Passive Transfer of Lambert-Eaton Myasthenic Syndrome Induces Dihydropyridine Sensitivity of $I_{Ca}$ in Mouse Motor Nerve Terminals

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Xu, You-Fen, Sandra J. Hewett, and William D. Atchison. Passive transfer of Lambert-Eaton Myasthenic Syndrome induces dihydropyridine sensitivity of $I_{Ca}$ in mouse motor nerve terminals. J. Neurophysiol. 80: 1056–1069, 1998. Mice were injected for 30 days with plasma from three patients with Lambert-Eaton Myasthenic Syndrome (LEMS). Recordings were made from the perineural sheath of motor axon terminals of triangularis sterni muscle preparations. The objective was to characterize pharmacologically the identity of kinetically distinct, defined potential changes associated with motor nerve terminal Ca$^{2+}$ currents ($I_{Ca}$) that were affected by LEMS autoantibodies. $I_{Ca}$ elicited at 0.01 Hz were significantly reduced in amplitude by $\sim$35% of control in LEMS-treated nerve terminals. During 100-Hz stimulation, $I_{Ca}$ amplitude was unchanged in LEMS-treated motor nerve terminals, but was depressed in control. During 20- or 100-Hz trains, facilitation of $I_{Ca}$ occurred in LEMS-treated nerve terminals whereas in control, no facilitation occurred during the trains at 20 Hz and marked depression occurred at 100 Hz. Saturation for amplitude and duration of $I_{Ca}$ in control terminals occurred at 2 and 4–6 mM extracellular Ca$^{2+}$, respectively; in LEMS-treated terminals, the extracellular Ca$^{2+}$ concentration had to increase by two to three times of control to cause saturation. Amplitude of the two components of $I_{Ca}$ observed when the preparation was exposed to 50 $\mu$M 3,4-diaminopyridine and 1 mM tetraethylammonium were both reduced by LEMS plasma treatment. The fast component ($I_{Ca,f}$) was reduced by 35%, whereas the slow component ($I_{Ca,s}$) was reduced by 37%. $\omega$-Agatoxin IVA ($\omega$-AgA-IVA; 0.15 $\mu$M) and $\omega$-conotoxin-MVIIC ($\omega$-CTx-MVIIC; 5 $\mu$M) completely blocked $I_{Ca}$ in control motor nerve terminals. The same concentrations of toxins were 20–30% less effective in blocking $I_{Ca}$ in LEMS-treated terminals. The residual $I_{Ca}$ remaining after treatment with $\omega$-AgA-IVA or $\omega$-CTx-MVIIC was blocked by 10 $\mu$M nifedipine and 10 $\mu$M Cd$^{2+}$. Thus LEMS plasma appears to downregulate $\omega$-AgA-IVA-sensitive (P-type) and/or $\omega$-CTx-MVIIC-sensitive (Q-type) Ca$^{2+}$ channels in murine motor nerve terminals, whereas dihydropyridine (DHP)-sensitive (L-type) Ca$^{2+}$ channels are unmasked in these terminals. Acute exposure (90 min) of rat forebrain synaptosomes to LEMS immunoglobulins (Igs; 4 mg/ml) did not alter the binding of $[^{3}H]$-nitrendipine or $[^{125}]\omega$-conotoxin-GVIA ($\omega$-CgTx GVIA) when compared with synaptosomes incubated with an equivalent concentration of control Igs. Conversely, LEMS Igs significantly decreased the $B_{max}$ for $[^{3}H]$-verapamil to $\sim$45% of control. The apparent affinity of verapamil ($K_{v}$) for the remaining receptors was not significantly altered. Thus acute exposure of isolated central nerve terminals to LEMS Igs does not increase DHP sensitivity, whereas it reduces the number of binding sites for verapamil but not for nifedipine or $\omega$-CgTx-GVIA. These results suggest that chronic but not acute exposure to LEMS Igs either upregulates or unmasks DHP-sensitive Ca$^{2+}$ channels in motor nerve endings.

 INTRODUCTION

Lambert-Eaton Myasthenic Syndrome (LEMS) is a presynaptic disorder of neuromuscular transmission, in which patients exhibit profound muscle weakness believed to be due to deficient Ca$^{2+}$-dependent release of acetylcholine (ACh) in response to nerve stimuli (Lambert et al. 1956; Lambert and Elmqvist 1971; Vincent et al. 1989). The electrophysiological features of this disorder are reduced quantal content at low frequencies of nerve stimulation with marked facilitation of end-plate potential amplitudes at high frequencies of stimulation (Lambert and Elmqvist 1971). LEMS is an autoimmune disease, which is associated frequently with small cell carcinoma of the lung (SCC) (Lang et al. 1981; Lennon et al. 1982). Mice injected with either plasma or immunoglobulins (Igs) derived from LEMS patients reproduce the electrophysiological and morphological features of the disease (Fukunaga et al. 1983; Kim 1985; Lang et al. 1983).

Indirect results from numerous studies suggest that the autoimmune reaction in LEMS targets the voltage-gated Ca$^{2+}$ channels in the presynaptic membrane that regulate ACh release (Hewett and Atchison 1991, 1992a,b; Kim and Neher 1988; Lang et al. 1987; Roberts et al. 1985). High-voltage-activated Ca$^{2+}$ currents ($I_{Ca}$) from chromaffin cells and neuroblastoma-glioma cells were reduced in amplitude by LEMS antibodies (Kim and Neher 1988; Peers et al. 1990). Three $I_{Ca}$ components (L, N, and T type) in human neuroblastoma (IMR 32) cells were markedly reduced in amplitude by LEMS Igs (Grassi et al. 1994). Depolarization-dependent uptake of $[^{4}Ca]^{2+}$ into isolated nerve terminals of rat forebrain was reduced by 40–50% by acute application of plasma and serum from patients with LEMS (Hewett and Atchison 1991, 1992b). In freeze-fracture micrographs of presynaptic membranes of mice injected with serum from LEMS patients, active zone particles were disorganized and reduced in number (Fukunaga et al. 1983). These particles are thought to be voltage-dependent Ca$^{2+}$ channels mediating ACh release. Thus the reduced neurotransmitter release by nerve impulses in LEMS could be related to a reduced ingress of Ca$^{2+}$ into the nerve terminals.

More recent studies have examined the effect of LEMS Igs on motor nerve $I_{Ca}$ directly. Focal recordings of currents from phrenic nerve terminals of mice in which LEMS was transferred passively revealed a reduction of $I_{Ca}$ with no change in either $I_{Na}$ or capacitative currents (Smith et al. 1995). Similarly, Garcia and Beam (1996) demonstrated a reduction in whole cell high-voltage-activated $I_{Ca}$ recorded from cultures of murine spinal motor neurons exposed semichronically to LEMS plasma. In each of these cases, currents sensitive to dihydropyridine (DHP)-type antagonists were either spared or unmasked after LEMS treatment.
Pharmacological studies suggest that the Ca\(^{2+}\) channels that regulate ACh release at mammalian neuromuscular junctions are predominantly of the P and/or Q type. \(\omega\)-Agatoxin (Aga)-IVA and \(\omega\)-conotoxin (CTx)-MVIIC almost completely block ACh release and presynaptic \(I_{\text{Ca}}\) in mouse motor nerve terminals (Uchitel et al. 1992; Xu and Atchison 1996; Yan et al. 1994). Although Hamilton and Smith (1992) found evidence for the presence of some N-type Ca\(^{2+}\) channels in rat motor nerve terminals, \(\leq 65\%\) of the current could not be blocked by \(\omega\)-conotoxin-GVIA (\(\omega\)-CgTx-GVIA). Similar insensitivity to \(\omega\)-CgTx-GVIA as well as to DHPs has been reported in mouse motor nerve terminals (Anderson and Harvey 1987; Penner and Dreyer 1986). Thus P/Q-type Ca\(^{2+}\) channels may reflect the predominant type of Ca\(^{2+}\) channels in mammalian motor nerve terminals targeted by LEMS antibodies. The sensitivity of \(I_{\text{Ca}}\) in mammalian motor nerve terminals to LEMS Igs after passive transfer with serum from LEMS patients was reported by Smith et al. (1995). However, sensitivity to \(\omega\)-Aga-IVA or \(\omega\)-CTx-MVIIC was not examined.

The present study had several goals. First, we sought to examine the specificity of LEMS Igs for the two components of \(I_{\text{Ca}}\) reported to exist in murine motor axon terminals (Penner and Dreyer 1986; Xu and Atchison 1996). Second, we sought to identify pharmacologically the types of high-voltage-activated \(I_{\text{Ca}}\) that were sensitive to LEMS Igs. Third, we sought to corroborate the striking observation that DHP-sensitive components of \(I_{\text{Ca}}\) were either unmasked (Smith et al. 1995) or spared (Garcia and Beam 1996) from block by LEMS Igs. These objectives were studied using perineurial measurements of \(I_{\text{Ca}}\) in \textit{trig\textit{e}n\textit{a}l\textit{i}r\textit{a}lis s\textit{te}\textit{r}\textit{n}i} muscle from mice injected with plasma from LEMS patients. As reported by several groups (Penner and Dreyer 1986; Shafer and Atchison 1992; Xu and Atchison 1996), it is possible to separate pharmacologically the current in the perineurial sheath into two kinetically and pharmacologically distinct components. We also performed binding studies for ligands reputed to interact with distinct populations of Ca\(^{2+}\) channels using rat cortical synaptosomes to assess if altered DHP sensitivity could be observed with acute application of LEMS Igs. This model was chosen because the pharmacological sensitivity of rat cortical synaptosomal Ca\(^{2+}\) channel function, faithfully mimics that at mammalian motor axon terminals (Anderson and Harvey 1987; Atchison et al. 1988; Nachshen and Blaustein 1979; Suszkiw et al. 1986; Uchitel et al. 1992). In addition, acute exposure of this system to LEMS Igs causes disruption of Ca\(^{2+}\) channel function (Hewett and Atchison 1991, 1992a,b) consistent with that seen during chronic passive transfer experiments.

Our results show that \(I_{\text{Ca}}\) was reduced and DHP-sensitive \(I_{\text{Ca}}\) appeared in the motor nerve terminals after chronic LEMS plasma treatment. The types of \(I_{\text{Ca}}\) affected were sensitive to \(\omega\)-Aga-IVA and \(\omega\)-CTx-MVIIC. Conversely, acute treatment of central nerve terminals did not result in an alteration in DHP sensitivity, although the number of binding sites associated with the high-voltage-activated channel blocker verapamil was reduced by LEMS Igs.

METHODS

Passive transfer of LEMS

Male ICR mice (20–50 g, Harlan Sprague-Dawley Laboratories, Madison, WI) were divided into three groups: a group receiving LEMS plasma, a control group receiving control plasma, and an untreated group that was kept for the same duration as the other two groups but that received no injections. Mice were injected intraperitoneally for 30 days with either 1.5 ml of control or LEMS plasma (Hewett and Atchison 1992b; Kim 1985). To suppress the immune response to the injected plasma, the mice were first injected with cyclophosphamide (300 mg/kg ip) 1 day in advance. This treatment regimen reliably suppresses the full immune response (Lang et al. 1983). Thirty-one days later, the left intercostal nerve with the triangularis sterni muscle was dissected from the mice for electrophysiological recording (McArdle et al. 1981).

Neuromuscular preparation and solutions

After euthanasia, the mouse’s rib cage was separated from the spinal column and bathed in an oxygenated solution of the following ionic composition (in mM): 135 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 11 d-glucose, 12 NaHCO\(_3\), and 1 NaH\(_2\)PO\(_3\). The solution was gassed with 95% O\(_2\)-5% CO\(_2\) to buffer it at pH 7.4 at 25°C. The bath volume was 20 ml and was entirely replaced every 2–4 min during the dissection. The muscle, along with its branch of intercostal nerve, was dissected carefully to avoid damage to superficial fibers and their associated nerve terminals (McArdle et al. 1981). A sufficient length of nerve trunk was dissected for external stimulation with a glass suction electrode. After dissection, the preparation was pinned out at its resting length in a small chamber of 5 ml. The solution circulated over the preparation at 5 ml/min. In all experiments, n-tubocurarine (20–50 μM) was used to abolish postsynaptic responses and procaine (100 μM) was used to prevent the repetitive firing of motor nerve terminals that occurs in the presence of K\(^+\) channel blockers.

\(\omega\)-Aga-IVA and \(\omega\)-CTx-MVIIC were stored frozen at −70°C as stock solutions. Before experimentation, the stock solutions were thawed and diluted to the desired final concentration. Before applying \(\omega\)-Aga-IVA and \(\omega\)-CTx-MVIIC, bovine serum albumin (0.01% wt/vol) was added to the superfusion solution to prevent nonspecific binding of the toxins to the tube and chamber. Nifedipine and Bay K 8644 were prepared as 10 mM stock solutions in ethyl alcohol and stored at 4°C. The final concentration of ethyl alcohol was 0.1% (vol/vol). Before each experiment, nifedipine and Bay K 8644 were diluted with buffer to the desired final concentration. All experiments with nifedipine and Bay K 8644 were done in a darkened environment to prevent photo-oxidation. In the experiments using Cd\(^{2+}\), the bicarbonate buffering system was replaced with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; 10 mM) to avoid precipitation of CdCl\(_2\). For the experiments in which Ca\(^{2+}\) concentrations were decreased or increased, the osmolarity was maintained by adjusting the NaCl concentration.

Perineurial recording

Electrophysiological recordings of voltage changes in the perineurial space induced by Ca\(^{2+}\) entry via voltage-gated Ca\(^{2+}\) channels were made from the isolated left triangularis sterni muscle preparations. Suprathreshold electrical stimuli were applied to the intercostal nerve trunk via a suction electrode and stimulator (S88, Grass Medical Instruments, Quincy, MA) with a stimulus isolation unit (SIU, Grass Medical Instruments). Stimulus frequencies ranged from 0.01 to 100 Hz. \(I_{\text{Ca}}\) was recorded using a glass micro-electrode (1.0 mm ID, WP Instruments, Sarasota, FL) filled with 2 M NaCl and having a resistance of 2–5 M\(\Omega\); the electrode was placed inside the perineurial sheath of one of the branches of the intercostal nerve near the endplate region. The perineurium then was penetrated, and a steady negative 2–6 mV deflection denoted effective placement of the electrode for recording perineurial currents. Superficial nerve terminals were observed at a magnification...
of 400× using a microscope (Olympus BH-2, Olympus Optical, Tokyo, Japan) fitted with a water immersion objective and interference contrast (Nomarski) optics. Electrode movement during an experiment affects the amplitude of both the positive and negative components. If there was any evidence of electrode movement during the course of an experiment manifest as a change in the negative Na+ spike amplitude, records from that site were excluded from further analysis. Once a stable recording site was obtained, it could be held for as long as 80–90 min.

The signals were amplified (M707, WP Instruments, Sarasota, FL), fed into a digital storage oscilloscope (Model 4090, Nicolet Instruments, Verona, WI) for inspection and recorded on magnetic tape using an FM instrumentation tape recorder (Model B, A. R. Vetter, Rebersburg, PA) for signal analysis using a microcomputer and software (SCAN, University of Strathclyde, Scotland) kindly provided by Dr. J. Dempster.

Preparation of synaptosomes and solutions

Synaptosomes were prepared from forebrains of male Sprague-Dawley rats (Harlan, 175–225 g) according to a modification of the method of Gray and Whittaker (1962), as described in detail by Atchison et al. (1988). After ultracentrifugation in a discontinuous sucrose gradient, the synaptosomal pellet was resuspended in physiological saline buffer to an approximate concentration of 15–25 mg/ml (Lowry et al. 1951).

Equal aliquots of synaptosomal suspension were incubated at 4°C under 100% O2 for 60 min with either physiological saline buffer alone, or buffer including 10% (vol/vol) serum, 4 mg/ml control, or LEMS Ig plus 10% (vol/vol) serum. This concentration of LEMS Ig and serum was shown previously to reduce uptake of 45Ca2+ into synaptosomes during depolarization (Hewett and Atchison 1992a,b). The proper volume of physiological saline buffer was added to maintain a constant incubation volume. Before the initiation of binding experiments, synaptosomes were allowed to equilibrate for 30 min at 37°C. Binding assays were carried out at room temperature.

The physiological saline buffer used for preparation of synaptosomes contained (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 10 d-glucose, and 10 HEPES. Incubation solution contained (in mM) 72.5 NaCl, 5 KCl, 1 MgCl2, 0.04 CaCl2, 10 d-glucose, 10 HEPES, and 72.5 choline chloride. Cold quench solution contained (in mM) 5 KCl, 2 MgCl2, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’’-tetraacetic acid, 10 d-glucose, 10 HEPES, and 145 N-methyl-d-glucamine. Stock solutions of the radiolabels were made in incubation buffer.

Binding experiments

Binding of [3H]-nitrendipine and [3H]-verapamil was initiated by addition of 40 μl of synaptosomal suspension (200–300 μg protein) to 400 μl of incubation buffer containing either 10–1,500 pmol of [3H]-nitrendipine or 0.2–12.8 nmol of [3H]-verapamil in ethanol for 90 min at 25°C in a room illuminated by a sodium lamp. The final concentration of ethanol was always <0.1% (vol/vol). In addition, the verapamil stock solutions and quench buffer contained 0.5 mg/ml bovine serum albumin to limit nonspecific binding of verapamil to test tubes and filters. Incubation was terminated by addition of 5 ml of cold quench solution followed by rapid filtration through glass fiber filters (Whatman GF) that had been presoaked in quench solution containing 0.1% (wt/vol) polyethyleneimine. The filters were rinsed twice with 5 ml of cold quench solution. Scintillation cocktail was added 12 h before the radioactivity trapped on the filters was estimated in a liquid scintillation counter having an approximate efficiency of 45% for 3H. Nonspecific binding was measured in the presence of 5 μM unlabeled nifedipine or 25 μM unlabeled verapamil, respectively. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. A 40-μl aliquot was taken from the stock solution to determine total ligand concentration. The average of triplicate values was used to determine the equilibrium dissociation constant (Kd) for binding and the maximum density of binding sites (Bmax) (Scatchard 1949).

Initiation of [125I]-ω-CgTx-GVIA binding was essentially identical to that described earlier except that synaptosomes were added to incubation buffer containing 30–480 pmol of [125I]-ω-CgTx-GVIA and 5 mg/ml bovine serum albumin in deionized water. Nonspecific binding was measured in the presence of 1 μM unlabeled ω-CgTx-GVIA. Radioactivity remaining on the filters was estimated using a gamma counter with an efficiency of 80% for 125I. Because binding of ω-CgTx-GVIA is essentially irreversible, Scatchard analysis of the data could not be performed. Thus the average of duplicate values was used for estimation of Bmax by saturation analysis.

Immunoglobulin isolation

Control and LEMS sera and/or plasma were stored at −20°C until used. After thawing, fibrin was removed from the plasma samples by clotting, which was facilitated by the addition of 1 M CaCl2. The clot was removed by centrifugation at 2,500 × g for 15 min. Total immunoglobulins (Igs) were isolated by serial precipitations of serum in 30 and 50% saturated ammonium sulfate at 4°C, as described previously (Hewett and Atchison 1992a). The LEMS Igs used in the synaptosomal studies were isolated from a patient with SCC. The plasma preparation from this patient already was shown to transfer the electrophysiological features of LEMS to mice and to reduce depolarization-dependent uptake of 45Ca2+ into rat forebrain synaptosomes (patient 3) (Hewett and Atchison 1992b). Igs from patients with SCC alone did not affect 45Ca uptake.

Materials

Control human plasma donated by healthy volunteers was obtained from the American Red Cross (Lansing, MI). Plasma from three patients with typical clinical and electromyographic features of LEMS was generously provided by Dr. Andrew Massey (University of Kentucky Medical Center, Lexington, KY), Drs. Eva Feldman and James Albers (University of Michigan Medical Center, Ann Arbor, MI), and Dr. Shin Oh (University of Alabama Medical Center, Birmingham, AL). ω-Aga-IVA was generously provided by Dr. Nicholas Saccamano, Pfizer (Groton, CT) and Research Biochemicals (Natick, MA). ω-Ctx-MVIIc was obtained from Bachem (Torrance, CA) and the Peptide Institute (Osaka, Japan). ω-CgTx-GVIA was purchased from Peninsula Laboratories (Belmont, CA). The radioisotopes [3H]-nitrendipine (74 Ci/mmol), [3H]-verapamil (66 Ci/mmol), and [125I]-ω-CgTx (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Nifedipine, Bay K 8644, verapamil, tetraethylammonium chloride (TEA), 3,4-diaminopyridine (3,4-DAP), d-tubocurarine hydrochloride, bovine serum albumin, procaine hydrochloride and polyethyleneimine were all obtained from Sigma Chemical (St. Louis, MO). All other reagents were of analytic grade or better.

Statistical analysis

Data from perineurial recordings were analyzed using Student’s unpaired t-test; significance was set at P < 0.01 for all experiments. Data from the equilibrium binding of [3H]-nitrendipine and [3H]-verapamil were analyzed by mixed design analysis of variance (ANOVA) followed by Student’s t-test for paired samples. Data from ω-CgTx-GVIA binding experiments were analyzed using a
randomized complete block ANOVA. Differences in binding study data were considered significant at \( P < 0.05 \).

**Results**

*Effect of \( K^+ \) channel block on motor nerve terminal \( Ca^{2+} \) currents*

\( I_{Ca} \) in mouse motor nerve terminals cannot be distinguished as a separate entity in standard Krebs solution, because its amplitude is obscured by voltage-gated \( K^+ \) currents (\( I_K \)) (Mallart 1986; Penner and Dreyer 1986). It is necessary, therefore, to block \( I_K \) using TEA and 3,4-DAP to reveal \( I_{Ca} \). Shown in Fig. 1A are perineurial recordings in the absence and presence of 3,4-DAP and TEA. In the absence of \( K^+ \) channel block, a double-notched downward voltage deflection is observed. The initial component is due to \( I_{Na} \), and the subsequent component is due to \( I_K \). Addition of 300 \( \mu M \) DAP and 10 mM TEA causes a time-dependent reduction in the downward voltage trace, leaving the component associated with \( I_{Na} \) unaffected, and gradually unmasking an upward voltage response associated with \( I_{Ca} \) (Fig. 1A, \( d \) and \( e \)). Figure 1B is obtained from the continuous recording of Fig. 1A, but the time base has been adjusted to allow more precise analysis of the upward voltage changes. Tracings 1B, \( a-c \), reflect additional time of the preparation in 300 \( \mu M \) DAP and 10 mM TEA and show a progressive increase of the \( Ca^{2+} \) voltage change plateau with nerve stimulation at 0.01 Hz. Maximal response to these \( K^+ \) channel blockers was attained at 20 min (Fig. 1Bc). Addition of further DAP and TEA did not increase further the maximal amplitude of the sustained upward voltage change (\( I_{Ca} \)), although it did slow its rate of decay somewhat. Thus as reported previously by Penner and Dreyer (1986), 10 mM TEA and 300 \( \mu M \) 3,4-DAP display maximally effective block of \( I_K \).

When the three \( I_K \) (fast, slow, and \( Ca^{2+} \) activated) of motor nerve terminals are completely blocked by use of 300 \( \mu M \) 3,4-DAP and 10 mM TEA, typical waveforms recorded from the perineurial sheath have a small upward (positive) component, followed by a large downward (negative) component and then a large and prolonged positive component (Fig. 1B). The early positivity has been ascribed to the spread of longitudinal capacitive currents into the terminal portions of the motor nerve—\( I_{Capacitative} \) (Brigant and Mallart 1982; Mallart 1985). The negative component is associated with \( Na^+ \) currents (\( I_{Na} \)) in the Nodes of Ranvier near the recording electrode, and the delayed positive waveform is related to a prolonged \( I_{Ca} \) at the nerve terminal. Perineurial signals result from the fact that the signal amplitude at any recording point increases with the number of endings that supply local circuit current flow to the recording site (see Mallart 1982) Thus the magnitude of both positive and negative components should vary depending on the position of the microelectrode tip within the perineurial sheath (Brigant and Mallart 1982; Mallart 1985; Penner and Dreyer 1986). Smith et al. (1995) found a strong linear relationship between the peak amplitudes of \( I_{Ca} \) and \( I_{Capacitative} \) of mouse motor nerve terminals using focal extracellular recordings, thus providing a convenient method to compare \( I_{Ca} \) amplitudes obtained from different terminals. In the present study, we also found a strong correlation between \( I_{Ca} \) and \( I_{Na} \) in control and LEMS-treated preparations (Fig. 2C). However, treatment with LEMS plasma caused a downward shift of the line from those of controls, indicating a reduced \( I_{Ca} \) in LEMS-treated terminals (Fig. 2C).

**Passive transfer of LEMS reduced \( Ca^{2+} \) currents**

After perfusing the preparations with 300 \( \mu M \) 3,4-DAP and 10 mM TEA, 0.01-Hz stimulation elicited \( I_{Ca} \) with long duration from motor nerve terminals of the mice treated with control plasma as well as those treated with plasma from three patients clinically diagnosed...
with LEMS. However, the peak amplitude of $I_{Ca}$ as well as the ratio of $I_{Ca}/I_{Na}$ in LEMS plasma-treated motor nerve terminals were both smaller than those in the nerve terminals treated with control plasma (Fig. 2A and Table 1). The peak amplitude of $I_{Ca}$ for LEMS plasma-treated mice was reduced by 36%: 39% for patient 1, 30% for patient 2, and 39% for patient 3. The ratio of $I_{Ca}/I_{Na}$ was reduced by ~32%: 32% for patient 1; 32% for patient 2, and 30% for patient 3. In contrast, there was no significant difference in the value of peak amplitude of $I_{Ca}$ or the ratio of $I_{Ca}/I_{Na}$ between untreated preparations or those treated with control plasma (Table 1). $I_{Na}$ in both the untreated motor nerve terminals and those treated with LEMS plasma from three patients was not significantly different from those treated with control plasma (Table 1).

Penner and Dreyer (1986) demonstrated the presence of two different $I_{Ca}$ currents in mouse motor nerve terminals based on their differences in frequency dependence and sensitivity to extracellular Ca$^{2+}$ concentration as well as inorganic and organic calcium-antagonists such as Cd$^{2+}$, verapamil, and diltiazem. Two kinetically distinct components also show differential sensitivity to $\omega$-Aga-IVA, a P-type Ca$^{2+}$ channel blocker, and $\omega$-CTx-MVIIC, a Q-type Ca$^{2+}$ channel blocker. $I_{Ca,f}$ appears to be more similar to a Q-type Ca$^{2+}$ channel, and $I_{Ca,s}$ appears more similar to a P-type Ca$^{2+}$ channel (Xu and Atchison 1996). The two $I_{Ca}$ components can be separated clearly by use of lower concentrations of K$^+$ channel blockers because they have different activation thresholds. Thus we could examine whether either current component was decreased preferentially by LEMS plasma treatment. As shown in Fig. 2B, 0.01-Hz nerve stimulation in the presence of 1 mM TEA and 50 $\mu$M 3,4-DAP elicited $I_{Ca,f}$ and $I_{Ca,s}$, which both were reduced significantly in amplitude in the LEMS plasma-treated mice. Table 2 summarizes the results from nine control and seven LEMS-treated preparations; it shows that in the LEMS-treated group $I_{Ca,f}$ and $I_{Ca,s}$ were reduced by 35 and 37%, respectively, whereas the
ratios of \( I_{Ca,f} \):\( I_{Na} \) and \( I_{Ca,s}:I_{Na} \) were reduced by 29 and 47%, respectively, compared with control.

### Frequency dependence of \( I_{Ca} \)

In motor nerve terminals treated with control plasma, \( I_{Ca} \) evoked in the presence of 10 mM TEA and 300 \( \mu \)M 3,4-DAP was reduced in both duration and amplitude by increasing the stimulation frequency from 0.01 to 10 Hz. For mice treated with LEMS plasma, 10-Hz frequency nerve stimulation decreased the duration of \( I_{Ca} \) but did not alter its amplitude (Fig. 3A and C). The peak amplitude of \( I_{Ca} \) for control preparations was 2.3 ± 0.17 (SE) mV at 0.01 Hz and 1.2 ± 0.23 mV at 10 Hz. In LEMS-treated preparations, the peak amplitude of \( I_{Ca} \) was unchanged as stimulus frequency was increased (1.86 ± 0.24 at 0.01 Hz and 1.78 ± 0.23 mV at 10 Hz). In the presence of low concentrations of \( K^+ \) channel blockers (1 mM TEA and 50 \( \mu \)M 3,4-DAP), increasing the stimulation frequency to 10 Hz had no effect on either amplitude or duration of \( I_{Ca,f} \) from either control or LEMS-treated mice. Conversely, at 10-Hz stimulation \( I_{Ca,s} \) for both groups was blocked almost completely (Fig. 3B).

In the presence of high concentrations of \( K^+ \) channel blockers, there was a striking difference between control and LEMS-treated mice in the response of \( I_{Ca} \) amplitude during short trains of nerve stimulation at 20 and 100 Hz. In most of the LEMS-treated mice, facilitation of \( I_{Ca} \) occurred not only at 20 Hz but also at 100 Hz. Conversely there was no facilitation of \( I_{Ca} \) at 20 Hz in any of the control preparations and marked depression occurred uniformly at 100 Hz (Fig. 4A and B). The amplitude of \( I_{Ca} \) in these trains was expressed as a percentage of the first response. The summaries of results from five control and six LEMS-treated preparations are shown in Fig. 4D. In the presence of low concentrations of \( K^+ \) channel blockers, there was no difference between control and LEMS-treated groups in the \( I_{Ca} \) amplitude during the train stimulation. \( I_{Ca} \) facilitation was seen in both groups at 100-Hz train stimulation (Fig. 4C).

### \( Ca^{2+} \) dependence of \( I_{Ca} \)

In preparations treated with control plasma, increasing the extracellular \( Ca^{2+} \) concentration ([\( Ca^{2+} \)]\(_e\)) enhanced both the amplitude and duration of \( I_{Ca} \). The peak amplitude of \( I_{Ca} \) saturated at 2 mM \( Ca^{2+} \), whereas the duration of \( I_{Ca} \) saturated at 4–6 mM. For LEMS plasma-treated preparations, a similar pattern was observed, however, the amplitude and duration of \( I_{Ca} \) were both increased further by raising the [\( Ca^{2+} \)]\(_e\), above that which was maximally effective for the controls. Saturation for the amplitude and duration of \( I_{Ca} \) occurred at 8–9 mM (Fig. 5, A–C). Therefore, in LEMS-treated mice, the [\( Ca^{2+} \)]\(_e\) had to increase by two to three times control levels to produce a maximal \( I_{Ca} \).

\( \omega-Aga-IVA \)- or \( \omega-CTx-MVIIC \)-sensitive \( Ca^{2+} \) channels as the target of LEMS autoantibodies

To determine whether LEMS autoantibodies affect a particular type of \( Ca^{2+} \) channel in motor nerve terminals, \( \omega-Aga-IVA \), \( \omega-CTx-MVIIC \), and nifedipine were applied to identify pharmacologically, the types of \( I_{Ca} \) in control and LEMS plasma-treated mice. In control preparations, perfusing the preparation with 150 nM \( \omega-Aga-IVA \) caused block of ∼90% of \( I_{Ca} \) within 15 min (Fig. 6A) The positive component remaining after treatment with \( \omega-Aga-IVA \) was not sensitive to \( Cd^{2+} \) even at 10 mM (results not shown). Thus the effect of \( \omega-Aga-IVA \) on \( I_{Ca} \) of control plasma-treated motor nerve terminals is similar to that of our previous observation in untreated motor nerve terminals (Xu and Atchison 1996). However, 150 nM \( \omega-Aga-IVA \) was less effective in LEMS-plasma-treated preparations; it reduced \( I_{Ca} \) of LEMS-treated terminals 22% less than in control preparations. The \( I_{Ca} \) component that was resistant to \( \omega-Aga-IVA \) was sensitive to low concentrations of \( Cd^{2+} \) (10 \( \mu \)M), thus indicating the involvement of another type of \( Ca^{2+} \)-sensitive channel in LEMS. The portion of \( I_{Ca} \) which remained after \( \omega-Aga-IVA \) was reduced further within 15 min of addition of 10 \( \mu \)M nifedipine (Figs. 6A and 8). Similar results were obtained using \( \omega-CTx-MVIIC \). At 5 \( \mu \)M, \( \omega-CTx-MVIIC \) completely blocked \( I_{Ca} \) in the control plasma-treated terminals (Fig. 6B); the same concentration of \( \omega-CTx-MVIIC \) was 37% less effective at blocking \( I_{Ca} \) in LEMS-plasma-treated terminals. The \( I_{Ca} \) that remained after \( \omega-CTx-MVIIC \) also was blocked by nifedipine (Figs. 6B and 8), and \( Cd^{2+} \) (results not shown).

To investigate whether the effects of \( \omega-Aga-IVA \) and \( \omega-CTx-MVIIC \) on \( I_{Ca} \) in LEMS-treated terminals were additive with those of nifedipine, we examined the effect of nifedipine, verapamil, and Bay K 8644 on \( I_{Ca} \) in the absence of \( \omega-Aga-IVA \) and \( \omega-CTx-MVIIC \). At 10 \( \mu \)M, nifedipine did not affect the amplitude or duration of \( I_{Ca} \) at 0.01 Hz (Fig. 6C). However, at 0.01–0.1 Hz, \( I_{Ca} \) was decreased by 150 nM nifedipine (Fig. 6C).

### Table 1. Amplitude of \( I_{Ca} \) and \( I_{Na} \) in mouse motor nerve terminals untreated or treated with control and LEMS plasma

<table>
<thead>
<tr>
<th>Group</th>
<th>( I_{Ca} ) mV</th>
<th>( I_{Na} ) mV</th>
<th>( I_{Ca}/I_{Na} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (15)</td>
<td>3.00 ± 0.13</td>
<td>3.41 ± 0.30</td>
<td>0.93 ± 0.05</td>
</tr>
<tr>
<td>Patient 1 (10)</td>
<td>1.83 ± 0.15</td>
<td>2.96 ± 0.33</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td>Patient 2 (6)</td>
<td>2.11 ± 0.29</td>
<td>3.51 ± 0.37</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>Patient 3 (11)</td>
<td>2.12 ± 0.15</td>
<td>3.31 ± 0.22</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>LEMS combined (27)</td>
<td>2.01 ± 0.10*</td>
<td>3.22 ± 0.17</td>
<td>0.63 ± 0.03*</td>
</tr>
<tr>
<td>Untreated (4)</td>
<td>3.20 ± 0.30</td>
<td>3.97 ± 0.46</td>
<td>0.80 ± 0.08</td>
</tr>
</tbody>
</table>

The recording was obtained in the presence of 300 \( \mu \)M 3,4-diaminopyridine and 10 mM tetraethylammonium. Values are the means ± SE of different preparations in control and Lambert-Eaton Myasthenic Syndrome (LEMS) plasma-treated mice and each preparation value is from different nerve branches. * Value significantly different (\( P < 0.01 \)) from control.

### Table 2. Comparison of \( I_{Ca} \) and \( I_{Ca,s} \) in control and LEMS plasma-treated motor nerve terminals

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>LEMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( I_{Ca} ) mV</td>
<td>4.26 ± 0.39</td>
<td>3.77 ± 0.29</td>
</tr>
<tr>
<td>( I_{Ca,s} ) mV</td>
<td>2.97 ± 0.23</td>
<td>1.95 ± 0.25*</td>
</tr>
<tr>
<td>( I_{Ca}/I_{Na} )</td>
<td>0.74 ± 0.07</td>
<td>0.52 ± 0.02*</td>
</tr>
<tr>
<td>( I_{Ca,s} ) mV</td>
<td>1.72 ± 0.188</td>
<td>1.05 ± 0.101*</td>
</tr>
<tr>
<td>( I_{Ca,s}/I_{Na} )</td>
<td>0.46 ± 0.06</td>
<td>0.24 ± 0.029*</td>
</tr>
</tbody>
</table>

The recording was obtained in the presence of 50 \( \mu \)M 3,4-DAP and 1 mM TEA. Values are the means ± SE of different preparations in control and LEMS plasma-treated mice. \( n = 9 \) for control and \( n = 7 \) for LEMS. * Value significantly different (\( P < 0.05 \)) from control mice.
FIG. 3. Effect of nerve stimulation frequency on $I_{Ca}$ in control and LEMS plasma-treated motor nerve terminals. A: current was recorded in the presence of 300 μM 3,4-DAP and 10 mM TEA. Superimposed recordings from the same site are depicted at stimulation frequencies of 0.01 Hz (1), 0.1 Hz (2), 1 Hz (3), and 10 Hz (4). Note that the amplitudes of $I_{Ca}$ are decreased in the control but not in LEMS-treated group as the stimulation frequency is increased. B: current was recorded in the presence of 50 μM 3,4-DAP and 1 mM TEA. Superimposed recordings from the same site are depicted at a stimulation frequency of 0.01 Hz (1), 0.1 Hz (2), 1 Hz (3), and 10 Hz (4). Note that there is no difference between control and LEMS groups during the increased stimulation frequency, where the amplitude of $I_{Ca}$ for both is not decreased. C: data were obtained in the presence of 300 μM 3,4-DAP and 10 mM TEA. Each value is the mean ± SE of 12 control preparations and 11 LEMS-treated preparations. $I_{Na}$ was increased slightly as the stimulation frequency was increased in control and LEMS-treated preparations but there was no difference between the 2 groups.

Effects of LEMS Igs on the binding of $Ca^{2+}$ channel antagonists to cortical synaptosomes

As indicated above, in the chronic LEMS plasma-treated mouse motor nerve terminals, $I_{Ca}$ was reduced, and this was accompanied by appearance of a DHP-sensitive $I_{Ca}$. In previous studies (Hewett and Atchison 1991, 1992a,b), we found that plasma or Igs isolated from patients with LEMS could reduce the uptake of $^{45}Ca^{2+}$ into synaptosomes during KCl-induced depolarization by 40–50%. We thus used this model to assess whether acute exposure to LEMS IgG would unmask a DHP-sensitive component in central nerve terminals. Specifically, we sought to determine whether in synaptosomes exposed to LEMS Igs, the pharmacologically distinct binding sites reputed to be associated with nerve terminal $Ca^{2+}$ channels are altered either in number or in their affinity for antagonist.

NITRENEDIPINE BINDING. When the binding of $[^{3}H]$-nitrendipine (10–1.00 pmol) was measured after incubation in buffer alone, a single high-affinity binding site was observed.

not affect $I_{Ca}$ in the control plasma-treated motor nerve terminals, but inhibited $I_{Ca}$ by 25–35% in LEMS plasma-treated terminals (Fig. 7A). The current resistant to nifedipine in these animals could be abolished by 150 nM ω-Aga-IVA just as in the controls (Fig. 7A). The same effect occurred when using ω-CTX-MVIIC after nifedipine (results not shown). Thus the inhibitory effects of ω-Aga-IVA or ω-CTX-MVIIC on $I_{Ca}$ were additive with those of nifedipine, suggesting the presence of ω-Aga-IVA- and ω-CTX-MVIIC-sensitive, nifedipine-sensitive L-type $I_{Ca}$ in LEMS plasma-treated but not control motor nerve terminals. The nifedipine-sensitive components of current could be increased to a level similar to that measured in nifedipine-free solution by addition of 10 μM Bay K 8644. There was no significant difference in the percent inhibition of verapamil on $I_{Ca}$ between control and LEMS-treated preparations (Figs. 7B and 8). At 10 μM, verapamil reduced $I_{Ca}$ by 50% for control plasma-treated and 57% for LEMS plasma-treated preparations.

Effects of LEMS Igs on the binding of $Ca^{2+}$ channel antagonists to cortical synaptosomes

As indicated above, in the chronic LEMS plasma-treated mouse motor nerve terminals, $I_{Ca}$ was reduced, and this was accompanied by appearance of a DHP-sensitive $I_{Ca}$. In previous studies (Hewett and Atchison 1991, 1992a,b), we found that plasma or Igs isolated from patients with LEMS could reduce the uptake of $^{45}Ca^{2+}$ into synaptosomes during KCl-induced depolarization by 40–50%. We thus used this model to assess whether acute exposure to LEMS Igs would unmask a DHP-sensitive component in central nerve terminals. Specifically, we sought to determine whether in synaptosomes exposed to LEMS Igs, the pharmacologically distinct binding sites reputed to be associated with nerve terminal $Ca^{2+}$ channels are altered either in number or in their affinity for antagonist.

NITRENEDIPINE BINDING. When the binding of $[^{3}H]$-nitrendipine (10–1.00 pmol) was measured after incubation in buffer alone, a single high-affinity binding site was observed.
Nonspecific binding accounted for \( \sim 25\% \) of total binding. Scatchard analysis of the data yielded a \( K_d \) value of \( 214 \pm 84 \) pM and an apparent \( B_{\text{max}} \) value of \( 62.0 \pm 12.0 \) fmol/mg protein (Table 3). The Hill slope was approximately equal to one. These values are consistent with those reported by others for \(^{[3]}H\)-nitrendipine binding in synaptosomes (Boles et al. 1984; Suszkiw et al. 1986; Turner and Goldin 1985). Administration of LEMS IgG did not affect either the \( K_d \) or apparent \( B_{\text{max}} \) value (Table 3).

\( \omega \)-CgTX-GVIA BINDING. The specific binding of \(^{[125]}I\)-\( \omega \)-CgTX GVIA to synaptosomes in the presence of control and LEMS IgG also was examined (Fig. 9). Nonspecific binding accounted for <\( 10\% \) of total binding. \( \omega \)-CgTX GVIA bound to synaptosomes with a half-saturation of between 0.1 and 0.2 nM. Because binding of \( \omega \)-CgTX GVIA is essentially irreversible, the maximum density of binding sites was estimated by extrapolation of the saturated component to the y axis. The apparent \( B_{\text{max}} \) value for \( \omega \)-CgTX binding in synaptosomes treated with control or LEMS IgG did not differ. The control value (260.6 \( \pm \) 6.7) was consistent with those reported by others (Cruz and Olivera 1986; Marqueze et al. 1988; Wagner et al. 1988).

VERAPAMIL BINDING. Over a concentration range of 0.2–12.8 nmol, \(^{[3]}H\)-verapamil bound a single high-affinity site in synaptosomes incubated with any IgG (Fig. 10). Nonspecific binding accounted for 40–50% of total binding. Scatchard analysis revealed that the \( K_d \) values for binding of \(^{[3]}H\)-verapamil in the presence of control and LEMS IgG plus serum were similar: 17.1 \( \pm \) 4.2 and 14.6 \( \pm \) 9.1 nM, respectively. However, \( B_{\text{max}} \) was reduced from 97.4 \( \pm \) 15.0 to 37.3 \( \pm \) 3.8 fmol/mg protein after incubation with LEMS IgG as compared with incubation with control IgG.

**DISCUSSION**

Passive transfer using plasma or sera from patients with LEMS produces the hallmark electrophysiological and ultrastructural characteristics of LEMS (Hewett and Atchison 1992b; Kim 1985; Kim and Neher 1988; Lambert and Elmqvist 1971; Smith et al. 1995). These include depletion of...
FIG. 5. Effects of extracellular Ca\(^{2+}\) concentration on \(I_{\text{Ca}}\) of motor nerve terminals of mice treated with control and LEMS plasma. A and B: superimposed recordings were obtained from the same site at extracellular Ca\(^{2+}\) concentrations from 0.5 to 6 mM for control preparations and from 0.5 to 10 mM for LEMS-treated preparations. Recordings were made in the presence of 10 mM TEA and 300 \(\mu M\) 3,4-DAP and continuously at a single terminal for the various concentrations of Ca\(^{2+}\) administered sequentially at the time that the effect of the previous concentration appeared to be maximal. Each record is an average of 5–10 successive sweeps at 0.01 Hz.

The reduced amplitude of \(I_{\text{Ca}}\), loss of frequency-dependent depression, and enhanced sensitivity of \(I_{\text{Ca}}\) to changes in [Ca\(^{2+}\)]\(e\) induced by passive transfer \(I_{\text{Ca}}\) to changes in [Ca\(^{2+}\)]\(e\) induced by passive transfer of LEMS to mouse motor nerve terminals all indicate a reduced Ca\(^{2+}\) entry in the nerve terminals during nerve impulses. This effect can account for the well-known reduction of ACh release in LEMS and is consistent with the previous findings that \(^{45}\)Ca\(^{2+}\) uptake was reduced in rat cortical synaptosomes (Hewett and Atchison 1991, 1992b) and that protein components, the sizes of which are consistent with those of the Ca\(^{2+}\) channel complex, are recognized by Igs of 14 patients with LEMS (Hajela and Atchison 1995).

A major objective of the present study was to examine the specificity of LEMS autoantibodies for pharmacologically separable components of \(I_{\text{Ca}}\) at mammalian motor axon terminals. Several studies have examined the pharmacological preferentiality of LEMS autoantibodies using indirect functional measures such as quantal content of endplate potentials or amplitude of somatic Ca\(^{2+}\) currents under voltage-clamp conditions, generally from cells not involved in motor neurotransmission. Although these studies have provided valuable clues as to the pathophysiology of LEMS, they suffer from the inability to examine the actual presumed target of LEMS- the motor axon terminal. This is potentially important insasmuch as the functional behavior of Ca\(^{2+}\) channels at nerve terminals may differ from that of channels in the soma (Cousin et al. 1997). The perineurial recording method used in the present study lacks the unequivocal nature of voltage clamp for resolving electrically distinct events, however, it does provide a reproducible, reliable estimation of events occurring at the mammalian motor axon terminal, a structure not amenable to voltage-clamp methodology. We have shown previously that the two components of \(I_{\text{Ca}}\) recorded at motor axon terminals of triangularis sterni are primarily sensitive to \(\omega\)-Aga-IVA and \(\omega\)-CTX-MVIIC.
FIG. 6. Effect of ω-agatoxin-IVA (ω-Aga-IVA) and ω-conotoxin-MVIIC (ω-CTX-MVIIC) on $I_{Ca}$ in control and LEMS plasma-treated motor nerve terminals. A: $I_{Ca}$ was recorded in the presence of 300 μM 3,4-DAP and 10 mM TEA. Superimposed recordings from the same site are depicted before and after application of ω-Aga-IVA (0.15 μM) and nifedipine (10 μM) for control and LEMS groups. B: current was recorded in the presence of 300 μM 3,4-DAP and 10 mM TEA. Superimposed recordings from the same site are depicted before and after application of ω-CTX-MVIIC (5 μM) and nifedipine (10 μM) for control and LEMS. Toxins were administered sequentially at the time that the effect of the previous toxin appeared maximal. Each record is an average of 6–7 successive sweeps at 0.01 Hz.

(Xu and Atchison 1996). In the present study, ω-Aga-IVA and ω-CTX-MVIIC inhibit $I_{Ca}$ from motor nerve terminals of mice treated with control plasma by 90–95%, confirming that channels sensitive to these peptide toxins (putative P- and/or Q-type Ca$^{2+}$ channels) are primarily responsible for the Ca$^{2+}$ influx in mammalian motor nerve terminals. In LEMS plasma-treated mouse motor nerve terminals, whereas $I_{Ca}$ is reduced by 30–40% compared with control, ω-Aga-IVA and ω-CTX-MVIIC again block the majority of the remaining $I_{Ca}$, except for the presence of 20–30% toxin-insensitive but DHP-sensitive L-type $I_{Ca}$. These results demonstrate a preferential effect of LEMS antibodies on P- or Q-type Ca$^{2+}$ channels in mammalian motor nerve terminals to cause their loss of function. This is consistent with the recent report that LEMS sera preferentially affects P- or Q-type Ca$^{2+}$ currents, but not L-type currents, of mouse motoneuron cell bodies (Garcia and Beam 1996).

In control plasma-treated motor nerve terminals, $I_{Ca}$ was not sensitive to DHP L-type Ca$^{2+}$ channel antagonists. This corroborates earlier findings (Atchison 1990; Atchison and O’Leary 1987), which suggest that DHP-sensitive Ca$^{2+}$ channels do not normally contribute to ACh release at murine neuromuscular junctions. However, apparently L-type Ca$^{2+}$ channels do contribute to the Ca$^{2+}$ influx in LEMS plasma-treated mouse motor nerve terminals. Similar findings have been reported by Smith et al. (1995), who

FIG. 7. Effect of nifedipine and verapamil on $I_{Ca}$ in control and LEMS plasma-treated motor nerve terminals. A: current was recorded in the presence of 50 μM 3,4-DAP and 1 mM TEA. Superimposed recordings from the same site are depicted before and after application of 10 μM nifedipine and 0.15 μM ω-Aga-IVA for control and LEMS groups. Toxins were administered sequentially at the time that the effect of the previous toxin appeared maximal. B: current was recorded in the presence of 50 μM 3,4-DAP and 1 mM TEA. Superimposed recordings from the same site are depicted before and after application of 10 μM verapamil for control and LEMS groups. Each record is an average of 6–7 successive sweeps at 0.01 Hz.
FIG. 8. Summary of the effects of Ca\(^{2+}\) channel antagonists on \(I_{Ca}\) in control and LEMS plasma-treated motor nerve terminals. Values are the means ± SE of 4–6 control and LEMS-treated preparations.

showed that 43% of \(I_{Ca}\) in mouse phrenic motor nerve terminals exposed to LEMS antibodies became sensitive to DHPs but insensitive to \(\omega\)-CgTx-GVIA. They proposed that activation of L-type Ca\(^{2+}\) channels in LEMS-treated preparations was due to depolarization of the terminal membrane resulting from inactivation of an \(I_{\text{K,Ca}}\) because intracellular Ca\(^{2+}\) concentrations were decreased by LEMS antibodies. The question arises whether this apparent L-type Ca\(^{2+}\) channel activation also would occur after acute exposure to LEMS Igs.

Acute application of LEMS Igs to rat cortical synaptosomes did not affect the binding characteristics of the DHP nitrendipine, whereas it reduced both KCl-induced uptake of \(^{45}\)Ca (Hewett and Atchison 1992a,b) and the apparent \(B_{max}\) for verapamil. No effect of LEMS plasma was seen on the apparent affinity of the remaining binding sites for ligand, suggesting that reduction of available verapamil binding sites occurs without effects on the functional characteristics of the remaining sites. The same concentration of Igs did not affect the binding characteristics of \([^{3}\text{H}]\)-nitrendipine.

**TABLE 3.** Characteristics of binding of \([^{3}\text{H}]\)nitrendipine to rat brain synaptosomes in the absence and presence of LEMS Igs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(B_{max}) (fmol/mg protein)</th>
<th>(K_d) (pm)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>62.0 ± 12.0</td>
<td>214.1 ± 84.0</td>
<td>3</td>
</tr>
<tr>
<td>Serum alone</td>
<td>62.5 ± 12.0</td>
<td>152.8 ± 48.0</td>
<td>3</td>
</tr>
<tr>
<td>Control Igs</td>
<td>75.2 ± 10.2</td>
<td>236.0 ± 49.0</td>
<td>6</td>
</tr>
<tr>
<td>LEMS Igs</td>
<td>72.4 ± 18.5</td>
<td>289.6 ± 77.0</td>
<td>6</td>
</tr>
<tr>
<td>Control Igs + serum</td>
<td>75.1 ± 14.5</td>
<td>256.0 ± 76.4</td>
<td>6</td>
</tr>
<tr>
<td>LEMS Igs + serum</td>
<td>65.2 ± 9.9</td>
<td>190.6 ± 33.0</td>
<td>6</td>
</tr>
</tbody>
</table>

Equal aliquots of synaptosomal suspension were incubated at 4°C under 100% O\(_2\) for 60 min with either physiologic saline buffer, 10% (vol/vol) serum, control or LEMS immunoglobulins (Igs) alone (4 mg/ml) or Igs (4 mg/ml) plus 10% (vol/vol) serum. Before the initiation of binding experiments, synaptosomes were allowed to equilibrate for 30 min at 37°C. Equilibrium binding assays were carried out at room temperature. Synaptosomes were added to non-depolarizing K\(^+\) buffer containing 10–1,500 pmol of \([^{3}\text{H}]\)nitrendipine for 60 min at 25°C. Incubation was terminated by addition of 5 ml of cold quench solution followed by suction filtration. The average of triplicate values was used for Scatchard analysis to determine the equilibrium dissociation constant \(K_d\) (pm) for binding and the maximum density of binding sites \(B_{max}\) (fmol/mg protein). \(n\) refers to separate synaptosomal preparations.

FIG. 9. Comparative effects of control (– – –) and LEMS Igs (– – –) on \(\omega\)-conotoxin-GVIA (\(\omega\)-CgTx-GVIA) binding to rat forebrain synaptosomes. Equal aliquots of synaptosomes were incubated with control or LEMS Igs (4 mg/ml) in the presence of 10% (vol/vol) human serum 90 min before initiation of binding. Specific binding of \([^{3}\text{H}]\)-CgTx was determined as described in METHODS. Values shown are the means ± SE of 3 experiments. When SE bars are not shown the SE is smaller than the size of the symbol.

FIG. 10. Comparative effects of control and LEMS immunoglobulins (Igs) on verapamil binding to synaptosomes. Equal aliquots of synaptosomes were incubated with control or LEMS Igs (4 mg/ml) in the absence (A) or presence of 10% (vol/vol) human serum (B) 90 min before initiation of binding. Specific binding of \([^{3}\text{H}]\)-verapamil was determined as described in METHODS. Values shown are the means ± SE of 6 experiments. When SE bars are not shown, the SE is smaller than the size of the symbol.
Thus acutely LEMS Igs appears to spare DHP-sensitive binding sites. Superficially, this result agrees with the observations of Garcia and Beam (1996) that DHP-sensitive components of I_{Ca} were spared by LEMS Igs. The question then is whether induction of DHP sensitivity to LEMS Igs represents an acute or adaptive response. Perhaps DHP-sensitive channels are simply unmasked because they are spared clustering by the antibodies and subsequent lysis, or they may be actively upregulated teleologically to help the terminal cope with reduced ingress of Ca^{2+} caused by loss of P/Q types of channels normally present. Evidently another mechanism is involved in the activation of L-type Ca^{2+} channels during chronic treatment of mouse motor nerve terminals with LEMS serum or plasma, an activation that does not occur during acute treatment. For instance, the appearance of DHP-sensitive, L-type Ca^{2+} currents in the LEMS-treated motor nerve terminals may be due to new protein and mRNA synthesis for L-type Ca^{2+} channels or covalent modifications of preexisting proteins for L-type Ca^{2+} channels to compensate for conductance lost from P- or Q-type Ca^{2+} channels due to LEMS antibody attack. The DHP-sensitive, L-type Ca^{2+} currents do not appear in the acutely treated preparations because feedback regulation requires time for accumulation of new proteins synthesized for L-type Ca^{2+} channels, and synaptosomes lack the necessary biosynthetic machinery needed to transcribe and translate new L channels. ω-Aga-IVA-insensitive, but nifedipine-sensitive, L-type Ca^{2+} currents involved in ACh release processes have been found at newly formed (Sugiura and Ko 1997) and reinnervating mammalian neuromuscular junctions (Katz et al. 1995). However, the pattern of effect of DHP antagonists at these channels is to increase ACh release rather than to decrease it. Nevertheless, it may be possible that the apparent activation of L-type Ca^{2+} channels in LEMS-treated motor nerve terminals results from regeneration of the nerve terminals after LEMS antibody-induced degradation and necrosis. However, morphological studies have not suggested that nerve terminal degeneration and/or sprouting occurs. Instead, it appears that LEMS antibodies only reduce the density and number of Ca^{2+} channels with the remaining Ca^{2+} channels aggregating into clusters; no change of other membrane structures was reported (Fukunaga 1983). Furthermore, in synaptosomes incubated with LEMS Igs, lactate dehydrogenase release was not increased, whereas depolarization-dependent uptake of 45Ca^{2+} was decreased (Hewett and Atchison 1992a,b), providing further support that the reduction in Ca^{2+} channel function by LEMS antibodies was not simply a result of disruption of nerve terminal membrane integrity. Thus we think it unlikely that motor nerve terminals degraded, necrosed and regenerated after injection of LEMS plasma.

In the present study, we find that LEMS Igs significantly decreased the density of verapamil binding sites on synaptosomes (B_{max}) while leaving its affinity for the remaining receptors (K_{d}) unaffected. In the experiment from the neuromuscular preparations, we find that there is no significant difference in sensitivity of I_{Ca} to verapamil between the chronic LEMS plasma-treated and control plasma-treated motor nerve terminals. These two results provide a consistent picture that the number of verapamil-sensitive channels is reduced by LEMS Igs, with no reduction in apparent affinity for verapamil of the remaining channels. Verapamil is presumed to act as an L-type antagonist, the binding site of which is separate from that of the DHPs (Carvalho et al. 1986; Glossmann and Striessnig 1988; Miller 1987). However, recent evidence suggests that verapamil may not be as selective for L-type channels as was previously thought. In cerebellar granule cells (Carboni and Wojcik 1988) and neocortical neurons (Mangano et al. 1991) in primary culture, Ca^{2+} influx resulting from cell depolarization is only partially inhibited by nitrendipine. In contrast, verapamil completely blocked the depolarization-induced influx, suggesting that verapamil either blocks all subtypes of voltage-sensitive Ca^{2+} channels (Carboni and Wojcik 1988) or blocks the activity of a separate, perhaps novel type Ca^{2+} channel (Mangano et al. 1991). A verapamil-sensitive I_{Ca} current also exists at mouse motor nerve terminals (Anderson and Harvey 1987; Penner and Dryer 1986; Smith et al. 1995), and nerve-evoked release of ACh at mammalian neuromuscular junctions is sensitive to verapamil as well (Nachshen and Blaustein 1979). We found that the I_{Ca} remaining after treatment with ω-Aga-IVA and ω-CTx-MVIIC could be blocked by 10 μM verapamil. Thus the best pharmacological correlation between the functional effectiveness of LEMS Igs on motor axon terminals and inhibition of ligand binding on nerve terminals exists for verapamil. Our results show that a portion of P- or Q-type I_{Ca} in motor nerve terminals is sensitive to verapamil as well; this is consistent with the results of Ishibashi et al. (1995), who showed that verapamil blocked current carried through P-type Ca^{2+} channels in rat dissociated Purkinje neurons. Thus the total effect of inhibition of verapamil-sensitive P- or Q-type I_{Ca} by LEMS antibodies and appearance of DHP-sensitive I_{Ca} cause no greater percent reduction in the verapamil-sensitive currents in the LEMS-treated motor nerve terminals than those of control plasma-treated motor nerve terminals.

In the presence of 300 μM 3,4-DAP and 10 mM TEA, the three K^{+} currents in motor nerve terminals were blocked completely. Under these conditions, in control plasma-treated motor nerve terminals the amplitude and duration of I_{Ca} were depressed with increases in stimulation frequency from 0.01 to 10 Hz. Conversely, in the LEMS-treated group, the amplitude of I_{Ca} wasn’t altered under such stimulation conditions, although the duration of I_{Ca} was obviously decreased. A similar effect was observed when using short train stimulation, for which I_{Ca} remained relatively constant in amplitude at 20 Hz but was markedly depressed at 100 Hz in control. The respective LEMS-treated preparations exhibited an apparent facilitation of I_{Ca} at these higher frequencies. This frequency-dependent decrease of I_{Ca} in normal mouse motor nerve terminals has been proposed to be due to Ca^{2+}-dependent Ca^{2+} channel inactivation. During high-frequency stimulation, the amount of Ca^{2+} entering the terminals may be high enough to saturate the cellular mechanisms for Ca^{2+} buffering and extrusion. Above a critical intraterminal [Ca^{2+}], Ca^{2+} stimulates a process at the internal membrane surface to inactivate Ca^{2+} channels (Standen and Stanfield 1982). Therefore in LEMS plasma-treated motor nerve terminals, the loss of frequency-dependent depression for I_{Ca} during high frequencies of stimulation may well reflect the low [Ca^{2+}], which is insufficient to trigger Ca^{2+}-induced inactivation. However, in the presence of low con-
centrations of K+ channel blockers, there was no difference in ICa between control and LEMS plasma-treated preparations during high-frequency stimulation or train stimulation; facilitation of ICa amplitude occurred in both control and LEMS-treated groups. A more likely explanation is that delayed rectifier K+ channels, spared in the presence of low concentrations of K+ channels blockers, repolarized the membrane promptly to prevent Ca2+ increase (Mallart 1985). Therefore these results demonstrate that caution is required when using perineural recording technique to assess and compare the frequency-dependent effects on Ca2+ channel function between control and LEMS-treated motor nerve terminals.

In conclusion, these studies provide direct evidence for the relatively specific action of LEMS plasma on P- or Q-type Ca2+ channels in mouse motor nerve terminals. Sparing of the DHP-sensitive component of ICa appears not due to an acute effect of antibodies but may reflect a teleologic compensatory mechanism to maintain transmitter release in the absence of normal control mechanisms.

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