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The Effects of Sera from Amyotrophic Lateral Sclerosis Patients on Neuromuscular Transmission and Calcium Channels in Mice

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Amyotrophic lateral sclerosis (ALS) is a degenerative neuromuscular disease of unknown etiology in which the upper and lower motor neurons are progressively destroyed. Recent evidences support the role of autoimmune mechanisms in the pathogenesis of ALS. This study investigated the effects of sera from ALS patients on neuromuscular transmission in phrenic nerve-hemidiaphragm preparations and on calcium currents of single isolated dorsal root ganglion (DRG) cells in mice. Mice were injected with either control sera from healthy adults or ALS sera from 18 patients with ALS of sporadic form, for three days. Miniature end plate potential (MEPP) and nerve-evoked end plate potential (EPP) were measured using intracellular recording technique and the quantal content was determined. Single isolated DRG cells were voltage-clamped with the whole-cell configuration and membrane currents were recorded. Sera from 14 of 18 ALS patients caused a significant increase in MEPP frequency in normal Ringer's solution (4.62±0.14 Hz) compared with the control (2.18±0.15 Hz). In a high Mg^{2+}/low Ca^{2+} solution, sera from 13 of 18 ALS patients caused a significant increase in MEPP frequency, from 2.18±0.31 Hz to 6.09±0.38 Hz. Sera from 11 of 18 patients produced a significant increase of nerve-evoked EPP amplitude, from 0.92±0.05 mV to 1.30±0.04 mV, while the other seven ALS sera did not alter EPP amplitude. In the ALS group, EPP quantal content was also elevated by the sera of 14 patients (from 1.49±0.07 to 2.35±0.07). MEPP frequency and amplitude in wobbler mouse were 4.03±0.53 Hz and 1.37±0.18 mV, respectively, which were significantly higher than those of wobbler controls (wobblers without the symptoms of wobbler). Sera from ALS patients significantly reduced HVA calcium currents of DRG cells to 42.7% at −10 mV. Furthermore, the inactivation curve shifted to more negative potentials with its half-inactivation potential changed by 6.98 mV. There were, however, significant changes neither in the reversal potential of I_{Ca} nor in the I-V curve. From these results it was concluded that: 1) The serum factors of sporadic ALS patients increase neuromuscular transmission and can alter motor nerve terminal presynaptic function. This suggests that ALS serum factors may play an important role in the early stage of ALS, and 2) Calcium currents in DRG cells were reduced and rapidly inactivated by ALS sera, suggesting that in these cells, ALS serum factors may exert interaction with the calcium channel.

Key Words: Amyotrophic lateral sclerosis, Neuromuscular junction, Quantal content, Evoked transmitter release, Calcium channels, Dorsal root ganglion, Wobbler mouse

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative neuromuscular disease in which the upper and lower motor neurons are progressively destroyed. Two forms of the disease are known to exist. The first is the genetically transmitted familial form which accounts for approximately 10% of the cases. The remaining 90% are sporadic. Their exact causes are still unknown.

Attempts have been made to explain the etiology and pathogenesis of both forms of ALS, and several hypotheses have been put forward (Eisen & Krieger, 1993; Brown, 1994). In familial ALS, approximately 20% of patients exhibit mutation in the Cu^{2+}/Zn^{2+} superoxide dismutase (SOD1) gene and the hypothesis that such mutation may be an underlying cause of motoneuron degeneration has recently been

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strengthened by the development of a transgenic animal model (Rosen et al, 1993; Gurney et al, 1994).

Although the etiology or pathogenesis of sporadic ALS remains unknown, several hypotheses have been developed. These include a possible role of viruses (Gibbs & Gajdusek, 1982; Viola et al, 1982; Brahic et al, 1985), of toxin (Spencer et al, 1987), of impaired aging (Bradley, 1984), of altered trophic-factor function (Appel et al, 1991), and of abnormal glutamate metabolism (Rowland, 1991; Rothstein et al, 1992). The autoimmune hypothesis has received considerable attentions for the past ten years (Uchitel et al, 1988, 1992b; Appel et al, 1991; Llinas et al, 1993; Mosier et al, 1995).

Circumstantial evidences provide suggestive but not conclusive supports for the autoimmune hypothesis. These evidences include the presence of other autoimmune disorders in ALS patients, the presence of paraproteinemias (Shy et al, 1986; Younger et al, 1990; Duarte et al, 1991), of lymphomas (Younger et al, 1991), of lymphocytic infiltration (Engelhardt et al, 1993), and of activated macrophages in ALS spinal cord and immunoglobulin (Ig) G within ALS motoneurons (Donnenfeld et al, 1984; Engelhardt & Appel, 1990). Lampson et al (1990) demonstrated phagocytes and small numbers of T cells in degenerating white matter of ALS spinal cords. Whether autoimmunity accounts for the majority or a minority of cases of sporadic ALS is not clear. The small number of cases with paraproteinemia supports a role for autoimmunity in only a limited number of patients, while the large number of cases with antibodies to the calcium channel (Smith et al, 1992) supports a role for autoimmunity in the majority of cases of sporadic ALS.

Although there are several findings suggesting the possible pathogenic actions of ALS autoantibodies, critical evidences came from experiments which examined the functional effects of Ig on motor nerve terminals. It was initially observed that enhanced spontaneous release of acetylcholine (ACh) from motor nerve terminals was present in nerve-muscle preparations exposed for 4 hours to ALS Ig (Uchitel et al, 1988). This phenomenon was confirmed in another study (Uchitel et al, 1992b) in which the animals received ALS Ig *in vivo* for 4～12 weeks. Further evidence for the autoimmune hypothesis was provided by the results of a passive transfer experiment, in which laboratory mice were innoculated with serum containing IgG from ALS patients (Appel et al, 1991). IgGs were found both in motor neurons and at the neuromuscular junctions (NMJs) of innoculated animals.

An increase in miniature endplate potential (MEPP) frequency was also observed, while MEPP amplitude and time course remained unaltered (Engelhardt & Joo, 1986). Furthermore, other authors have demonstrated the cytotoxic effects of ALS IgG on a motor neuron cell line *in vitro* (Alexianu et al, 1994; Smith et al, 1994) and morphological alterations in motor neurons following passive transfer *in vivo* (Uchitel et al, 1992b).

The first biochemical evidence that anti-VGCC antibodies occur in ALS was reported by Smith and his colleagues (Smith et al, 1992). Using an enzyme-linked immunosorbent assay (ELISA), they demonstrated that antibodies binding to L-type calcium channels from rabbit skeletal muscle were present in sera from 75% of ALS patients. Further investigation showed that the antibodies were bound to the channel's α_1 subunit and not to α_2, β, δ, or γ (Kimura et al, 1994). In addition, in an immunoprecipitation assay study, 23% of the samples of sporadic ALS exhibited autoantibodies to P/Q-type calcium channels (Lennon et al, 1995), a predominant calcium channel subtype found in mammalian motor neurons (Umemiya & Berger, 1994). These antibodies affected calcium currents. Delbono and his colleagues used the voltage-clamp technique and rat extensor digitorum longus muscle preparations to demonstrate that peak calcium current (I_{Ca}) was reduced in dihydropyridine (DHP)-sensitive L-type calcium channels (Delbono et al, 1991b). Further investigation observed that ALS IgG reduced mean channel open time and amplitude in mammalian single skeletal muscle DHP-sensitive calcium channels (Magnelli et al, 1993). This finding, the inhibition of L-type calcium activity, seemed inconsistent with the effects on motor nerve terminals. Recent studies, however, have reported that neuronal P-type Ca^{2+} channels in mammalian cerebellar Purkinje cells and in isolated channel protein in lipid bilayer which were exposed to ALS immunoglobulins showed an enhancement of Ca^{2+} current (Llinas et al, 1993). Similarly, ALS immunoglobulins increased I_{Ca} in hybrid motoneuron cell line VSC4.1. (Mosier et al, 1995): these currents were blocked by the polyamine funnel-web spider toxin, FTX.

It has recently been shown that mammalian neuromuscular transmission (Atchison, 1989; Protti et al, 1991) and excitatory and inhibitory neuromuscular transmission at the crayfish opener muscle (Araque et al, 1994) are insensitive to both ω-conotoxin (ω-CgTX) and DHPs. Furthermore, transmission in those systems is inhibited by FTX and ω-agatoxin IVA (ω-Aga-IVA). On the other hand ω-CgTX decreased the

frequency and amplitude of MEPP and had no effect on quantal content at the NMJ of mouse diaphragm (Protti et al, 1991). The presynaptic N-type voltage dependent calcium channels (VDCCs) which mediate transmitter release were irreversibly blocked by ω-CgTX at the electromotoneuron-electrocyte synapses of the weakly electric fish *Gymnotus carapo* (Sierra et al, 1995).

The primary objective of present work is to further define the action of ALS sera on the presynaptic process of neuromuscular transmission and to assess the effects of ALS sera on calcium currents. Since calcium channel activity in mammalian motor nerve terminals is difficult to test directly, DRG cells were selected for study. The focus is on the short term *in vivo* effects of ALS humoral factors on neurally evoked EPPs and on quantal content and calcium channels. Using the mouse passive transfer model, the ability of serological samples from 18 ALS patients to alter pre- and postsynaptic parameters of neuromuscular transmission was tested, and the results were compared with those produced by control sera from healthy subjects. In addition, neuromuscular transmission in the wobbler mice, which is an autosomal recessive mutant and characterized by progressive weakness and atrophy of forelimb, shoulder girdle and bulbar muscles (Duchen et al, 1968; Bradley, 1984), was compared. Parts of these results were presented elsewhere (Yan et al, 1997).

METHODS

Passive transfer and animal preparation

ICR mice of either sex, weighing 25~30 g were used in the experiments. Whole blood was collected from human test subjects and spun in a centrifuge; the sera was removed and stored at −20°C until needed. Passive transfer model mice were given an intraperitoneal injection of 0.5 ml of ALS or control serum daily, for 3 days. On the fourth day the animals were anesthetized with urethane (1 g/kg), and the diaphragm with intact phrenic nerve was removed. The diaphragm was then cut in half and prepared for the *in vitro* recording of synaptic biopotentials at the NMJ.

Neuromuscular junction recording and analysis

Biopotentials at the NMJ were recorded using conventional intracellular microelectrode recording techniques (Fatt & Katz, 1951). Glass microelectrodes filled with 3 M KCl had resistances in the range of 5~10 MΩ. The first hemi-diaphragm muscle was placed in the bottom of a Sylgard-covered recording chamber containing normal Ringer's solution (12 ml) consisted of (mM) NaCl 135, $NaHCO_3$ 15, Na_2HPO_4 1, $CaCl_2$ 2, $MgCl_2$ 1, and glucose 11, with pH of 7.2 to 7.4. All solutions were aerated with 95% O_2 and 5% CO_2 throughout the experiment, and the recording chamber was maintained at 30±2°C. The second hemi-diaphragm was placed for 40~60 min in Ringer's solution containing low $[Ca^{2+}]_o$ (1.2 mM) and high $[Mg^{2+}]_o$ (8 mM) in which isoosmolarity was maintained by adjusting $[Na^+]_o$. The $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ values were chosen in order to eliminate muscle twitching in response to electrical stimulation. Comparison of data from ALS and control mice was made between preparations exposed to the same $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$. In order to ensure focal recordings from the endplates, the electrode was inserted into fibers near the endplate region (Fig. 1A), and data were excluded if the mean rise-time of the MEPPs was greater than 1 ms or if the initial resting membrane potential (RMP) was more positive than −70 mV. The neuromuscular recording system is shown in Fig. 1B. Recorded biopotentials were fed into a microprobe amplifier (GeneClamp 500, Axon Instruments, U.S.A), and output signal from the amplifier was fed into two channels. One of these was fed into an oscilloscope (Tektronix 2430 A, U.S.A), and then into an Analog/Digital converter (PP-50 Lab, Warner Instrument, U.S.A), which was used to record both the MEPP and EPP waveforms; the other channel was fed into the Analog/Digital converter, which was set to allow DC voltages and was used to record the RMP. The oscilloscope amplified the biopotential signals, and these were then recorded into a computer. To stimulate the phrenic nerve and evoke EPPs, a stimulator (S88, Grass Instruments, U.S.A) was used. One channel of this was delivered to a bipolar stimulating electrode via a stimulation isolation unit (SIU5, Grass Instruments, U.S.A), while the other served as a trigger for the computer and oscilloscope.

An automated computer system, which was developed by O'Shaughnessy & Kim (1995) based on an IBM-PC compatible computer for complete analysis of 45 parameters of neuromuscular transmission, was used for on-line acquisition and signal processing of focally recorded spontaneous MEPPs and evoked EPPs. The program calculated MEPP and EPP amplitudes, rise-times, and half-decay times. Quantal content at each junction was computed by three different methods: the direct method (m_d), the indirect

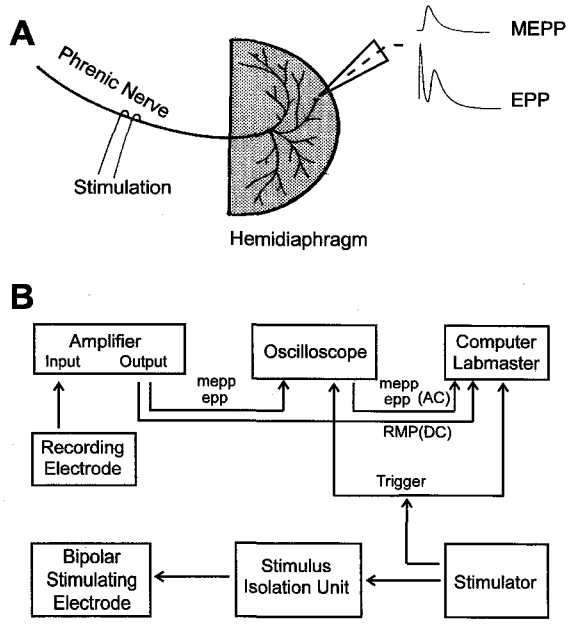

Fig. 1. Equipment configuration of the neuromuscular recording system. The biopotentials were recorded from a hemi-diaphragm with intracellular recording electrode filled with 3 M KCl. It was inserted into muscle fibers near endplate region (A). The biopotentials were fed into the microprobe amplifier (B). One of the amplifier output, DC signal, was monitored and recorded as resting membrane potential (RMP). The other output, miniature endplate potential (MEPP) or endplate potential (EPP), was fed into the oscilloscope and processed as AC signal. One channel of the stimulator was used to elicit EPPs through the bipolar stimulating electrode, while the other served as a trigger for the computer and oscilloscope.

method (m_i) and the failure method (m_f):

$$m_d = EPP/MEPP$$

$$m_i = EPP/q = (EPP)^2/v = (EPP/\sigma)^2$$

$$m_f = \log_e (N/N_f),$$

where EPP is the mean corrected amplitude of 30 evoked potentials, excluding the first 10 EPPs; MEPP is the mean corrected amplitude of 20 consecutive MEPPs, excluding 'giant' or 'slow' MEPP which does not participate in the evoked quantal release process; q is the quantal size; v and σ are the variance and standard deviation of a series of corrected EPP failures, respectively.

Preparation of single DRG cells

Acutely dissociated dorsal root ganglia neurons were prepared from mice (25~30 g, ICR). The animals were anesthetized with urethane (1 g/kg), and the spinal column was removed and placed in a culture dish containing (in mM) NaCl 140, KCl 5, HEPES 10, and glucose 25, with pH of 7.4 (Solution A). The isolated ganglia were washed three times with solution A and were incubated at 37°C for 20 min with a 1 ml Ca^{2+}-free collagenase solution (Collagenase IA 0.15%, Hyaluronidase IS 0.1%, Sigma, U.S.A.). They were then transferred to solution A and washed three times with this solution, to which 0.25% trypsin had been added, and incubated for a further 10 min. Ganglia were removed and placed in media (Dulbecco's modified Eagle's medium with supplements of 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml of streptomycin, 0.5 ml), which were then isolated by trituration of the ganglia using fire-polished pasteur pipette with progressively finer tips. Isolated cells were plated on polyethyleneimine (Sigma, U.S.A.)-coated glass coverslips. After waiting for about an hour until surface cells settled down, the coverslips were flooded with media and kept for up to 2 days in CO_2 incubator at 37°C.

Whole cell patch-clamp recordings

Voltage-clamp recordings were obtained using the standard giga-seal, whole-cell patch-clamp recording techniques (Hamill et al, 1981). Single cell were voltage clamped, and membrane currents were measured using the whole-cell configuration of patch clamp technique (Fig. 2). Aliquots of single DRG cells in suspension (0.5 ml) were added to the recording chamber mounted on an inverted microscope (Nikon Diaphot, Japan). Solutions were superfused through the chamber by gravity at a rate of 2~3 ml/min. I_{Ca} from mouse DRG cell was measured using a patch-clamp amplifier (EPC-7, List, Germany). Electrodes were fabricated from borosilicate capillary tubes (Kimax-51, 1.5 mm inner diameter, Kimble, U.S.A) by pulling on a microelectrode puller (Narishige, pp-83, Japan). The resistance of electrodes ranged from 2 to 5 MΩ. Electrode tips were coated with Sylgard (Dow Corning, U.S.A.) to reduce capacitive current, and remnant capacitive transients were routinely cancelled using the EPC-7's cancellation circuitry. For patch-clamp experiment, DRG cells on the culture coverslips were transferred to the recording chamber filled with bath solution containing (in mM) N-methyl-D-glucamine (NMG) 140, $CaCl_2$ 2.5, $MgCl_2$ 0.5, KCl 2.7, tetraethylammonium chloride (TEA-Cl) 2.7, glucose 5, and HEPES 10, buffered to pH 7.4. Osmolarity was adjusted to 310 mOsm by sucrose. The patch pipettes filling solution contained (in mM) CsCl 120, TEA-Cl 20, $MgCl_2$ 2, EGTA 11, HEPES 10, and K-ATP 2, and pH was buffered to 7.4 with CsOH. Osmolarity was adjusted

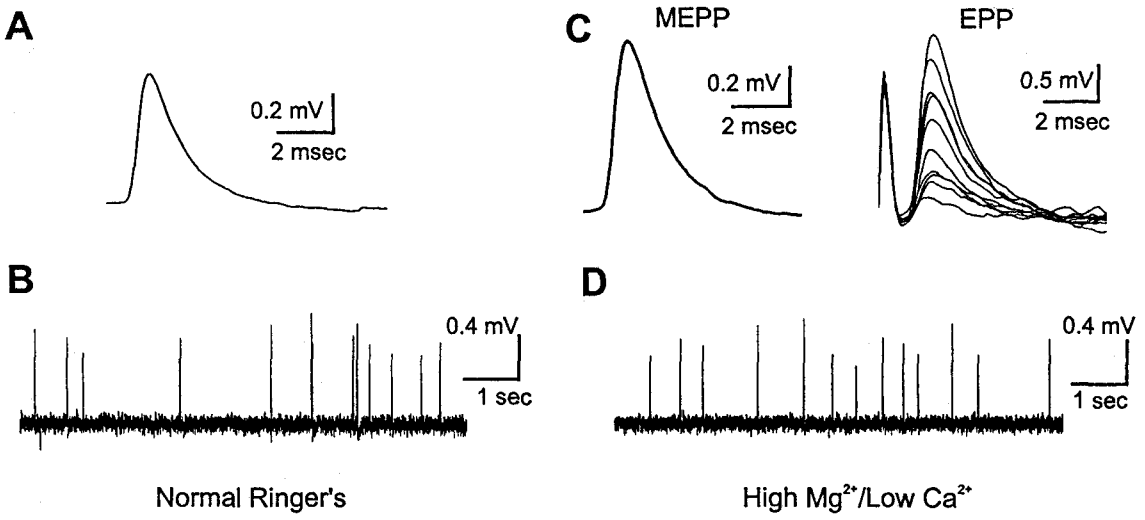

Fig. 2. Examples of MEPP and EPP waveforms. They were recorded either in normal Ringer's solution (A and B) or in high Mg^{2+}/low Ca^{2+} solution (C and D). A. Sixty MEPPs were recorded from an endplate and ensemble-averaged. B. MEPPs were recorded continuously to measure the frequency. C. Ensemble-averaged waveforms of sixty MEPPs were shown. Phrenic nerve-evoked EPPs were superimposed. D. MEPPs were continuously recorded.

Table 1. Clinical features and electrodiagnostic findings from ALS patients

ALS patient No.	Age	Sex	Duration (month)	Lower motor Neuron[a]	Upper motor Neuron[b]	Electrophysiology[c]
1	75	M	15	+++	+++	+++
2	48	F	20	+	++	++
3	44	F	7	+	0	+++
4	69	M	8	++	+	++
5	35	F	18	++	+	++
6	71	M	24	++	++	+++
7	54	F	18	+++	+	++
8	18	M	6	++	++	++
9	48	M	6	+	+	++
10	31	M	6	+	0	++
11	47	M	18	+	++	++
12	64	M	8	++	+++	++
13	59	F	31	+++	++	+++
14	55	F	18	++	++	++
15	42	M	24	++	+	+++
16	70	M	12	++	+	++
17	57	M	18	+	+	++
18	31	F	4	+++	+++	+++

[a]: +++, prominent muscle atrophy and fasciculations; ++, mild to moderate muscle atrophy and fasciculations; +, minimal or absent muscle atrophy, rare fasciculations.
[b]: +++, marked spasticity, hyperreflexia, and extensor-plantar responses; ++, only two of above symptoms or moderate spasticity and hyperreflexia; +, only one of above symptoms.
[c]: Wide spread denervation potentials: +++, marked; ++, moderate; +, minimal.

to 300 mOsm. To allow sufficient time for the diffusion of internal solution into the cell, the current recording was started 1 min after the rupture. All experiments were performed at room temperature (20~25°C).

Depolarizing voltage pulses were generated using

an IBM-compatible computer and pClamp software v.5.5.1. (Axon Instruments, U.S.A); data were displayed on a digital oscilloscope (PM 3350, Philips, The Netherlands) and a computer monitor. The following stimulating protocols were used. 1) Activation protocol: 150 msec depolarizing pulses with 10 mV increments from −80 mV to 60 mV; holding potential was −100 mV. 2) Inactivation protocol: from a holding potential of −80 mV, steady state inactivation was induced by 5 sec pre-pulses varying from −110 mV to 10 mV, with 10 mV increments. After 2.5 msec interval at −80 mV, 10 mV test pulses were given for 200 msec.

Control and ALS patients

The sera used in this study were obtained from 18 ALS patients who had been clinically and electromyographically diagnosed as suffering from ALS in the Department of Neurology, Seoul National University Hospital or in Samsung Medical Center. Their ages ranged from 31 to 75 years, eleven were male, and seven were female. The clinical features and electrodiagnostic findings of these patients are shown in Table 1. In the control group, the sera were obtained from seven healthy persons (six males and one female), aged between 28 and 47 years.

Statistics

All data are expressed as the mean±standard error (SEM) and the error bars in figures also indicate SEM. The statistical significance of difference was determined by non-paired student's t-test, and a p-value of <0.05 was considered to be significant.

RESULTS

Characterization of MEPPs and nerve evoked EPPs in normal mice

Spontaneous quantal release was determined by measuring MEPP, which was recorded from mouse phrenic nerve-diaphragm in normal Ringer's solution and high Mg^{2+}/low Ca^{2+} solution. For each fiber, data were gathered from 20 spontaneous MEPPs. Fig. 2 shows an example of MEPPs recorded in normal Ringer's solution. An ensemble averaged wave form of 60 MEPPs is shown in Fig. 2A, and a continuous recording of MEPPs in Fig. 2B. MEPP wave parameters including amplitude, rise time and half-decay time were obtained from 62 end plates of six animals. Average MEPPs amplitude was 0.84±0.04 mV, rise time was 0.84±0.02 ms, and half-decay time was 1.19±0.02 ms. Mean MEPP frequency was 2.19±0.15 Hz, and resting membrane potential was −79.80± 0.98 mV.

Nerve-evoked EPP and quantal content were recorded in a high Mg^{2+} (8 mM) and low Ca^{2+} (1.2 mM) solution to prevent muscle twitch. EPPs were evoked by a concentric bipolar electrode that stimulated the phrenic nerve with a square wave pulse of between 5 and 20 V, 0.06 ms in duration, at the frequency of 1 Hz. For each fiber, data from 20 spontaneous MEPPs and 30 evoked EPPs were gathered. EPP and MEPP waveforms were nearly identical in shape and differed only in amplitude. Examples of EPP and MEPP trace are shown in Fig. 2C. MEPPs were continuously recorded for 8 sec, as shown in Fig. 2D. Average values from 53 endplates (11 animals) were as follows: for MEPP, amplitude 0.60±0.03 mV, rise time 0.82±0.02 msec, half-decay time 1.07±0.03 msec, frequency 1.75±0.22 Hz, RMP −81.7±0.94 mV, and for EPP, amplitude 0.92±0.08 mV, rise time 0.80±0.02 msec, half-decay time 1.07±0.03 msec, RMP −76.8±0.91 mV, evoke failure 22.64±2.19, direct quantal content (M_d) 1.68±0.18, indirect quantal content (M_i) 2.12±0.31, quantal content obtained by failure method (M_f) 1.58± 0.10.

Observed MEPP frequency and amplitude were normally distributed around the mean population (Fig. 3). In normal Ringer's solution, MEPP amplitude distributed between 0.5 and 1.0 mV population, and frequency distributed between 1.5 and 2.5 Hz population (Fig. 3A). In high Mg^{2+}/low Ca^{2+} solution, MEPPs amplitude distributed between 0.3 and 0.75 mV population, and frequency distributed between 1 and 2 Hz population (Fig. 3B).

Effects of ALS sera on MEPP frequency and amplitude

In a previous study where mice were subjected to subcutaneous injection of ALS Ig for 4 to 12 weeks, Uchitel et al (1992b) found that evoked transmitter release was highly variable. In the present work, injection of ALS sera was performed for a shorter period in order to assess the early effects of the sera on neuromuscular transmission.

ALS sera tended to produce an increase in MEPP frequency when MEPPs were recorded in normal Ringer's solution. In Fig. 4A, an example of MEPP recorded in a mouse treated with sera from control

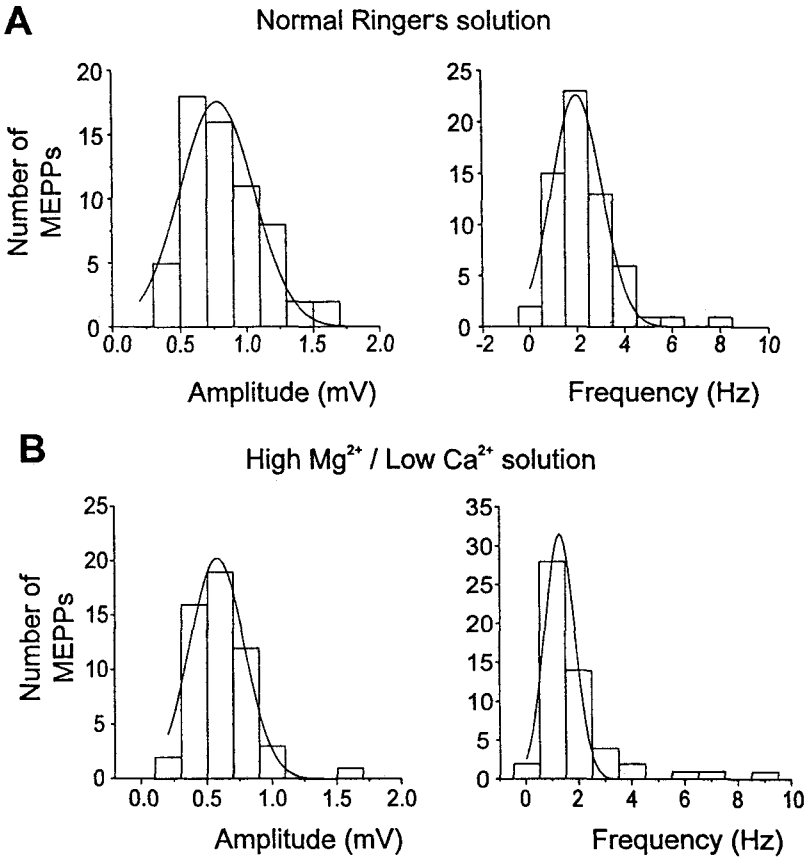

Fig. 3. Amplitude and frequency histograms of MEPPs in mice. The histogram were fitted with Gaussian distribution function. A. Data were obtained from six animals in normal Ringer's solution. B. Data from 11 animals were recorded in high Mg^{2+}/low Ca^{2+} solutions. The concentrations of Mg^{2+} and Ca^{2+} were 8 mM and 1.2 mM, respectively.

and ALS patient in normal Ringer's solution is shown. MEPP traces were continuously recorded for 8 sec. The effects of individual sera from ALS patients on MEPP frequency are shown in Fig. 5. In normal Ringer' solution, sera from 14 of 18 ALS patients caused a statistically significant increase in MEPP frequency (Fig. 5A); sera of the remaining four patients did not alter the frequency. Mean MEPP frequency in the ALS group was 4.62 ± 0.14 Hz (n=681), a significant increase compared with the control group (2.18 ± 0.15 Hz, n=129, $p < 0.001$, Table 2). It was also seen in MEPPs recorded in high Mg^{2+}/low Ca^{2+} solution that MEPP frequency tended to increase. The increase caused by sera from 13 patients was significant (Fig. 5B). Mean MEPP frequency in the ALS group (6.09 ± 0.38 Hz, n=422) showed a significant increase, compared with the control group (2.18 ± 0.31 Hz, n=104, $p < 0.001$, Table 2). Sera from three ALS patients caused no consistent effects on MEPP frequency in normal Ringer's and high Mg^{2+}/low Ca^{2+} solution. Three ALS samples increased MEPP frequency in normal Ringer's solution, but had no effect in high Mg^{2+} /Low Ca^{2+} solution. Likewise, three ALS samples increased MEPP frequency in high Mg^{2+}/low Ca^{2+} solution, but had no effect in normal Ringer's solution.

A plot of the distribution of MEPP frequencies in fiber populations treated with ALS sera revealed a distinct shift to the right compared with the control curve which more closely follows a skewed Gaussian pattern (Fig. 5C). This suggests that increases in the spontaneous transmitter release are relatively uniformly distributed among the ALS sera-treated motor nerve terminals.

In contrast to their effects on MEPP frequency, the ALS sera caused no consistent effects on MEPP amplitude (Table 2). Some of these sera caused an increase, whereas others had no effect on MEPP amplitude. Furthermore, the range of effects on MEPP amplitude is narrower compared with the frequency of release, producing changes of less than

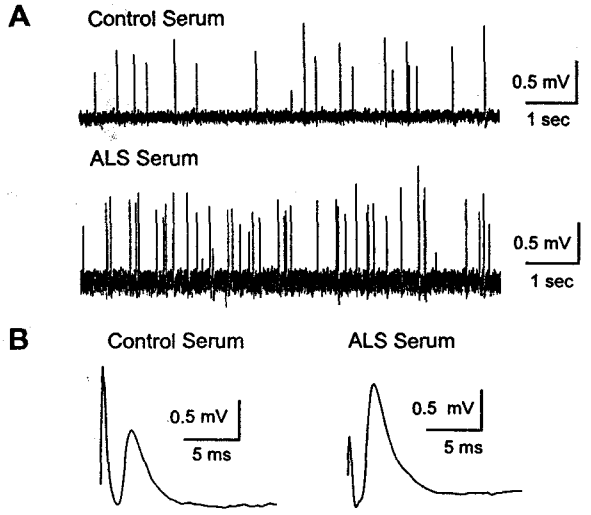

Fig. 4. Effects of ALS sera on MEPP and nerve-evoked EPP. A. An example of MEPPs recorded from diaphragm of mouse treated with sera from control (upper trace) and ALS patients (lower trace) in normal Ringer's solution. Mean frequency of MEPP from the ALS group was significantly increased compared with the control group. B. An example of averaged EPPs (n=30) recorded from the control (left) and ALS (right) group. Mean amplitude of EPPs from ALS group was significantly increased compared with the control group.

50%. MEPPs measured in high Mg^{2+}/low Ca^{2+} solution similarly exhibited no consistent difference in their amplitudes compared with controls (Table 2). Sera in the ALS group did not cause any increase in MEPP amplitude compared with the control group.

As with MEPP amplitude, characteristics of the time course of MEPP waveforms did not appear to be altered by the treatment with ALS sera (Table 3). Rise time and half-decay time, measured either in normal Ringer's or high Mg^{2+}/low Ca^{2+} solution, obtained values similar to the control. Likewise, RMP recorded from the endplate region remained unchanged from the control. These findings suggest that the sera did not modify postjunctional sensitivity to ACh or the function of ACh receptors.

Effects of ALS sera on EPP amplitude and quantal content:

In order to determine the mean number of ACh quanta released per nerve impulse, we exposed the diaphragm muscle preparations to high Mg^{2+}/low Ca^{2+} solution. Under these conditions, repetitive stimulation of the phrenic nerve caused intermittent failures of EPPs in animals receiving control sera, the

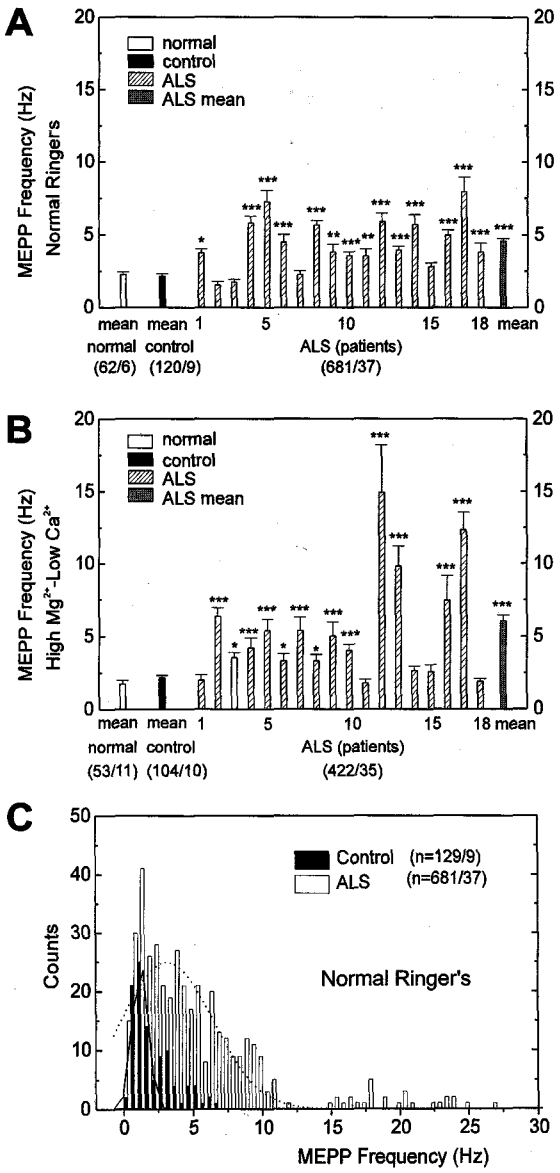

Fig. 5. Effects of sera from each ALS patient on MEPP frequency. MEPPs were recorded from diaphragm in normal Ringer's solution (A) or high Mg^{2+}/low Ca^{2+} solution (B). Each bar represents mean MEPP frequency (±SEM). The data of 'normal group' were recorded from normal (untreated) mice. The 'control group' was from mice injected with sera from seven healthy humans. The 'ALS group' was from mice injected with sera from 18 ALS patients. The 'mean' values are the averages of total MEPP frequencies in each group. The frequencies of each ALS patient or mean frequency of total ALS group were compared with mean frequency of the control group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (Student's t-test). C. All the data from animals treated with control and ALS sera were plotted. The curves are Gaussian fits to the histogram. The MEPPs were recorded in normal Ringer's solutions. n = number of endplates / number of animals.

Table 2. Effects of ALS patients' sera on MEPP, EPP, and quantal content (QC) in mice hemidiaphragm preparations (mean±SEM)

	Normal Ringer's			High Mg^{2+}/Low Ca^{2+}					
	MEPP			MEPP			EPP		
	Frequency (Hz)	Amplitude (mV)	N	Frequency (Hz)	Amplitude (mV)	N	Amplitude (mV)	QC (M_d)	N
Normal	2.28±0.17	0.84±0.04	62/6	1.75±0.22	0.60±0.03	53/11	0.92±0.08	1.68±0.12	53/11
Control	2.18±0.15	0.95±0.03	120/9	2.18±0.31	0.64±0.05	104/10	0.98±0.05	1.49±0.07	105/10
ALS	4.62±0.14*	0.88±0.01	681/37	6.09±0.38*	0.58±0.01	422/35	1.30±0.04*	2.35±0.07*	338/33

Normal: Untreated normal mice, Control: Mice injected with sera from seven healthy humans, ALS: Mice injected with sera from 18 ALS patients. N: number of endplates / number of animals
*$p<0.001$ (Student's t-test)

Table 3. Neuromuscular junction parameters from animals treated with control and ALS sera

		Normal Ringer's		High Mg^{2+}/Low Ca^{2+}	
		Control	ALS	Control	ALS
MEPP	Amplitude (mV)	0.95±0.06	0.88±0.01	0.64±0.02	0.58±0.01
	Rise time (ms)	0.88±0.01	0.84±0.01	0.82±0.02	0.82±0.01
	Half-decay time (ms)	1.29±0.02	1.21±0.01	1.11±0.02	1.11±0.01
EPP	Rise time (ms)			0.78±0.02	0.80±0.01
	Half-decay time (ms)			1.05±0.02	1.12±0.01
	Resting membrane potential (mV)	−76.13±0.26	−80.25±0.33	−75.74±0.59	−72.67±0.36
	Number of fibers	120	681	105	338

average percentage of failure being 17.46±1.35%, though in the ALS group this decreased to 12.59±0.69% ($p<0.001$). The rise-times, half-decay times and resting membrane potentials for EPPs were not significantly altered (Table 3). Examination of nerve-evoked EPPs showed that some ALS sera caused a marked increase in EPP amplitude. In Figure 4B, an example of EPP trace recorded from the control and ALS group is shown. It was an ensemble averaged waveform of 30 EPPs. The sera from 11 of 18 patients produced a significant increase in EPP amplitude (Fig. 6A), while sera from the remaining seven caused no change. Average EPP amplitude in the ALS group was 1.30±0.04 mV (n=338), which was significantly higher than that in the control group (0.92±0.05 mV, n=105, $p<0.001$) (Table. 2). While EPP amplitudes were increased by ALS sera, MEPP amplitude remained relatively unchanged. Thus, there were only negligible postjunctional effects of ALS sera; this result is similar to that observed when normal Ringer's solution was used.

In the ALS group, EPP quantal content measured using the direct method (m_d) also showed a trend toward elevated levels. Sera from 14 patients produced significant rises in quantal content (Fig. 6B). Mean quantal content for these ALS group mice was 2.35±0.07 (n=338), while the mean control group value was 1.49±0.07 (n=105). The value in the ALS group was significantly higher than that in the control group ($p<0.001$, Table 2). When plotted as histograms, the distributions of quantal content exhibited a difference between the control and ALS population (Fig. 6C). As was the case with MEPP frequency, ALS endplates generated a curve that shifted towards higher values of m_d, and the distribution was spread over a wider range. Increases in quantal content in sera from 10 of 14 patients were accompanied by a significant increase in the EPP amplitude. Further-

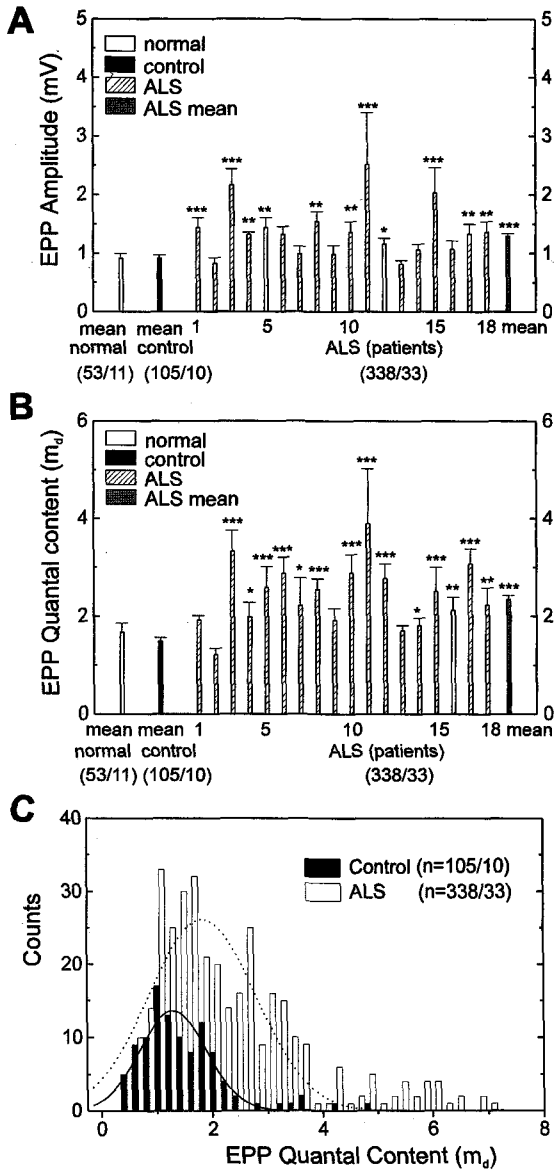

Fig. 6. Effects of sera from each ALS patient on amplitude and quantal content of nerve-evoked EPP. EPPs were recorded from diaphragm in high Mg^{2+}/low Ca^{2+} solution. Each bar represents mean EPP amplitude (A) or quantal content (B) (±SEM). The treatment of normal, control, and ALS group were same as that of Fig. 5. The 'mean' values are averages of total EPP amplitudes or quantal content in each group. The amplitude and quantal content of each ALS patient or those of total ALS group were compared with mean values of the control group. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test). C. All the data from animals treated with control and ALS sera were plotted. The curves are Gaussian fits to the histogram. n = number of endplates / number of animals.

more, in sera from 11 of these 14, the increases correlated with significant increases in MEPP frequency recorded in normal Ringer's solution and high Mg^{2+}/low Ca^{2+} solution, respectively. Patterns of MEPP, EPP and quantal content which were altered

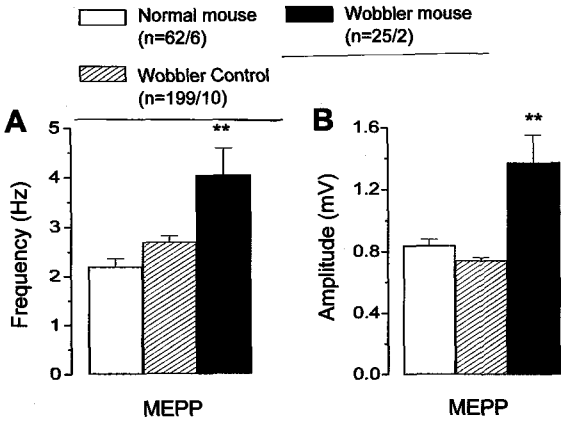

Fig. 7. MEPP were recorded from normal or wobbler mouse diaphragms in normal Ringer's solution. MEPP frequency (A) and amplitude (B) of wobbler mice were significantly increased compared with those of normal or wobbler control (genetically wobbler mice, but without distinct symptoms) mice. **$p<0.01$. n= number of endplates / number of animals.

by sera from 18 ALS patients are illustrated in Table 4.

We repeated the same comparison of quantal content estimates obtained using the indirect variance (m_i) and failure (m_f) methods, and this led to essentially the same conclusion. The fact that EPP quantal content values estimated by these methods are in close agreement suggests that evoked transmitter release in both ALS and control sera-treated junctions follows a Poisson process. In conclusion, the results of our EPP amplitude and quantal content measurements clearly indicate that ALS sera acts presynaptically and that this leads to an enhanced number of ACh quanta released per motor nerve impulse.

MEPP recording in wobbler mice

In the study using the wobbler mouse diaphragm, it tended to produce an increase in MEPP frequency and amplitude when MEPPs were recorded in normal Ringer's solution. Mean MEPP frequency of two wobbler mice was 4.03±0.53 Hz (n=25), and amplitude was 1.37±0.18 mV. We used ten wobbler mice without symptoms as a control group for symptomatic wobblers. Observed mean MEPP frequency and amplitude in control group was 2.69± 0.12 Hz and 0.74 mV±0.02 mV (n=199), respectively. MEPP frequency and amplitude recorded in wobbler mice were significantly higher than in control and normal group mice (Fig. 7). This suggests that in wobbler mice there is an increased spontaneous transmitter release at the NMJ. Because of muscle twitch, nerve-evoked EPP and quantal content

Table 4. Patterns of changes in MEPP, EPP, and quantal content (QC) of mice diaphragm injected with 18 ALS patients' sera

	Normal Ringer's		High Mg^{2+}/Low Ca^{2+}			
	MEPP		MEPP		EPP	
	Frequency	Amplitude	Frequency	Amplitude	Amplitude	QC(M_d)
Increase	14	1	13	1	11	14
Decrease	0	6	1	4	0	0
No effect	4	11	4	13	7	4
Sum	18	18	18	18	18	18
N	681/37	681/37	422/35	422/35	338/33	338/33

Each number except N indicates the number of patients.
N= number of endplates / number of animals

were difficult to record in wobbler mice.

Effects of ALS sera on Ca^{2+} currents in DRG cells

The calcium currents were recorded from single isolated DRG cells of mice in normal, passively transferred control and ALS sera. DRG cells between 20 and 30 μm in diameter were selected, and elicited voltage dependent Ca^{2+} currents in response to depolarizing test potentials applied in the whole-cell patch clamp configuration. Mouse DRG cells displayed transient and sustained Ca^{2+} currents separable into low voltage activated (LVA) and high voltage activated (HVA) Ca^{2+} current components. Of the 135 cells sampled, approximately 5.9% (n=8/135, normal=4/47, control=2/36, ALS=2/50) had classical T currents. HVA currents were observed in most cells. In the present study, if DRG cells elicited LVA currents, they were not included in statistical treatment.

The effect of ALS sera on the HVA Ca^{2+} current is shown in Fig. 8. In mice treated with sera from ALS patients, HVA Ca^{2+} currents were reduced. Fig. 8A shows examples of Ca^{2+} current traces recorded from DRG cells in the normal, control, and ALS groups. Peak I_{Ca} values in seven normal animals and in seven control animals were at -10 mV, -2189 ± 140 pA (n=43) and -2131 ± 160 pA (n=34), respectively. I_{Ca} in the ALS group (10 animals) at this potential was -911 ± 49 pA (n=50). The ALS sera significantly reduced peak I_{Ca} ($p<0.001$), but there was no change in reversal potential for I_{Ca}. The I-V curve was not shifted by ALS sera (Fig. 8B). In the ALS group, peak I_{Ca} at -20 mV, -10 mV, and 0 mV test potential was -24.74 ± 2.50 pA/pF, -45.8 ± 2.92 pA/pF, and -44.4 ± 3.25 pA/pF, respectively. In the control group, the values at these potentials were -67.2 ± 6.83 pA/pF, -98.3 ± 5.73 pA/pF and -80.6 ± 4.63 pA/pF, respectively. ALS sera significantly decreased normalized Ca^{2+} current ($p<0.001$). This observation indicates that ALS sera could affect the calcium channel in DRG cells and reduce calcium channel activities.

To further characterize the Ca^{2+} currents in DRG cells, we determined the voltage dependence of activation and steady-state inactivation (Fig. 8C). The activation curve was determined from normalized conductance in which the conductance at a given potential was divided by the maximum conductance (holding potential, -100 mV). The curve was fitted with the Boltzman equation, $h_\infty = [1+\exp\{(V- V_{1/2})/k\}]^{-1}$. The currents began to activate at -40 mV. Half-activation voltages were -18.42 mV, -21.51 mV and -18.30 mV in the normal, control and ALS group, respectively. The slope factor was 3.63, 3.87 and 4.70 in these same respective groups. Values in the ALS group were not significantly different from those in the control group. As previously shown, I_{Ca} amplitude was reduced by ALS sera, but the activation process was not affected. To determine the voltage dependence of steady-state inactivation, I_{Ca} values at the test pulse were normalized to their maximum (-110 mV conditioning pulse). The observed curve was fitted by Boltzman equation: half-inactivation potentials were -35.43 mV, -36.76 mV and -43.74 mV, and slope factor (k) values were 14.75, 13.36 and 14.65, in the normal, control and ALS groups, respectively. ALS sera produced a change in half-inactivation potential ($p<0.05$), and the steady-state inactivation curve shifted towards

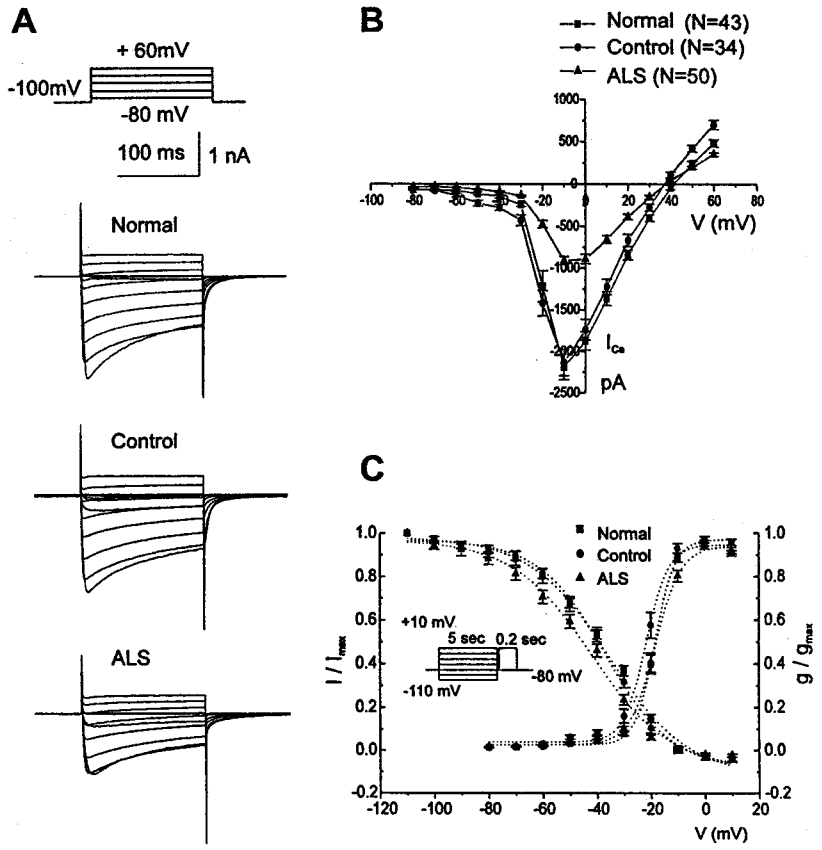

Fig. 8. Whole cell patch-clamp recordings of I_{Ca} from mouse DRG cells. The currents of the 'Normal' were recorded from normal (untreated) mice. 'Control' and 'ALS' were from mice treated with sera from four healthy humans and five ALS patients, respectively. A. The traces of I_{Ca} were produced by 150 msec depolarizing pulses with 10 mV increments from −80 mV to 60 mV. Holding potential was −100 mV. B. Current-voltage relationship in the normal, control, and ALS groups. C. Inactivation and activation characteristics of I_{Ca} in DRG cells. The left ordinate axis indicates the inactivation parameters and the right axis indicates the normalized conductance. The currents were obtained by an inactivation protocol: from the holding potential of −80 mV, the steady state inactivation was induced by 5 sec prepulses varying from −110 mV to 10 mV with 10 mV increments. After 2.5 msec interval at −80 mV, 10 mV test pulses were given for 200 msec. The smooth curves were drawn by fitting to Boltzman equation.

more negative potentials. These results suggest that the inactivation processes of HVA Ca^{2+} currents in DRG cells are altered by ALS sera.

DISCUSSION

The aim of the present study was to assess the short-term *in vivo* effects of the ALS humoral factors on neuromuscular transmission and calcium channels in DRG cells. The first step of assessing the effects of ALS sera on synaptic function at the NMJ was an evaluation of spontaneous transmitter release. Fourteen ALS sera out of 18 sera tested caused an increase in the frequency of MEPPs in normal Ringer's solution but produced no change in MEPP amplitude. These results agree with previous studies which showed that Ig from ALS patients, while not affecting MEPP amplitude or time course, caused a significant increase in MEPP frequency (Uchitel et al, 1988; Appel et al, 1991; Uchitel et al, 1992b). The spontaneous endplate activity observed in high Mg^{2+} /low Ca^{2+} solution, on the other hand, differed from the findings of Uchitel et al (1988), which indicated

that the increase in MEPP frequency was attenuated in the presence of high $[Mg^{2+}]_o$. In the present study, MEPP frequency in high Mg^{2+}/low Ca^{2+} solution was increased.

It has previously been reported that in animals subjected to subcutaneous injection of ALS Ig, quantal content was affected (Uchitel et al, 1992b), but after 4~12 weeks of Ig injection, synaptic response was highly variable. Significant increases, as well as decreases, in quantal content were produced in muscles treated with the same ALS Ig. In some cases, a complete lack of evoked neuromuscular transmission was noted. This synaptic failure was probably due to neuronal death rather than the direct action of ALS Ig. This was supported by a morphological study (Uchitel et al, 1992b) which showed that muscles with decreased quantal content were also the ones showing the greatest extent of morphological change. In the present work, however, we examined the effect of ALS sera on evoked ACh release at a much earlier time. After three days of passive transfer of ALS sera, the effect on nerve-evoked transmitter release was found to be increased. A rise in quantal content, accompanied by increased EPP amplitudes and unchanged MEPP amplitudes, supports the view that ALS humoral factors act on presynaptic calcium channels.

In the present study it was observed that HVA Ca^{2+} currents were significantly reduced and rapidly inactivated by ALS sera. It is possible that ALS antibodies targeted Ca^{2+} channels, then further blocked Ca^{2+} channel opening, or reduced the number of Ca^{2+} channels. These results agree with those of a previous study showing that ALS IgG reduced DHP-sensitive I_{Ca} amplitude and charge movement in rat extensor digitorum longus muscle (Delbono et al, 1991a, b). Our finding of the effects of ALS sera on I_{Ca} activation and inactivation processes differed from the findings of Delbono et al, which observed that I_{Ca} was slowly activated by ALS IgG and that the inactivation process was unaltered. The observed difference in activation and inactivation kinetics between skeletal muscle fibers and DRG neurons could be related to different characteristics of Ca^{2+} channel activation and inactivation. Another study supported our results, in which ALS IgG reduced mean channel open time and amplitude in mammalian single skeletal muscle DHP-sensitive calcium activity (Magnelli et al, 1993). In these studies, calcium entry was reduced by IgG and sera from ALS patients. Our study is consistent with the finding that Lambert-Eaton myasthenia syndrome (LES) antibodies inhibit I_{Ca} in chromaffin cells (Kim & Neher, 1988) and DRG cells (Garcia et al, 1996).

Enhancement of MEPP frequency, of nerve-evoked EPP amplitude, and of quantal content, would be expected to result in excitation, rather than inhibition, of calcium entry. The apparent discrepancy between these results rather suggests an unnoticed aspect of pathophysiology of ALS. The same serum factor could affect both motor and sensory system resulting in opposite responses, which is determined by the different expression of Ca^{2+} channel subtypes.

The relevant Ca^{2+} channels at motor nerve terminal are N-type and P-type, rather than L-type. Ca^{2+} influx through VDCCs is the trigger which releases neurotransmitters from the nerve terminals (Katz, 1969; Llinas et al, 1993). The evoked release of neurotransmitters was to be dependent on Ca^{2+} influx through N-type VDCC in sympathetic neurons from the finding that ω-CgTX inhibited the release, and DHP did not affect the release (Hirning et al, 1988). Substance P release from DRG neurons (Perney et al, 1986), and catecholamine release from chromaffin cells (Cena et al, 1983) are strongly inhibited by DHP, which is consistent with the suggestion that L-type Ca^{2+} channels play a major role in this process. Mammalian motor nerve terminals are, however, normally insensitive to either ω-CgTX or DHP (Olivera et al, 1985; Atchison, 1989; Protti et al, 1991). Neurotransmitter release in mammalian synapses was mediated by P-type Ca^{2+} channels, and was insensitive to N- or L-type Ca^{2+} channel blockers (Uchitel et al, 1992a; Araque et al, 1994). Another study observed that N-type VDCCs mediated transmitter release (Sierra et al, 1995). Furthermore, the cytotoxic effect induced by ALS IgG also suggested an increase of calcium entry, via N-type and/or P-type channels (Smith et al, 1994). In the case of mouse NMJ, these channels are primarily P-type (Uchitel et al, 1992a; Kim et al, 1994). The notion that increased quantal content and MEPP frequency are mediated by increased current flow through P-type calcium channels is thus consistent with the results of the study by Llinas et al (1993) that showed an increase of P-type current in Purkinje cells treated with ALS Ig. This work revealed that ALS antibodies act on individual P-type calcium channels by prolonging the open time of channel, not by increasing single channel conductance or the frequency of channel opening; it was further demonstrated that ALS Ig's increased calcium currents in a hybrid motoneuron cell line, VSC4.1. These currents were blocked by the polyamine funnel-web spider toxin FTX, which has previously been shown to block Ca^{2+} currents and evoke transmitter release at mammalian motoneuron

terminals (Mosier et al, 1995). These results suggest that ALS antibodies might produce an increase in calcium flux through P-type channels, could have the opposite effect on calcium flux through L-type channels, and could bind to all types of channel (P, N, and L). Since the increased calcium flux occurred through P type channels, these might represent the main site of ALS antibodies which induce the entry of calcium into motor neurons.

Another plausible explanation for the observed prejunctional effect is that ALS antibodies may also interfere with intracellular Ca^{2+} metabolism; the increased concentration of intraterminal Ca^{2+} ions facilitates spontaneous neurotransmitter release. An elevation in internal Ca^{2+} concentration could be caused by either increased external Ca^{2+} ion influx to the motor nerve terminal or the release of internal Ca^{2+} ions which are stored mainly in the endoplasmic reticulum. One apparent possibility is that the ALS serum factors may cause elevation of motor nerve terminal $[Ca^{2+}]_i$ by augmenting the release of Ca^{2+} from intracellular Ca^{2+} stores. Such enhanced intraterminal Ca^{2+} mobilization would manifest itself as a marked rise in spontaneous, as well as evoked, transmitter release. This explanation fits particularly well with the observed increase in MEPP frequency, a parameter which is not expected to change significantly with the presumed insignificant opening of voltage-gated Ca^{2+} channels at non-depolarized resting membrane potential (Matthews & Wickelgren, 1977). Finally, it may be that ALS antibodies produce NMJ effects by combined action on calcium channels and the intracellular release of Ca^{2+} ions.

ALS autoantibodies may also act on voltage-dependent potassium channels to produce the functional effects characterized in the present experiments. It is well known that when applied to the NMJ, K^+ channel blockers cause a distinctive rise in the nerve-evoked release of ACh, but do not significantly alter spontaneous discharge (Molgo et al, 1977; Kim et al, 1980). This effect is caused by an increased influx of Ca^{2+} during action potential-induced depolarization of the motor nerve terminal membrane, which is longer than normal due to the loss of their hyperpolarizing effects by the blocked K^+ channels. It is noteworthy that fasciculation, a symptom of ALS, may be due to the reduced potassium channel currents in motor neurons and that this blockade could lead to the destruction of motor neurons via an increased level of $[Ca^{2+}]_i$ resulting from the depolarization of the motoneuron membrane (Bostock et al, 1995). We did not, however, find evidence of consistent depolarization of endplate membranes in animals injected with ALS sera.

The present study found that sera from 14 out of 18 ALS patients tested on the NMJ produced a significant effect on EPP quantal content. An increase in calcium entry into the motor nerve terminals produced by ALS Ig is consistent with the proposal that calcium-mediated neurotoxicity is the cause of sporadic ALS (Brown, 1994). The apparent lack of effect in a small number of patients has yet to be explained, though there are some possibilities. The first is simply that in the remaining patients, the effect is too subtle to be detected by the tests. Secondly, the differential effects seen among various ALS patients may correlate with the clinical severity and phase of the disease. A lack of effect in some patients may also indicate that an autoimmune response could be only one of the several possible causes of the sporadic form of ALS. This is quite possible, given that the sporadic and familial forms of ALS already indicate multiple causes of the disease (Younger et al, 1990). The final possibility is that in the ALS patients, autoimmune response is actually a secondary effect, possibly mounted against the P-type calcium channels of motor neurons that are being destroyed by the true cause of disease.

In this study, we observed that in the wobbler mouse, MEPP frequency and amplitude increased. Recent studies on the functions of excitatory amino acids, glutamate and aspartate, in the central nervous system have led to the suggestion that disturbed glutamate transmission may play a key role in the pathophysiology of neurodegenerative diseases. There is an increasing number of evidences that glutamate dysfunction may also cause motor neuron degeneration in ALS patients (Jansen et al, 1990). Reduced glutamate content has been observed in the spinal cord of the wobbler mouse (Tomiyama et al, 1994), and other investigation have shown that motoneuron degeneration in the wobbler mouse is associated with glutamine synthetase (GS) negativity (Blondet et al, 1995), thyrotrophin-releasing hormone (TRH) (Shi & Vacca-Galloway, 1993), and acetyltransferase (ChAT) activities (Yung et al, 1994). These factors (glutamate, GS, TRH and ChAT) may play significant roles in wobbler disease, possibly affecting motoneuron terminals, and enhanced neuromuscular transmission in the diaphragms of these mice.

The above results therefore suggest that ALS serum factor affects neuromuscular transmission and may play an important role in the early stage of ALS. Furthermore, ALS serum factor may interact directly or indirectly with the calcium channel in DRG cells. Any calcium or other channels in DRG cells are

targets for ALS serum factor, and interaction with any receptor and changes in intracellular calcium which trigger the process of motoneuron cell death in ALS are currently under investigation.

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