagate from the endplate region to both tendon ends of the muscle fiber. The rising phase of the skeletal muscle AP results from the rapid opening of voltage-gated Na⁺ channels. Na⁺ current (I_{Na}) passing through the open Na⁺ channels depolarizes the muscle fiber. I_{Na} amplitude for a region of membrane depends upon the density of Na⁺ channels in the membrane, how much I_{Na} a single channel conducts (single channel conductance) and the fraction of Na⁺ channels that open in response to membrane depolarization.

The safety factor (SF) for neuromuscular transmission can be defined as:

$$SF = EPP / E_{AP}$$

where EPP is the endplate potential amplitude and E_{AP} is the voltage difference between the resting potential (RP) and the AP threshold (Ruff and Lennon, 1998). The high concentration of voltage-gated Na⁺ channels at the endplate increases the safety factor for neuromuscular transmission by lowering the threshold of depolarization needed to generate an AP (Ruff, 1996c; Ruff and Lennon, 1998; Wood and Slater, 1995).

Endplate I_{Na} is reduced in the muscle fibers of patients with MG and rats with PTMG (Ruff and Lennon, 1998). We previously established that the gating properties of Na⁺ channels away from the endplate were not altered in MG or PTMG. It appeared, therefore, that pathogenic antibodies in MG or PTMG did not target extrajunctional Na⁺ channels (Ruff and Lennon, 1998). An unresolved issue is whether the anti-AChR antibodies reduce I_{Na} at the endplate due to a direct action of the antibodies on Na⁺ channels. An additional issue is how much the reduction in endplate I_{Na} contributes to the reduction of the safety factor in patients with MG.

METHODS

Patient descriptions

The protocol for studying human intercostal muscle was approved by the Institutional Review Board of the Department of Veterans Affairs Medical Center in Cleveland. All biopsies were obtained with the subjects' informed consent. Five male patients with MG, aged 35, 42, 44, 46 and 47 years, donated intercostal muscle biopsies at the time of thymectomy. All had moderately severe generalized MG (Osserman class 2B) and were seropositive for AChR binding antibodies. The mean AChR antibody titres were 52.8 ± 9.9 nmol/L (range 35 – 90 nmol/L), but seronegative for striational antibodies targeting sarcomeric antigens. Each patient received five courses of plasmapheresis before thymectomy (2–3 L removed per session). Seven control male subjects, aged 35–52 years and lacking evidence of neuromuscular disease, donated control intercostal muscle biopsies at the time of a thoracotomy for treatment of cardiac or pulmonary disease. Criteria for excluding a muscle biopsy were: 1) muscle disease other than MG, 2) inability to give informed consent, 3) elevated serum creatine kinase values, 4) evidence of exposure to HIV infection, 5) history of viral hepatitis or 6) intravenous drug abuse.

PTMG Protocol

Female Lewis rats, weighing 180 g to 200 g, were injected intraperitoneally once with a myasthenogenic rat monoclonal anti-AChR IgG (McAb3, 1×10^{-10} M) or a control rat monoclonal IgG (McAb1), which recognizes an epitope specific to *Torpedo* AChR and does not induce PTMG (Lennon and Lambert, 1980). Rats were killed by sodium pentobarbital overdose 24 h after injection. Omohyoid muscles were removed for I_{Na} recordings to determine if AChR antibodies altered the gating properties and single Na^+ channel con. The rat omohyoid muscle contains mostly type IIb fibers (Ruff et al., 1982). Selection of these muscles allowed dissection of bundles one to two fiber layers thick. This enabled precise definition of the endplate regions by light microscopy using Nomarski optics (Ruff, 1992). In some experiments omohyoid muscle bundles were incubated in tyrode solution containing 4 μ M of McAb1 or McAb3 antibodies for 4 hours before recording I_{Na} . As a positive control for the *in vitro* application of McAb1 and McAb3 we also studied omohyoid muscle bundles that were incubated for 4 hours with tyrode solution containing an antibody against mouse and rat skeletal muscle Na^+ channels, S9568 (Sigma-Aldrich, St. Louis, MO, USA). Most studies used 4 μ M S9568.

Tyrode solution

for human and rat muscle contained, mM: NaCl-135, KCl-3.5, $MgCl_2$ -1, $CaCl_2$ -6, HEPES-10 and glucose-10. Solutions were gassed with O_2 and the pH was adjusted to 7.4. Dissections and experiments were performed *in vitro* at $19 \pm 1^\circ C$.

Tissue preparation

Superficial connective tissue was gently dissected from rat and human muscles in Tyrode solution. The rat muscles that were studied with a patch clamp to evaluate single channel I_{Na} were incubated for 45 minutes in oxygenated circulated Tyrode solution containing collagenase 2 mg/ml (type I, Sigma-Aldrich) and bovine albumin 1 mg/ml (fraction V, Sigma-Aldrich). For further dissection, bathing solution with 2 mg/ml collagenase and 1 mg/ml albumin was flowed over the central portion of the muscle, and bathing solution without collagenase was circulated across the tendon regions. The finally dissected muscle regions were one to two fibers thick, permitting visualization of the endplates by a Nikon Diaphot inverted phase contrast microscope equipped with Nomarski optics (Nikon Inc., Instrument Group, Melville, NY). To remove the nerve terminals from the endplates, the central portions of the muscles were perfused with bathing solution containing 2 mg/ml collagenase (Sigma type 1) and 1 mg/

ml of bovine albumin, under continuous observation by Nomarski optics. At 45 to 60 minutes, nerve terminals began to separate from the muscle fibers. Enzyme perfusion was stopped a few minutes later when postsynaptic membranes were exposed (Ruff, 1992). We washed collagenase from the bathing chamber before recording currents. Additional details of the dissections are described elsewhere (Ruff, 1992; Ruff and Whittlesey, 1992; Ruff and Whittlesey, 1993a; Ruff and Whittlesey, 1993b).

Direct visualization of the neuromuscular junction by Nomarski optics enabled precise positioning of the micropipette on the muscle fiber membrane with respect to the nerve terminal. To record I_{Na} from the endplate border, we positioned the pipette on the muscle fiber sarcolemma next to the margin of the nerve terminal. Recordings from extrajunctional membrane were made $> 200\mu\text{m}$ away from the endplate border.

MEPPs and resting membrane potentials

(RPs) were measured in human intercostal muscle fibers at the endplate and membrane potentials were also measured on extrajunctional membrane using intracellular microelectrodes filled with 3M KCl with resistances of 10–20 M Ω (Ruff and Lennon, 1998). MEPPs were recorded from the endplate border after collagenase treatment with the endplate directly visualized with Nomarski optics. MEPP amplitudes were scaled to a membrane potential of -80 mV by multiplying the MEPP amplitudes by $(-80/\text{RP})$ to adjust for differences in fiber membrane potentials and to enable comparison with prior studies of MG (Engel et al., 1977; Lennon and Lambert, 1980; Lindstrom et al., 1976b).

To record EPPs

bundles of muscle fibers along with motor nerve branches were incubated in μ -conotoxin GIIIB (μCTX) (6 μM , Bachem California Inc., Torrance, CA) for one hour to selectively block skeletal muscle Na^+ channels. EPPs were measured at the endplate border using intracellular microelectrodes filled with 3M KCl with resistances of 10–20 M Ω (Ruff and Lennon, 1998). To record EPPs, the motor nerve of a muscle bundle was stimulated using a suction electrode (0–20 V, 0–1 ms, 1 Hz). The recording protocol required EPP rise times (10–90% of peak amplitude) had to be $< 1\text{ ms}$ to assure the integrity of EPP stimulation and recording. We recorded EPPs and AP characteristics from different muscle fiber bundles because complete wash out of the μCTX could not be assured.

AP thresholds and maximum rates of rise

were measured using two microelectrodes, one for passing current the other for recording membrane potential. The electrodes were positioned within 50 μm of each other and connected to a voltage clamp amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA). The microelectrodes and muscle fiber were visualized with Nomarski optics to enable the microelectrodes to be positioned on the endplate border or $> 200\mu\text{m}$ away from the endplate border on extrajunctional membrane. AP threshold was determined using depolarizing current pulses of gradually increasing amplitude (Lennon and Lambert, 1980; Ruff, 1996a; Ruff, 1996c).

Giga-Ohm Seal Patch Clamping

was used to evaluate single channel I_{Na} from the denuded endplates of control and PTMG rat omohyoid muscle fibers. We chose to use standard cell-attached patch clamp techniques (Hamill et al., 1981; Ruff, 1996b) rather than excised patches because cell-attached patches survive longer and there is a gradual change in voltage dependence of Na^+ channel gating in excised patches. Others also have reported changes in Na^+ channel gating changes in excised

patches and whole-cell recordings. (Aldrich et al., 1983; Aldrich and Stevens, 1983; Aldrich and Stevens, 1987; Kunze et al., 1985; Wendt et al., 1992)

The giga-ohm seal patch pipettes were made from borosilicate glass with a two-stage Flaming-Brown micropipette puller. The pipettes were coated with a double layer of Sylgard (Dow Corning 184, Midland, MI) to within 100 μm of the tip and then fire polished. The fire polished pipettes had tip diameters $\leq 1 \mu\text{m}$ and, when filled with Tyrode solution, resistances $< 5 \text{ M}\Omega$.

Single channel currents were recorded using a List EPC-7 patch clamp amplifier (Hamill et al., 1981; Ruff, 1996b) with customized settling time $< 100 \mu\text{sec}$. The amplifier output was lowpass filtered at 5 kHz (-3 dB) with an 8-pole Bessel filter (Krohn-Hite). The signal was digitized at 100 kHz with a 12 bit A/D converter. Single channel records were corrected for leak currents and capacity transients by digital subtraction of templates fitted to records with no openings. Missed brief events and false channel openings were corrected for as previously described. (Ruff, 1996b) Current records were converted to an idealized form by the half-height criteria (Colquhoun and Sigworth, 1983) to construct ensemble averages or histograms. We selected patches that had 5 or less active Na^+ channels and no other channel types. With 5 or fewer Na^+ channels in a membrane patch, it was possible to discern the morphology of single channel currents. (Horn, 1991; Ruff, 1996b) Pulse protocols for eliciting currents were previously described (Ruff, 1996b). Each test pulse is preceded by a 20 msec hyperpolarizing prepulse to remove fast inactivation.

Loose patch voltage clamp

was used to measure macroscopic I_{Na} on the endplate and extrajunctional regions of control and PTMG rat omohyoid muscle fibers. Technical details of the loose patch voltage clamp technique used to measure I_{Na} were described previously (Ruff, 1992; Ruff et al., 1987; Ruff et al., 1988; Ruff and Whittlesey, 1992). To record membrane currents, a micropipette with a fire-polished tip was pressed against the sarcolemma to form a resistive seal between the pipette and the membrane. The seal electrically isolated the patch of membrane under the pipette. The potential of the membrane patch was controlled by the potential within the pipette. Currents elicited from the membrane patch were recorded by the patch pipette. The seal between the membrane and pipette was 3–10 $\text{M}\Omega$; consequently, a fraction of the current produced by the ionic channels within the membrane patch passed across the seal resistance rather than through the pipette. We corrected for the fraction of membrane current lost across the seal by analog and digital compensation. We used micropipettes with tip diameters after fire polishing of 5 to 10 μm and resistances of 200 to 300 $\text{k}\Omega$. We chose pipettes of these sizes to permit sampling from a sufficiently large patch of membrane to minimize local variations in I_{Na} density, and yet not stimulate too large a current to maintain voltage control of the membrane patch. To reduce capacitive coupling between the bath and pipette, we coated the pipettes with a double layer of Sylgard (Dow Corning 184, Midland, MI) to within 100 μm of the tip. We applied minimal suction to the micropipettes to avoid the formation of membrane blebs (Ruff and Whittlesey, 1993a).

Histochemical fiber types

were determined for rat and human muscle preparations, using previously described techniques (Ruff and Whittlesey, 1992; Ruff and Whittlesey, 1993a; Ruff and Whittlesey, 1993b). All intracellular microelectrode tips were coated with carbon black to mark an impaled fiber for later histochemical identification. A muscle bundle containing a fiber marked with carbon black was placed in skinning solution that contained (mM): free Mg-2.5; MgATP-10; K-138; creatine phosphate-15; 12,ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-10; and 3-(N-morpholino) propanesulfonic acid (MOPS)-15. The marked fiber was identified with a Nikon Diaphot microscope and a 4 to 6 mm length of the marked fiber was

dissected free and divided into three segments. Each segment was placed on an albumin-coated coverglass and stored at -70°C . To determine where the fiber was fast or slow twitch, we preincubated a portion of it at pH 4.3 and at pH 10.3 and stained for ATPase; NADH-tetrazolium reductase staining determined if the fiber was oxidative or glycolytic. Stain intensity was determined by examining the slide microscopically and comparing the fiber to muscle of known fiber type. The fibers were classified into three groups, slow-twitch-oxidative (type I), fast-twitch-oxidative-glycolytic (type IIa) and fast-twitch-glycolytic (type IIb). For studies of EPP and MEPP amplitudes or AP characteristics, we analyzed data only from histochemically characterized type IIb fibers.

Statistical analyses

Data were subjected to analysis of variance (ANOVA) testing using two-tailed tests with alpha set at 0.05. Subsets of data were studied with ANOVA to determine significant differences among groups of fibers. When significant interactions were present, post hoc comparisons between different groups were made using Tukey's Honestly Significant Difference Test for pairwise comparisons, Scheffe's S method when more than two means were compared and the Mann-Whitney U test for comparisons of groups that did not satisfy normality criteria (Kirk, 1968; Milliken and Johnson, 1984). Values are expressed as means $\pm$ standard error of the mean.

RESULTS

The threshold for triggering an AP at the endplate was larger for patients with MG

Resting potentials in fibers that were used to record AP properties were similar for control and MG patients (Table 1). The resting membrane potentials of human muscle fibers did not change appreciably during the experiments, which lasted up to 7 hours. The mean resting potentials varied by less than 2 mV from the beginning to the end of an experiment.

For control human muscle fibers, the threshold depolarization for initiating an AP was lower and the AP rate of rise was greater on the endplate border compared with extrajunctional membrane (Table 1). The reduction in the AP threshold on the endplate border for control human muscle fibers was 11.8 mV. The rate of rise of the AP was greater at the endplate border compared with extrajunctional membrane, which indicates that the density of Na^+ channels was greater at the endplate border. The values for AP threshold and rate of rise measured on extrajunctional membrane were similar for MG and control patients. In contrast, compared to controls, the fibers from patients with MG had lower values for AP rate of rise and more positive values for the AP threshold at the endplate. The depolarization needed to trigger an action potential (E_{AP}) at the endplate was 13.5 mV for controls and 21.6 mV for patients with MG. E_{AP} away from the endplate was similar for patients with MG and controls (Table 1).

EPPs and MEPPs were reduced in patients with MG

We recorded MEPPs using intracellular electrodes that were positioned at the endplate border. To record EPPs we blocked skeletal muscle Na^+ channels using μCTX . This toxin has been used by others to selectively block Na^+ channels in order to record EPPs (Ermilov et al., 2007; Wood and Slater, 1997). Both the MEPPs and EPPs were reduced in fibers from patients with MG compared to controls. MEPPs and EPPs from patients with MG were reduced to a similar extent. For patients with MG, MEPPs were 53% of the control value and EPPs were 58% of the control value (Table 2). The similar fractional reductions of the MEPPs and EPPs is consistent with the reduction in the EPP being due to reduction in the sensitivity of the postsynaptic membrane to the transmitter ACh.

Decrease in EPP and increase in E_{AP} both contribute to reduce the SF for patients with MG

The SF for type IIB intercostal fibers from patients with MG was almost non-existent (Table 3). In contrast the SF for control fibers was almost 3. The SF in MG was only 37% of the control SF. We can evaluate the contributions of reduction in the numbers of AChRs and diminished endplate I_{Na} to the reduced SF by comparing the EPP and E_{AP} values of MG and control fibers. If the EPP was the same as control, the SF for MG fibers would have been 1.86 rather than the observed value of 1.09. If E_{AP} for MG fibers were the same as control fibers, the SF for MG fibers would have been 1.74. The reduction in EPP accounted for 59% of the reduction in SF and the increase in EAP produced 40% of the SF reduction.

AChR antibodies did not directly alter the gating or conductance of endplate Na^+ channels

The finding that AP properties away from the endplate for MG and control patients suggested that the antibodies that induce MG do not interfere with Na^+ channels away from the endplate. The increase in the AP threshold and reduction in AP dV/dt at the endplate indicate that myasthenogenic antibodies interfere with endplate Na^+ channels. There are several ways that antibodies in MG could alter I_{Na} at the endplate. The myasthenogenic antibodies could indirectly affect endplate Na^+ channels by triggering complement-mediated damage to endplate membrane. The loss of endplate membrane could result in the loss of Na^+ channels as well as AChRs. In this scenario the remaining endplate Na^+ channels would function normally. Alternatively, the higher density of Na^+ channels at the endplate and their proximity to AChRs could facilitate interactions between antibodies binding to AChRs and endplate Na^+ channels. Therefore, in MG or PTMG anti-AChR antibodies could reduce endplate I_{Na} by altering the properties of endplate Na^+ channels. Possible antibody-induced alterations in Na^+ channels include changes in: 1) voltage dependence of gating that reduce macroscopic I_{Na} , 2) reduced probability of a channel opening and 3) reduced single channel conductance. We previously reported that the gating properties of Na^+ channels were not altered in MG or PTMG (Ruff and Lennon, 1998). We advanced that study by evaluating the effects of McAb1 and McAb3 on rat Na^+ channels. In PTMG, endplate I_{Na} was reduced, whereas extrajunctional I_{Na} was not altered (Table 4). Injecting rats with the inactive McAb1 did not alter I_{Na} at the endplate or on extrajunctional membrane compared with recordings from animals injected with no antibody. In contrast, incubating muscle bundles with 4 μ M McAb3 did not reduce endplate I_{Na} . *In vitro* application of McAb3 did reduce MEPP amplitude, but not to the extent seen in PTMG. MEPP size was 0.906 ± 0.018 mV ($n=18$) for rats injected with McAb1, 0.201 ± 0.019 mV ($n=19$) for rats with PTMG and 0.680 ± 0.022 mV ($n=22$) for muscle incubated *in vitro* with McAb3 ($p<0.001$ compared with either *in vivo* application or McAb1). McAb3 applied *in vivo* reduced MEPPs by 78%, whereas applied *in vitro* McAb3 reduced MEPPs by only 25%. To check that an antibody could block endplate I_{Na} in the *in vitro* conditions we used, we incubated omohyoid muscle bundles with 4 μ M S9568, which is a commercially available of an antibody, also called L/D3, that specifically reacts with the isoform of skeletal muscle Na^+ channels found on the endplate and surface membrane of mature rat and mouse skeletal muscle fibers (Casadei et al., 1984; Cohen and Barchi, 1992). At a concentration of 4 μ M, S9568 completely blocked I_{Na} at the endplate border and on extrajunctional membrane. At 0.1 μ M concentration, the S9568 antibody blocked 52% of I_{Na} at the endplate border and 62% of I_{Na} on extrajunctional membrane compared with muscle incubated with McAb1.

To further examine the interaction of McAb3 with Na^+ channels at the endplate, we recorded single channel Na^+ currents from the endplate membrane of rat omohyoid muscle fibers. To obtain giga-ohm resistance seals between the recording pipette and the bare endplate membrane we needed to treat the muscle collagenase to remove the surface connective tissue and the dissociate the nerve terminal from the endplate. Muscle fibers from rats with PTMG were more fragile. For control rat muscles, collagenase treatment lysed < 4% of the fibers in a bundle. In contrast, collagenase treatment lysed between 30% to 40% of omohyoid fibers from PTMG

rats. Figure 1 shows current records from the endplates of a fiber from a rat with PTMG and from a rat injected with McAb1. The currents are similar to those recorded from the endplate membranes of rats that had not been treated with an antibody. Figure 2 shows the single channel current voltage relationships for type IIb rat skeletal muscle fibers obtained from rats that had been treated with the myasthenogenic IgG McAb3 or with the inactive rat IgG McAb1. Figure 2 additionally shows the current-voltage relationships of omohyoid fibers that were incubated *in vitro* with either McAb1 or McAb3. The single Na⁺ channel conductances were not altered by exposure to McAb3 antibodies. Table 5 lists the single Na⁺ channel conductances recorded on fibers from rats injected with inactive antibody, rats with PTMG, omohyoid muscles incubated *in vitro* with McAb3 and fibers incubated *in vitro* with McAb1. The single Na⁺ channel conductances were similar to each other and to the value of 17.2 pS that was previously reported by this laboratory for Na⁺ channels from untreated rat omohyoid muscle fibers (Ruff, 1996b).

We examined whether exposure to McAb3 could reduce endplate I_{Na} by reducing single channel open time or by changing the probability that a channel would open with depolarization. Table 5 shows that Na⁺ channels from rats with PTMG had similar open times and opening probabilities compared to Na⁺ channels from control rats. In addition, incubation with McAB3 did not alter Na⁺ channel opening probability or open time.

DISCUSSION

This study demonstrated that the impaired neuromuscular transmission in MG reflects impaired function of both endplate AChRs and endplate Na⁺ channels. AChR loss reduces the sensitivity of the postsynaptic membrane to the transmitter ACh, which reduces the EPP size. The impairment of endplate Na⁺ channels raised the threshold depolarization needed to trigger an AP or E_{AP}. The combination of the diminished EPP and increased E_{AP} reduced the safety factor from 2.98 to 1.09. AChR loss had a greater impact on reducing the safety factor for neuromuscular transmission. However, the reduction of I_{Na} at the endplate was responsible for 40% of the safety factor reduction. Using the same technique we used to measure the safety factor, other investigators reported similar values for the safety factor for neuromuscular transmission in control type IIb rat skeletal muscle fibers (Ermilov et al., 2007; Wood and Slater, 1997).

We have shown previously showed I_{Na} loss in muscle fibers of patients with MG and rats with PTMG is selective for the endplate region (Ruff and Lennon, 1998). If endplate membrane loss in MG and PTMG reduced only AChR numbers, then the concentration of Na⁺ channels and I_{Na} at the endplate should have increased. We attribute the reduction in endplate border I_{Na} (reflecting loss of Na⁺ channels) to destruction of endplate membrane containing both AChRs and Na⁺ channels initiated by the binding of a complement-activating AChR-specific IgG *in vivo*.

This study excluded the possibility that myasthenogenic AChR-specific IgG reduces I_{Na} by reacting directly with Na⁺ channels at the endplate. Rats with PTMG had selective reduction of endplate I_{Na}. However, when we applied the active antibody, McAb3, *in vitro* in the absence of complement activation, the IgG did not reduce endplate I_{Na}. *In vitro* applied McAb3 did reduce MEPP amplitudes, but not to the extent seen in PTMG, which is consistent with most of the impact of the antibody being mediated by complement activation (Lennon and Lambert, 1980; Lennon et al., 1978). In addition, we recorded single channel I_{Na} directly from the endplate to evaluate whether the reduction of endplate I_{Na} was in part due to altered Na⁺ channel function. The single channel properties of Na⁺ channels at the endplate including the single channel conductance, the duration of time that a channel was open and the probability that a channel would open with depolarization were the same for channels at the endplates of rats

with PTMG as for control animals. In addition, incubating fibers with active or inactive antibody did not alter Na⁺ channel function. We therefore concluded that the most likely cause of diminished I_{Na} was loss of endplate border Na⁺ channels rather than alteration of Na⁺ channel properties by antibody.

In the studies reported here, we considered how the loss of AChRs and Na⁺ channels from the endplate contributed to a reduction in the safety factor for neuromuscular transmission. An additional factor that can contribute to reduce the efficiency of neuromuscular transmission is the simplification of the secondary synaptic folds as a result of complement-mediated destruction of postsynaptic membrane (Engel, 1994; Engel and Fumagalli, 1982; Engel et al., 1977; Engel and Santa, 1971; Maselli et al., 1991; Santa et al., 1972). As current enters the postsynaptic membrane with opening of AChRs at the top of the synaptic folds, the secondary synaptic folds focus it directly to the Na⁺ channels at the base of the secondary synaptic folds (Martin, 1994; Slater, 2007; Wood and Slater, 1997). Thus the secondary synaptic folds assure that most of the endplate current resulting from AChR opening is directed to depolarizing the membrane region with the highest concentration of Na⁺ channels. In the absence of secondary synaptic folds (as in established MG) some endplate current exit the muscle fiber through membrane that does not contain a high concentration of Na⁺ channels. Thus, it is likely that simplification of the secondary postsynaptic folds further reduces the effectiveness of neuromuscular transmission in MG. This study addressed the impact of losing Na⁺ channels at the base of the synaptic folds, but not the impact of altering the geometry of cation channel organization in the folds.

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Figure 1.

Single channel Na^+ currents recorded from endplate membrane of rat omohyoid type IIb muscle fibers at 19° C using a cell-attached giga-ohm seal patch clamp. Current tracings from fibers from: 1) a rat injected 24 hours earlier with McAb3 antibody in the PTMG animal model of MG and 2) a control rat injected similarly with the inactive McAb1 antibody. The potential of each test pulse eliciting the single channel current is shown with the tracing.

Figure 2.

Voltage dependence of mean single channel I_{Na} amplitudes for the PTMG fiber ($\square$) and control fiber ($\square$) shown in Figure 1. In addition, the graph also shows mean single channel I_{Na} amplitudes from a muscle fiber incubated *in vitro* with McAb3 ($\bullet$) and from a fiber incubated *in vitro* with McAb1 ($\blacksquare$). The single channel conductances were: Control ($\square$) - 17.2 pS, PTMG - 17.3 pS, *in vitro* McAb3 - 17.1 pS and *in vitro* McAb1 - 16.9 pS.

Table 1

Resting membrane potential (RP) and action potential (AP) properties on the endplate border compared to extrajunctional membrane of type IIb intercostal muscle fibers from 7 control subjects and 5 patients with MG. AP properties are threshold, maximum rate of rise (AP dV/dt) and membrane depolarization required to reach threshold (E_{AP}).

	RP (mV)	AP Threshold (mV)	AP dV/dt (Vs^{-1})	E_{AP} (mV)
ENDPLATE BORDER				
Control	-85.4	-71.9	617	13.5
(n = 38 fibers)	± 1.1	± 2.2	± 23	± 2.4
MG	-83.9	-62.3	412	21.6
(n = 34 fibers)	± 1.5	± 2.7	± 18	± 2.9
		$p < 0.001$	$p < 0.001$	$p < 0.01$
EXTRAJUNCTIONAL MEMBRANE				
Control	-85.6	-60.1	364	25.5
(n = 38 fibers)	± 1.2	± 2.3	± 19	± 3.1
MG	-85.1	-59.7	351	25.4
(n = 34 fibers)	± 1.3	± 2.5	± 17	± 3.0

Table 2

EPP and MEPP size recorded from type IIb external intercostal muscles of 5 patients with MG and 7 control patients.

	MEPP (mV)	EPP (mV)
Control	0.91	40.2
(n = 38 fibers)	± 0.03	± 1.3
MG	0.48	23.5
(n = 34 fibers)	± 0.02	± 1.7
	$p < 0.001$	$p < 0.001$

Table 3

The safety factor (SF) for neuromuscular transmission for type IIb external intercostals muscles of 5 patients with MG and 7 control patients. $SF = EPP/E_{AP}$ using data from Table 1 and Table 2.

	SF
Control	2.98
MG	1.09

Macroscopic I_{Na} (mA/cm²) on rat type IIb muscle fibers measured with loose patch voltage clamp. For *in vivo* columns rats were injected with active (McAb3) or inactive (McAb1) antibodies 24 hours before the measurements. *In vivo* treatment with McAb3 produced PTMG. For the *in vitro* columns, muscle bundles were incubated for 4 hours with antibodies before measurements. The number of fibers studied are indicated in parentheses.

Table 4

	Antibody applied <i>in vivo</i>		No Antibody	Antibody applied <i>in vitro</i>	
	McAb1	McAb3		McAb1	McAb3
Endplate	108 ± 2.6(21)	31.5 ± 2.1(21)*	110 ± 2.7(21)	112 ± 2.8(21)	109 ± 2.9(21)
Extrajunctional	14.5 ± 1.2(21)	14.6 ± 1.4(21)	14.1 ± 1.3(21)	13.9 ± 1.6(21)	13.8 ± 1.7(21)

*
p < 0.001

Table 5

Myasthenogenic antibodies did not alter the single channel properties of endplate sodium channels. The single channel conductance was the cord conductance obtained by analyzing records as shown in Figure 2. The open time was the mean period that channels were open for depolarizations to a membrane potential associated with a maximum amplitude of the macroscopic I_{Na} , called $V_{INa,max}$. $V_{INa,max}$ was typically -25 mV. P_O was the probability that a channel would open when the membrane was depolarized to $V_{INa,max}$.

	Antibody applied <i>in vivo</i>		Antibody applied <i>in vitro</i>	
	McAb1	McAb3	McAb1	McAb3
Single channel	16.8	17.9	17.4	16.9
conductance (pS)	± 1.0	± 0.9	± 1.1	± 1.0
Open Time at	1.66	1.71	1.72	1.69
$V_{INa,max}$ (mS)	± 0.07	± 0.06	± 0.08	± 0.08
P_O at $V_{INa,max}$	0.53	0.52	0.54	0.53