Journal of Bioenergetics and Biomembranes, Vol. 35, No. 6, December 2003 (© 2003)

Ca^{2+} Channels as Targets of Neurological Disease: Lambert–Eaton Syndrome and Other Ca^{2+} Channelopathies

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In the nervous system, voltage-gated Ca^{2+} channels regulate numerous processes critical to neuronal function including secretion of neurotransmitters, initiation of action potentials in dendritic regions of some neurons, growth cone elongation, and gene expression. Because of the critical role which Ca^{2+} channels play in signaling processes within the nervous system, disruption of their function will lead to profound disturbances in neuronal function. Voltage-gated Ca^{2+} channels are the targets of several relatively rare neurological or neuromuscular diseases resulting from spontaneously-occurring mutations in genes encoding for parts of the channel proteins, or from autoimmune attack on the channel protein responses. Mutations in CACNA1A, which encodes for the α_{1A} subunit of P/Q-type Ca^{2+} channels, lead to symptoms seen in familial hemiplegic migraine, episodic ataxia type 2, and spinocerebellar ataxia type 6. Conversely, autoimmune attack on Ca^{2+} channels at motor axon terminals causes peripheral cholinergic nerve dysfunction observed in Lambert–Eaton Myasthenic Syndrome (LEMS), the best studied of the disorders targeting voltage-gated Ca^{2+} channels. LEMS is characterized by decreased evoked quantal release of acetylcholine (ACh) and disruption of the presynaptic active zones, the sites at which ACh is thought to be released. LEMS is generally believed to be due to circulating antibodies directed specifically at the Ca^{2+} channels located at or near the active zone of motor nerve terminals (P/Q-type) and hence involved in the release of ACh. However, other presynaptic proteins have also been postulated to be targets of the autoantibodies. LEMS has a high degree of coincidence (∼60%) with small cell lung cancer; the remaining 40% of patients with LEMS have no detectable tumor. Diagnosis of LEMS relies on characteristic patterns of electromyographic changes; these changes are observable at neuromuscular junctions of muscle biopsies from patients with LEMS. In the majority of LEMS patients, those having detectable tumor, the disease is thought to occur as a result of immune response directed initially against voltage-gated Ca^{2+} channels found on the lung tumor cells. In these patients, effective treatment of the underlying tumor generally causes marked improvement of the symptoms of LEMS as well. Animal models of LEMS can be generated by chronic administration of plasma, serum or immunoglobulin G to mice. These models have helped dramatically in our understanding of the pathogenesis of LEMS. This "passive transfer" model mimics the electrophysiological and ultrastructural findings seen in muscle biopsies of patients with LEMS. In this model, we have shown that the reduction in amplitude of Ca^{2+} currents through P/Q-type channels is followed by "unmasking" of an L-type Ca^{2+} current not normally found at the motor nerve terminal which participates in release of ACh from terminals of mice treated with plasma from patients with LEMS. It is unclear what mechanisms underlie the development of this novel L-type Ca^{2+} current involved in release of ACh at motor nerve terminals during passive transfer of LEMS.

KEY WORDS: Lambert–Eaton syndrome; myasthenic syndromes; motor nerve terminal; neuromuscular junction; neuromuscular diseases; Ca channelopathy; acetylcholine release; dihydropyridine; neuromuscular transmission.

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0145-479X/03/1200-0697/0 © 2003 Plenum Publishing Corporation

INTRODUCTION

Because of the critical role which Ca^{2+} channels play in signaling processes within the nervous system, disruption of their function will lead to profound disturbances in neuronal function. Several neurological disorders have been attributed to impaired function of Ca^{2+} channels—especially those associated with release of neurotransmitters. The purpose of this review is to describe in detail the alteration of Ca^{2+} channel function occurring in the Lambert–Eaton Myasthenic Syndrome (LEMS), particularly with respect to recent studies suggesting that adaptive changes occur in the process controlling acetylcholine (ACh) release from motor axon terminals. We will also discuss other channelopathies which are thought to result from naturally-occurring mutations in genes coding for Ca^{2+} channel subunits. These mutations cause a variety of Ca^{2+} channelopathies resulting in neuronal and neuromuscular disorders such as familial hemiplegic migraine, spinal cerebellar ataxia type 6, congenital stationary night blindness, and episodic ataxia type 2. Mutations of Ca^{2+} channels involved in excitation–contraction coupling have been linked to muscle disorders such as: hypokalemic periodic paralysis, malignant hyperthermia, and central core disease. The specifics of these channelopathies have been described in several recent thorough reviews (see reviews by Lorenzon and Beam, 2000; Meir and Dolphin, 2002; Pietrobon, 2002).

LAMBERT–EATON SYNDROME

LEMS is an autoimmune neurological disorder in which the nerve-stimulated release of ACh from peripheral cholinergic nerves is reduced (Lambert *et al.*, 1961; Lambert and Rooke, 1965; O'Neill *et al.*, 1988). Patients with LEMS primarily exhibit skeletal muscle weakness, decreased tendon reflexes, and various dysautonomias. The pathology underlying LEMS is believed to be due to circulating antibodies. This conclusion is based upon the findings that plasma exchange or immunosuppression transiently alleviates symptoms in patients with LEMS and the ability to transfer passively the disease to mice via injection of IgG from patients with LEMS (Lang *et al.*, 1981; Lennon *et al.*, 1982; Newsom-Davis and Murray, 1984). Approximately 60% of patients with LEMS have a small cell lung carcinoma (SCLC); the generation of circulating antibodies is believed to be initiated as an immune response to the tumor (Lambert and Rooke,1965; O'Neill *et al.*, 1988).

Although the clinical signs of LEMS could reflect actions at several sites, including the postsynaptic nicotinic receptor, unlike the situation seen in myasthenia gravis, sensitivity of muscle end-plates to ACh does not appear to be altered in LEMS (Cull-Candy *et al.*, 1980; Lang *et al.*, 1987). In addition, cholinergic vesicle size and contents and intraterminal processes involved in release of ACh at the motor nerve terminal are also unaltered in LEMS (Lang *et al.*, 1984; Molenaar *et al.*, 1982). On the other hand, active zone particles, which are believed to represent voltage-dependent Ca^{2+} channels (VDCC) aligned adjacent to active zones of neurotransmitter release are disorganized and fewer in number at motor nerve terminals from patients with LEMS. Furthermore, immunoprecipitation and functional studies have provided additional evidence supporting the role for circulating antibodies directed against VDCC in patients with LEMS.

Entry of Ca^{2+} through VDCC into nerve terminals is a necessary step coupling the action potential to release of ACh (Augustine *et al.*, 1987; Katz and Miledi, 1970; Llinas *et al.*, 1976). Although multiple subtypes of VDCC exist, the specific channel subtype involved in release of ACh from motor nerve terminals is both species and age-dependent. During maturation, motor nerve terminals appear to possess multiple subtypes of VDCC (Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001), however, mature motor nerve terminals contain primarily one subtype of VDCC involved in release of ACh. Mature mammalian motor nerve terminals utilize P/Q-type (Katz *et al.*, 1995; Protti *et al.*, 1996), whereas amphibians (Sano *et al.*, 1987) and birds (De Luca *et al.*, 1991) rely mainly on N-type Ca^{2+} channels to control release of ACh.

Among the multiple subtypes of VDCC, antibodies in patients with LEMS appear to target preferentially the P/Q-type channel (Lennon *et al.*, 1995; Pinto *et al.*, 2002). Thus, based upon the vital role of VDCC and antibody specificities, the decrease in nerve-stimulated release of ACh in patients with LEMS appears to be due to a decrease in the number of functional VDCC, particularly those of the P/Q-type.

CLINICAL FEATURES OF LEMS

LEMS occurs in two general populations: those with and without detectable carcinomas (Elmqvist and Lambert, 1968; O'Neill *et al.*, 1988). Among those with cancer, small cell, or oat cell lung carcinoma (SCLC) is the most common type; approximately 60% of all patients with LEMS exhibit SCLC (Eaton and Lambert, 1957; Lambert *et al.*, 1961; Lambert and Elmqvist, 1971; Lambert and Rooke, 1965; O'Neill *et al.*, 1988). Symptoms of LEMS almost always precede the diagnosis of cancer by 5 months to 3.8 years and in the majority of

patients occur over the age of 40 years. However the onset of LEMS has been reported to range from 17 to 79 years (O'Neil *et al.*, 1988). Males are more predominately affected than females in both cancer and noncancer groups (Elmqvist and Lambert, 1968; O'Neill *et al.*, 1988).

The most common clinical symptom and sign observed in patients with LEMS is that of skeletal muscle weakness, mainly limited to proximal and truncal muscles and most dramatically affecting the lower limbs (Erlington and Newsom-Davis, 1994; Lambert *et al.*, 1961; Lambert and Rooke, 1965; O'Neill *et al.*, 1988; Wise and MacDermot, 1962). Although arm weakness is often described, it is usually restricted to the proximal muscles and much less severe than that of the lower leg (Erlington and Newsom-Davis, 1994; O'Neill *et al.*, 1988). During sustained maximal muscle contractions there is a brief progressive augmentation in strength followed by increasing weakness and fatigue. This is a hallmark sign of LEMS, and the basis for an older alternate name for the disease—"facilitating myasthenia."

Tendon reflexes are often depressed and/or absent in most patients, but augment or reappear following maximal voluntary muscle contraction (Lambert *et al.*, 1961; Lambert and Rooke, 1965; Wise and MacDermot, 1962).

Autonomic dysfunction is a complaint in approximately 80% of the patients. Clinical symptoms include mainly dry mouth, sexual impotence, and to a lesser extent decreased sweating, constipation, difficulty with micturition and blurred vision (Henriksson *et al.*, 1977; Khurana *et al.*, 1988; Lambert and Rooke, 1965; O'Neill *et al.*, 1988; Rubenstein *et al.*, 1979). Most autonomic abnormalities can be ascribed to defects of the parasympathetic (cholinergic) system, however, two reports have described patients with sympathetic dysfunction. Clinical signs included orthostatic hypotension and increased sensitivity to exogenous sympathetic agonists (Khurana *et al.*, 1988; Mamdani *et al.*, 1985). The findings of sympathetic dysautonomias most likely reflect an effect induced by the underlying neoplasm in the described patients and not that of LEMS; for example, cardiovascular autonomic dysfunction is commonly found in patients with bronchial neoplasms and SCLC without LEMS (Gould *et al.*, 1986; Maier and Sommers, 1986; Park *et al.*, 1972) as well as breast cancer patients (Bruera *et al.*, 1986; Heath *et al.*, 1988). Impaired sensory nerve function is not a prominent finding in LEMS (Heath *et al.*, 1988; Khurana *et al.*, 1988; O'Neill *et al.*, 1988). However, cranial nerves are also affected in LEMS and patients often exhibit diplopia and ptosis, dysarthria, dysphagia, and dysphonia. The symptoms related to cranial nerve involvement, however, are often mild and transient (O'Neill *et al.*, 1988).

The symptoms of patients with LEMS can be dramatically reduced by pharmacological agents that enhance release of ACh (Newsom-Davis and Murray, 1984; Oh *et al.*, 1997; Sanders, 1998; Sanders *et al.*, 2000). For instance, guanidine and 4-aminopyridine, which prolong the duration of action potentials and thereby enhance entry of Ca^{2+} into the nerve terminal and consequently enhance release of ACh produce symptomatic relief, albeit frequently accompanied by serious side effects related to the central nervous system. On the other hand, the actions of a similar compound, 3,4-diaminopyridine (3,4 DAP), are generally limited to the peripheral nervous system. Thus this agent offers symptomatic relief without serious side effects. Although it may be theoretically possible that 3,4 DAP alters cardiac function by decreasing the rate of ventricular myocyte repolarization, patients taking 20 mg of 3,4 DAP three times daily for 6 days did not exhibit any changes in blood pressure or QT interval (which represents the time of ventricular myocyte repolarization) as measured using EKG (Sanders *et al.*, 2000). Drugs that prolong the actions of ACh at motor end-plates by inhibiting acetylcholinesterase and which alleviate symptoms in patients with myasthenia gravis, a neuromuscular disorder in which functional ACh receptors on skeletal muscles are reduced, offer little benefit in patients with LEMS (Eaton and Lambert, 1957; O'Neill *et al.*, 1988).

ELECTROPHYSIOLOGICAL CHARACTERISTICS

Clinical

Although several neuromuscular disorders (for example, myasthenia gravis) are characterized by skeletal muscle weakness, the differential diagnosis in patients with suspected LEMS is aided by electromyography (EMG), which utilizes surface recording electrodes to detect compound muscle action potentials (CMAP) generated from the muscles comprising the motor unit (the motor nerve and all muscle fibers innervated by it) (Eaton and Lambert, 1957). The typical EMG findings from patients with LEMS are depicted in Fig. 1. CMAP amplitudes in response to low frequency (usually $\leq$5 Hz) stimulation of the motor nerve are depressed and can be as low as 10% of normal in patients with LEMS (Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Jablecki, 1984; O'Neill *et al.*, 1988). During repetitive stimulation, CMAPs also decrement in amplitude. High frequency ($\geq$10 Hz) stimulation of the motor nerve, on the other hand, induces a progressive increase in the amplitude of the CMAP; this phenomenon is referred to as facilitation

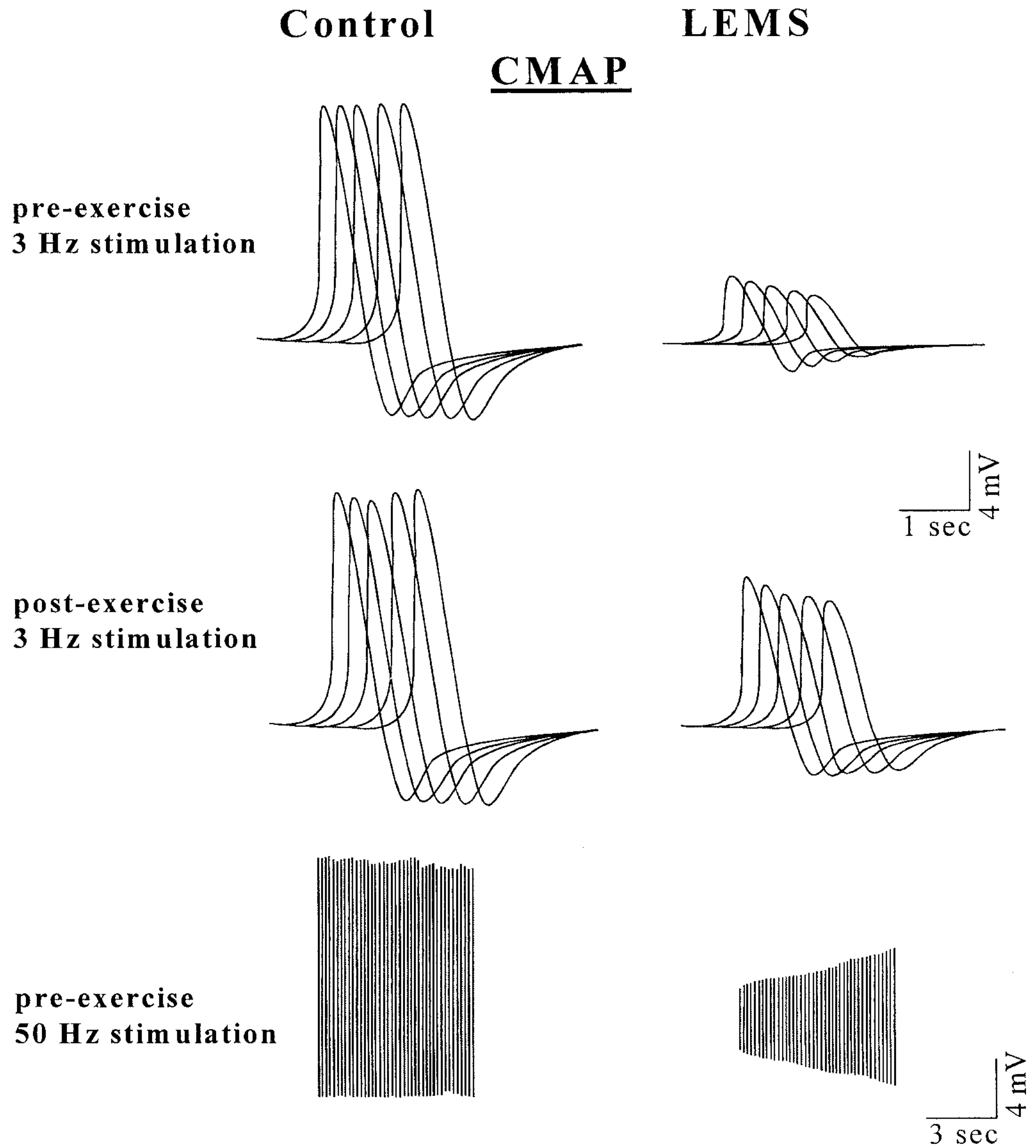

Fig. 1. Cartoon depicting representative EMG recordings of CMAP from control and LEMS patients. CMAP elicited during stimulation of the motor nerve at 3 Hz (low frequency) are smaller in amplitude from LEMS patients in comparison to controls. Following maximal voluntary muscle contractions (postexercise), CMAP increases in amplitude in comparison to preexercise recordings in LEMS patients during 3 Hz stimulation of the motor nerve. Prior to exercise, high frequency stimulation (50 Hz) of the motor nerve leads to a progressive increase in amplitudes of the CMAP from LEMS, but not control patients.

(Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Jablecki, 1984). Maximal voluntary muscle contraction also increases the CMAP amplitudes compared to that prior to muscle contraction. It has been postulated that facilitation of the CMAP amplitude is due to build-up of Ca^{2+} in the nerve terminal that normally does not occur to the same extent as that in patients with LEMS. Generally, sensory and motor nerve conduction velocities and latencies are unchanged, as are sensory nerve action potential amplitudes in patients with LEMS (Heath *et al.*, 1988; Jablecki, 1984; Lambert and Rooke, 1965; O'Neill *et al.*, 1988). These findings suggest that mechanisms involved in action potential generation and conduction are unaffected in LEMS.

Single-fiber EMG (SFEMG), a technique used to measure neuromuscular transmission in individual

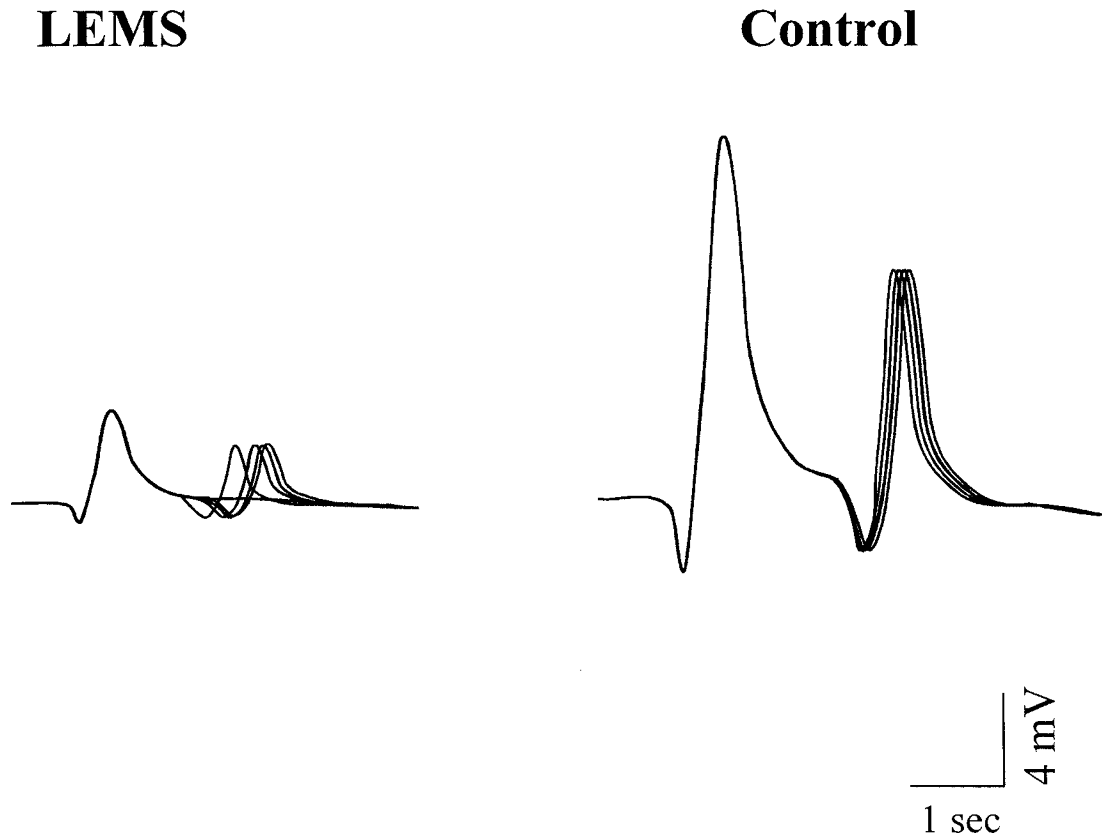

Fig. 2. Cartoon depicting typical single-fiber EMG recordings from two muscle fibers innervated by the same motor nerve. Tracings represent superimposed potentials following low frequency single shock stimulation of a motor nerve from control of LEMS patients. The duration of time between the two potentials recorded from each muscle fiber innervated by the same motor nerve varies between superimposed tracings and is known as "jitter." Failure of the second muscle fiber to generate a potential is known as "blocking." Tracings from LEMS patients exhibit a higher degree of jitter and blocking in comparison to control patients.

end-plates is also abnormal in patients with LEMS. This is shown schematically in Fig. 2 (Keesey, 1989; Sanders, 1994). SFEMG is often performed during voluntary muscle contractions and allows measurements of action potentials from two separate muscle fibers that are innervated by the same motor nerve. Normally, the time interval between the two action potentials varies from consecutive nerve discharges, producing response referred to as "neuromuscular jitter." In some conditions, generation of an action potential in the second muscle fiber fails; this is referred to as "neuromuscular block." In LEMS, both jitter and block are increased in comparison to controls and characteristically decrease as the rate of nerve stimulation increases (Sanders, 1994).

Experimental

Intracellular recordings of synaptic transmission from neuromuscular tissue isolated from patients with LEMS show electrophysiological characteristics consistent with those found using EMG: end-plate potential (EPP) amplitudes in response to single shock stimulation of the motor nerve are smaller than those of control preparations; following high frequency nerve-stimulation EEP amplitudes increase progressively in size (Cull-Candy *et al.*, 1980; Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971). Miniature end-plate potential (MEPP) amplitudes are not appreciatively changed at neuromuscular junction of tissue biopsy preparations from LEMS patients nor are their rise or decay times affected (Cull-Candy *et al.*, 1980; Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971). Although spontaneous quantal release of ACh (measured as changes in MEPP frequency) is normally unaltered from LEMS preparations exposed to solutions containing physiological concentrations of KCl, K^+-induced depolarization of the nerve terminal membrane, which increases the frequency of asynchronous release of ACh in an extracellular Ca^{2+}-dependent

manner is significantly lower than that observed from controls.

MORPHOLOGY OF NEUROMUSCULAR JUNCTIONS IN LEMS

Histological analysis of the light microscopic level of tissue biopsies from patients with LEMS does not reveal any gross abnormalities at motor nerve terminal or end-plate regions (Engel and Santa, 1971). Electron microscopic examination of neuromuscular junctions from patients with LEMS also reveals normal morphology of the motor nerve terminals, but alterations in postsynaptic regions. Engel and Santa (1971) found the following using tissue biopsies obtained from patients with a history of LEMS: mean nerve terminal area and number of synaptic vesicles were not significantly different form controls, mean vesicle diameter calculated for each biopsy was also unchanged, and the postsynaptic region was hypertrophied with increased area and length of the folds and clefts.

Although other investigators have described similar findings at motor nerve terminals, postsynaptic membrane areas and lengths appeared decreased from patients diagnosed with LEMS in the reports of Hesselmans *et al.* (1992) and Tsujihata *et al.* (1987). This discrepancy with the findings of Engel and Santa (1971) may reflect differences in the duration of LEMS in the patients used in the respective studies. Whereas the patients in the study performed by Engel and Santa (1971) had a history of LEMS for up to 10 years, the other studies used patients diagnosed as having LEMS for no longer than 1.5 years prior to their tissue biopsies (Hesselmans *et al.*, 1992; Tsujihata *et al.*, 1987). Expansion of the postsynaptic membrane is a well-known response to denervation, and may reflect an apparant compensatory response to the chronic impairment of presynaptic function in LEMS.

AUTOIMMUNITY

Initial conclusions that an autoimmune component underlies the pathology in LEMS were based on the observations that many patients with defects of neuromuscular transmission had associated autoimmune disorders (Gutmann *et al.*, 1972; Norris and Panner, 1966; Takamori *et al.*, 1972). More recent work has shown a higher than normal incidence of autoimmune disorders, such as vitilligo, pernicious anemia, celiac disease, juvenile-onset diabetes, and other organ-specific antibodies (Lennon *et al.*, 1982; O'Neill *et al.*, 1988) in patients with LEMS. Further support for an autoimmune component was gleaned from the observations that plasma exchange and immunosuppressive therapy induced marked improvements of symptoms in patients with LEMS with or without SCLC (Lang *et al.*, 1981; Newsom-Davis and Murray, 1984). The autoimmune basis for LEMS was established definitively by the findings that injection of mice with IgG from patients with LEMS not only duplicated the electrophysiological characteristics observed from EMG recordings and intracellular electrophysiological recordings of muscle preparations from patients with LEMS (Lang *et al.*, 1981), but ultrastructural studies showed IgG located at motor nerve terminals as well (Fukunaga *et al.*, 1983). Early work examining passive transfer of LEMS to mice using IgG in comparison to whole plasma fractions with slightly lower IgG concentrations showing a greater reduction in release of ACh from motor nerve terminals exposed to IgG alone (Lang *et al.*, 1981). These findings support the notion that the primary factor involved in the pathology of LEMS is the autoantibody.

ANTIGENIC TARGETS

It was originally proposed that the autoantibody in patients with LEMS acts at the motor nerve terminal and most likely interferes in the utilization of Ca^{2+} normally involved in evoked-release of ACh. This was based upon the findings that repetitive stimulation of the nerve or increases in extracellular Ca^{2+} levels, which increase the probability of quantal ACh release improve synaptic transmission (Lambert and Elmqvist, 1971). Although the specific site of action of LEMS antibodies was unclear, entry of Ca^{2+} through voltage-dependent channels or subsequent intraterminal processes involved in exocytosis or Ca^{2+} metabolism represented potential sites of disruption. However, in conditions of physiological extracellular K^+ (5 mM) and reduced Ca^{2+} concentrations (no added Ca^{2+}), the rate of spontaneous release of ACh from LEMS motor nerve terminals was normal (Lang *et al.*, 1987). This finding suggests that in LEMS, intraterminal processes, which are independent of extracellular Ca^{2+} levels are unaffected. If this was not the case, the frequency of occurrence of spontaneous release of ACh (MEPPs) would be reduced. In the presence of high extracellular Ca^{2+} concentrations which increase the amount of Ca^{2+} entering the terminal during nerve-stimulation, nerve-evoked release of ACh also appeared normal, suggesting that exocytotic mechanisms that occur following Ca^{2+} entry are also unaffected in LEMS. Thus, based upon these findings, it appeared that LEMS antibodies most likely reduce Ca^{2+} entry into the terminal, presumably by affecting the function of voltage-dependent Ca^{2+} channels.

Experiments involving the cardiac glycoside ouabain have provided further evidence against an intraterminal target in LEMS (Wray *et al.*, 1987). Incubation of motor nerve terminals with ouabain which blocks Na^+/K^+ exchange increases the MEPP frequency via a mechanism independent of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Baker and Crawford, 1975). Ouabain-induced release of ACh is not altered following exposure of motor nerve terminals to LEMS antibodies; thus intraterminal mechanisms are not affected in LEMS (Lang *et al.*, 1987; Wray *et al.*, 1987). Alternatively, the characteristics of LEMS could be explained by impaired ACh synthesis, storage, and uptake, however, based upon biochemical and electrophysiological techniques these appear intact (Lang *et al.*, 1984; Meyer *et al.*, 1986; Molenaar *et al.*, 1982).

Other possible targets in LEMS have also been excluded. The observation that release of ACh is impaired following K^+-induced depolarization of motor nerve terminals from patients with LEMS, suggests that action potential generation and conduction are not affected and the antibody effects are limited to nerve terminals. This idea (as described above) has also been confirmed by direct measurements of conduction velocity and action potentials from muscle and nerve preparations (Elmqvist and Lambert, 1968; Kim, 1985). Postsynaptic sensitivity to ACh also appears unaltered and MEPP amplitudes are not appreciably affected in LEMS (Lang *et al.*, 1987; Prior *et al.*, 1985). Additional observations consistent with the idea that LEMS antibodies interfere with Ca^{2+} entry through voltage-dependent Ca^{2+} channels included the findings that electrical stimulation of motor nerves exposed to LEMS antibodies exhibit strikingly similar electrophysiological characteristics to those seen after exposure of neuromuscular preparations to divalent heavy-metal ions, which are known to block the function of Ca^{2+} channels directly (Jenkinson, 1957; Silinsky, 1985).

A number of studies have provided more direct support implicating VDCC as the putative target in LEMS. Incubation of cultures of SCLC with IgG from patients with LEMS inhibits uptake of $^{45}Ca^{2+}$ during K^+-induced depolarization (De Aizpurua *et al.*, 1988). This inhibition is dependent upon the concentration of IgG used and time of exposure of the SCLC to LEMS IgG. Electrophysiological recording of Ca^{2+} currents from SCLC cells exposed to LEMS plasma have also corroborated the finding from the K^+-induced $^{45}Ca^{2+}$ uptake studies; SCLC cells exposed to LEMS IgG for 24 h reduced the amplitude of current through voltage-dependent Ca^{2+} channels by ~58% (Viglione *et al.*, 1995) when compared to SCLC cells exposed to control IgG. In another study, exposure of SCLC cells in culture to LEMS IgG for 24 h reduced the voltage-dependent Ca^{2+} entry by up to 70% (Meriney *et al.*, 1996). Voltage-dependent Ca^{2+} currents in bovine adrenal chromaffin cells (BAC) are also reduced following exposure to LEMS IgG; however, direct injection of Ca^{2+} into the cell, which bypasses the need for VDCC, induced normal exocytosis (Engisch *et al.*, 1999; Kim, 1987; Kim and Neher, 1988). Similarly, ionomyocin, which forms pores in membranes that are specific for Ca^{2+} entry and also bypasses the need for VDCC, triggers equal release of ACh from electric ray nerve terminals exposed to LEMS or control IgG (Satoh *et al.*, 1998). Although these finding all support the notion that voltage-dependent Ca^{2+} channel function is impaired by IgG from patients with LEMS, they do not provide direct evidence that Ca^{2+} channels located on mammalian nerve terminals were affected by LEMS antibodies. Inasmuch as it is clear that different phenotypes of Ca^{2+} channels are distributed differentially in neurons it was important to ascertain whether Ca^{2+} channel function at nerve terminals was specifically affected by LEMS IgGs. Several subsequent studies were designed to test this issue specifically. Uptake of $^{45}Ca^{2+}$ into isolated enriched rat nerve terminal preparations (synaptosomes of rat cerebral cortex) was reduced during KCl-induced depolarization following acute exposure to sera from patients with LEMS (Hewett and Atchison, 1991, 1992a). Conversely, uptake of ^{22}Na or ^{86}Rb as a surrogate for K^+ were not affected by the same IgGs. More directly, Ca^{2+} currents recorded from the perineuriun of motor nerves obtained from mature mice are also reduced following injection of LEMS patient sera for 30 days (Smith *et al.*, 1995; Xu *et al.*, 1998). Taken together, these observations reinforce the notion that LEMS antibodies act on VDCC in multiple systems without altering intracellular mechanisms involved in exocytosis.

VOLTAGE-DEPENDENT Ca^{2+} CHANNEL MODULATION

Electrophysiological Features

Although Ca^{2+} entry into BAC cells exposed to LEMS antibodies is reduced, properties of single Ca^{2+} channels, such as activation and inactivation kinetics, ion conductance, and channel open time are unaltered (Grassi *et al.*, 1994; Kim and Neher, 1988; Magnelli *et al.*, 1996). This finding suggests that LEMS antibodies react with VDCC in an all or none fashion, and as such either irreversibly block or induce the elimination of functional channels. This is further supported by the observations that the relationship between K^+-induced release of ACh and increasing extracellular Ca^{2+} concentrations is altered at motor nerve terminals exposed to LEMS antibodies in

a manner resembling the actions of an irreversible antagonist (Lang *et al.*, 1987). Nerve-stimulated release of ACh from LEMS-treated motor nerve terminals, on the other hand, appears to reflect the actions of a competitive antagonist (i.e. heavy metals). As such, the effect of LEMS antibodies on reducing maximum release of ACh can be overcome in the presence of high Ca^{2+} concentrations following electrical nerve-, but not K^+-induced stimulation. This discrepancy can be explained by differences in the methods used to elicit release of ACh. Unlike, high K^+ concentrations, electrical stimulation of the nerve produces much larger simultaneous entry of Ca^{2+} into the terminal, thus saturating intraterminal release mechanisms before saturation of Ca^{2+} entry through VDCC occurs (Lang *et al.*, 1987). Raising extracellular Ca^{2+} concentrations, which leads to an increase in the amount of Ca^{2+} entering the terminal following nerve-stimulation competes with the saturated release mechanisms and thus, gives the false appearance that LEMS antibodies act on VDCC in a manner resembling that of a competitive antagonist (Lang *et al.*, 1987). K^+-evoked entry of Ca^{2+}, on the other hand does not saturate intraterminal mechanisms and, thus maximum release of ACh becomes limited by saturation of Ca^{2+} entry instead. Taken together, these observations support the role of LEMS antibodies as an irreversible antagonist, reducing function of voltage-dependent Ca^{2+} channels in an all or none fashion.

Ultrastructural Features

Early investigations in which ultrastructural or freeze-fracture analysis was applied to motor nerve terminals provided compelling evidence that voltage-dependent Ca^{2+} channels are eliminated in LEMS. Normally, ultrastructural analysis of motor nerve terminals reveals the presence of large intramembraneous structures in the active zone region known as active zone particles (AZPs). Several ideas support the idea that voltage-dependent Ca^{2+} channels comprise active zone particles. (Llinas *et al.*, 1976; Pumplin *et al.*, 1981; Robitaille *et al.*, 1990). First, the rapid and precise nature of neuromuscular transmission necessitates a close proximity of Ca^{2+} channels with synaptic vesicles ready to be released at active zones. Consequently, the latency between Ca^{2+} influx and synaptic vesicle exocytosis is quite small (Llinas *et al.*, 1976) and exocytotic events can occasionally be resolved via quick freezing to occur near AZPs (Heuser *et al.*, 1979). Furthermore, maximum amplitude Ca^{2+} currents in the motor nerve terminal are related to the number of AZPs (Pumplin *et al.*, 1981). More direct support for the association of VDCC and AZPs is provided by the observation that fluorescent staining with ω-conotoxin GVIA, which presumably represents labeling of N-type VDCC at frog motor nerve terminals occurred in regular patterns apparently corresponding to AZPs, which precisely matched the pattern of fluorescence associated with α-bungarotoxin (a high affinity ligand for the muscle type nicotinic receptor) labeled ACh receptors on the postsynaptic membrane in a manner consistent with ultrastructural findings (Robitaille *et al.*, 1990). Furthermore, imaging of fluorescently-labelled Ca^{2+} ions in conjunction with carbon-fiber elctrode techniques to map spatially the release of catecholamines from BAC cells reveals that voltage-dependent Ca^{2+} entry occurs in distinct localized regions ("hotspots") and release of catecholamines mainly occurs within 1.4 μm of these "hotspots." (Robinson *et al.*, 1995). Conversly, catecholamine release rarely occurred at sites away from these "hotspots." These findings provide further support for the close proximity of VDCC and transmitter release sites.

Tissue biopsies from patients with LEMS displayed a paucity and disorganization of active zones and AZPs, and clustering of AZPs in comparison to control tissues (Fukunaga, 1982). Similar findings were obtained at motor nerve terminals of mice following chronic injection of IgG from patients with LEMS (Fukuoka *et al.*, 1987a). These observations not only provided one of the first pieces of evidence implicating VDCC as the antigenic target, but also suggested that loss of VDCC ultimately leads to the pathology of LEMS.

Radioligand Binding

Other findings have also provided further evidence that VDCC are eventually lost in LEMS. Incubation of IMR cells for 24 h with LEMS antibodies decreases binding of ^{125}I-ω-CgTxGVIA (Sher *et al.*, 1989). This situation could arise if LEMS antibodies bind to the same site on the VDCC as ^{125}I-ω-CgTx GVIA or if the number of VDCC in the plasma membrance is reduced. However, subsequent studies have shown that LEMS antibodies and ^{125}I-ω-CgTx GVIA bind to discrete sites on VDCC, and induce the eventual loss of VDCC (as described below).

Mechanism of Modulation

Although the mechanism involved in reducing functional VDCC is unclear, it has been proposed that antigenic modulation is a necessary step. Ultrastructural analysis of neuromuscular junction preparations incubated with LEMS antibodies indicates that a reduction in the

distance between adjacent AZPs initially occurs followed by disorganization and clustering, eventually leading to loss of AZPs (Engel *et al.*, 1987; Fukuoka *et al.*, 1987a). Furthermore, the decrease in distance between AZPs is consistent with the width of binding regions on the IgG molecule (Fukuoka *et al.*, 1987b). These findings suggest that modulation of AZPs and thus presumably VDCC occurs by cross linking with LEMS IgG (Engel *et al.*, 1987; Fukuoka *et al.*, 1987b). This idea was tested by exposing motor nerve terminals for 24 h to antibodies that were either capable {divalent IgG ($F(ab')_2$ fragments} or incapable {monovalent IgG (F(ab)) fragments} of cross-linking VDCC (Nagel *et al.*, 1988; Peers *et al.*, 1993). Following incubation with LEMS IgG $F(ab)_2$, but not LEMS IgG F(ab) fragments, AZPs appeared aggregated and depleted (Nagel *et al.*, 1988). In conjunction with this study, nerve-stimulated release of ACh is also reduced after motor nerve terminals are exposed to LEMS IgG $F(ab)_2$, but not LEMS IgG F (ab) fragments (Peers *et al.*, 1993). Thus, antigenic cross-linking appears to be a necessary prerequisite for functional loss of VDCC in LEMS.

Functional Antagonism of Voltage-Dependent Ca^{2+} Channels

In addition to a reduction in the number of VDCC, some studies suggest that irreversible blocking of the channels by LEMS IgG contributes to the reduction in release of ACh. Binding of LEMS antibodies to VDCC has been reported to occur very rapidly (Martin-Moutot *et al.*, 1995) and as such, K^+-induced uptake of $^{45}Ca^{2+}$ into mammalian nerve terminal preparations is significantly reduced following incubation with sera from patients with LEMS for either 3 min (Meyer *et al.*, 1986) or 1 h (Hewett and Atchison, 1991, 1992a). Similarly, exposure of SCLC or BAC cells in culture for 90 min or 30 min, respectively, to LEMS antibodies reduces voltage-dependent Ca^{2+} entry, which becomes more pronounced after 24 h (Johnston *et al.*, 1994; Kim and Neher, 1988). In contrast, exposure of mammalian neuromuscular junction preparations in vitro to LEMS antibodies or sera for 2 h does not consistently affect nerve-evoked release of ACh (Kim *et al.*, 1988; Prior *et al.*, 1985). Similarly, the $t_{1/2}$ of reduction in release of ACh following a single i.v. injection of mice with antibodies from one patient with LEMS was observed to be approximately 4–6 h (Lambert and Lennon, 1988). It is possible that access to motor nerve terminals in such preparations is limited, thus increasing the time required for LEMS antibodies to induce an observable effect. However, in cell cultures and nerve terminal preparations antibody-induced reduction in Ca^{2+} entry occurs too rapidly to be completely explained by elimination of VDCC and thus, loss of function is in part most likely the result of direct block of the channel, which may be an obligatory step prior to cross linking.

ROLE OF COMPLEMENT

In addition to antibody-induced antigenic modulation, other immune components could be involved in LEMS. For example, complement has been observed to participate in the pathogenesis of other neuromuscular disorders and complement components have been found localized at end-plate regions of biopsies from patients with myasthenia gravis and in animal models of myasthenia gravis. Thus, it is possible that complement is involved in LEMS as well.

Elimination of foreign bodies is aided by complement, which is comprised of serum proteins that act sequentially and in concert with one another to form two interconnected pathways—the classical and alternative pathway. Although both pathways share common components, they are activated differently (Benjamini *et al.*, 1996; Goetz, 1987; Loos, 1987). The classical pathway is activated by the formation of antibody and antigen complexes, while the alternative pathway is activated by various other substances, such as cobra venom and some bacteria cell walls and yeast. Many of the protein complexes formed in each pathway act as enzymes that catalyze the next step and/or aid directly in elimination of the initiating factor. Most of the complexes are given a number designation preceded by the letter "C." Two key protein complexes of complement are C3 and C5. The enzymatic reaction of C3 by C3 convertase produces factors C3a and C3b, which are vital components that enhance phagocytosis, immune adherence, and degranulation of mast and basophils (Hostetter *et al.*, 1984). C3b acts as common intermediary between the two complement pathways; it activates the alternative pathway and leads to its own amplification (Goetz and Muller-Eberhand, 1971). In addition, C3b also leads to the production of C5 convertase, which enzymatically cleaves C5 into C5a and C5b. Whereas C5a is important for mast and basophil cellular degranulation and as an attractant for other immune components, C5b initiates the formation of the C5–C9 complex, which possess cytotoxic properties (Porter and Reid, 1978). The role of complement in LEMS has been investigated using mice either genetically deficient in C5 or treated with cobra venom, which leads to the depletion of C3 by continual activation of the alternative pathway (Lambert and Lennon, 1988; Prior *et al.*, 1985). Chronic injection of mice deficient in C5 and C3 and mice with intact complement systems with LEMS antibodies

produced similar impairment of nerve-evoked release of ACh from motor nerve terminals. Also, muscle biopsies of patients with LEMS examined using immunoelectron microscopy do not exhibit localized C3 components on the nerve terminal (Engel *et al.*, 1977; Tsujihata *et al.*, 1987). These findings suggest that complement is not involved in LEMS. However, reduction in K^+-induced $^{45}Ca^{2+}$ uptake into mammalian synaptosomal preparations exposed to LEMS antibodies for 1 h is dependent upon C3, but not C5 (Hewett and Atchison, 1992b). In addition, factor B was observed to be required for low concentrations of LEMS antibodies to impair K^+-induced $^{45}Ca^{2+}$ uptake in these preparations (Hewett and Atchison, 1992b). Although complement components do not appear to be involved in LEMS, it is possible that in certain model systems complement enhances the effects of LEMS antibodies. As such, it has been noted that many of the effects of antibodies on cells is increased in the presence of complement (Benjamini *et al.*, 1996).

NONVOLTAGE-DEPENDENT Ca^{2+} CHANNEL ANTIGENIC TARGETS

In addition to VDCC, other proteins have been proposed to serve as antigenic targets in LEMS. In particular, the synaptic vesicle protein, synaptotagmin has been implicated. Synaptotagmin is believed to act as the Ca^{2+} sensor and hence play a vital role in the docking and fusion of synaptic vesicles during exocytosis (Brose *et al.*, 1992; Perin *et al.*, 1990). Several observations support the role of synaptotagmin as a target in LEMS. First, Western blots of VDCC partially purified from rat brains probed with IgG and plasma from LEMS patients labeled a 58-kDa antigen, which was putatively shown by partial amino acid sequence to represent synaptotagmin (Leveque *et al.*, 1992). However, only IgG and plasma from LEMS patients with high antibody titer specific for ^{125}I-ω-conotoxin GVIA-precipated VDCC labeled the 58-kDa protein on Western blots. On the basis of similar molecular weights, it was unclear if the 58-kDa protein actually represented synaptotagmin or the β subunit that is normally a component of VDCC (el Far *et al.*, 1995; Leveque *et al.*, 1992). The exclusion of the β subunit was shown by the finding that six out of 20 LEMS patients sera used reacted with synaptotagmin I expressed using *E. coli* (Takamori *et al.*, 1995, 2000a). However, only three out of six of these sera reacted specifically with expressed synaptotagmin alone, whereas the other remaining patient sera exhibited cross-reactivity to purified P/Q-or N-type VDCC (Takamori *et al.*, 1995, 2000a). In addition, SCLCs, which are believed to express the antigenic sites that initiate the development of autoantibodies in the majority of LEMS patients have also been observed to possess synaptotagmin (David *et al.*, 1993). Lastly, quantal content of EPP was reduced significantly at 50% of diaphragm preparations obtained from rats immunized with synthetic synaptotagmin residues quantal content was increased when these preparations were exposed to solutions containing elevated Ca^{2+} concentrations (Takamori *et al.*, 1994). However, none of the rats injected with synthetic synaptotagmin were clinically weak or exhibited alterations in CMAPs. This lack of a pronouced effect may reflect differences between synthetic peptides as immunogens because they lack normal tertiary or quanternary structure, which may often be recognized only weakly (Takamori *et al.*, 1990, 1992) and intact antigens that normally are responsible for initiating LEMS.

However, there are several deficits supporting a role for synaptotagmin as a potential antigenic target in LEMS. In its native state, the C-terminus of synaptotagmin extends into the cytoplasm of the nerve terminal, whereas the N-terminus lies within the synaptic vesicle (Perin *et al.*, 1990) and thus, normally is not accessible to the actions of LEMS antibodies except potentially during exocytosis (Shoji-Kasai *et al.*, 1992) assuming a complete exocytotic event occurs, as opposed to so-called “kiss and run” incomplete exocytosis. As such, it has been postulated to represent a potential antigenic sites (el Far *et al.*, 1995; Leveque *et al.*, 1992; Takamori *et al.*, 1995, 2000a), however, this idea has yet has to be proven. However, not all investigations have been able to identify synaptotagmin positive antibodies in LEMS patients’ sera; IgG from 14 patients with LEMS was used to probe western blots containing recombinant synaptotagmin, and human membrane and rat synaptosomal proteins; this study did not label bands corresponding to synaptotagmin, whereas it did label a large band of ~220 KDa (Hajela and Atchison, 1995). This finding, however, may reflect a heterogenous specificity among antibodies from different patients with LEMS. Furthermore, immunoprecipitation studies that identified synaptotagmin as a putative binding site for LEMS antibodies relied upon denaturation of partially purified Ca^{2+} channels and therefore, in such a system, epitopes common to both synaptotagmin and VDCC may exist. It has been shown that immunopreipitation of VDCC with antisynaptotagmin antibodies was dependent upon the detergent used for partial purification of the VDCC; in the presence of nondenaturing detergents, antisynaptotagmin antibodies do not immunoprecipitate VDCC (Lennon *et al.*, 1995). It is also possible that sera of LEMS patients contain other specific antibodies that only recognize proteins associated with VDCC in intact, nondenatured configurations (Leveque

et al., 1992). Furthermore, the specific synaptotagmin congener present in SCLC cells may not be identical to that at mammalian motor nerve endings and the putatively synaptotagmin congener believed to be affected by LEMS antibodies. Lastly, use of ionomyocin or direct injection of Ca^{2+} as a means to increase intracellular Ca^{2+} concentrations without the need for VDCC overcomes the inhibition of evoked-transmitter release from synaptosomes (Satoh *et al.*, 1998) or BAC cells (Kim *et al.*, 1998) exposed to LEMS antibodies. This finding also provides support against synaptotagmin as a putative target in LEMS unless release induced by these methods bypasses the role of synaptotagmin. Thus, while there is some evidence consistent with a role for synaptotagmin in LEMS, the etiology of the lack of seropositivity for synaptotagmin antibodies and in most patients' plasma and lack of conclusive evidence regarding its role in LEMS suggests that is does not play a major role in the disease.

ANTIBODY ETIOLOGY

The involvement of SCLC as a potential etiologic source underlying LEMS was originally suspected based upon the findings that at least 60% of LEMS patients have a detectable SCLC and these SCLCs exhibit Ca^{2+} spike electrogenesis in culture (McCann *et al.*, 1981). This idea was later supported by several other findings: SCLC possess multiple subtypes of VDCC, including L-, N-, and P/Q-type channels that potentially contain antigenic sites responsible for antibody development in LEMS (Barry *et al.*, 1995; Codignola *et al.*, 1993; De Aizpurua *et al.*, 1988; McCann *et al.*, 1981; Meriney *et al.*, 1996; Pancrazio *et al.*, 1989; Roberts *et al.*, 1985; Sher *et al.*, 1990; Viglione *et al.*, 1995); the subtypes of VDCCs expressed on SCLC correlate with the channel subtypes affected in LEMS (Barry *et al.*, 1995; De Aizpurua *et al.*, 1988; Sher *et al.*, 1990; Viglione *et al.*, 1995); exposure of SCLCs in culture to antibodies from LEMS patients reduced evoked-Ca^{2+} influx (De Aizpurua *et al.*, 1988; Lang *et al.*, 1989; Roberts *et al.*, 1985; Viglione *et al.*, 1995); LEMS antibodies precipitate VDCC on SCLC (Sher *et al.*, 1990); and finally, clinical improvement of LEMS follows treatment or removal of SCLC (Chalk *et al.*, 1990; Darnell and DeAngelis, 1993). Thus, the possibility of SCLC as etiologic antigenic source for LEMS appears quite high. Although the reason that SCLCs contain VDCC is uncertain, it is believed that Ca^{2+} influx through these channels is necessary for mitosis and antibodies most likely develop against these VDCC in order to prevent or arrest tumor growth (Hafner and Petzelt, 1987).

Approximately 40% of LEMS patients lack a detectable tumor. Thus, the antigenic etiology is less clear in these cases. Perhaps the tumor is subclinical or in remission due to autoimmune attack. However, many patients with LEMS have other autoimmune disorders or have first-blood relatives with autoimmune diseases (Gutmann *et al.*, 1972; Norris and Panner, 1966; O'Neill *et al.*, 1988; Takamori *et al.*, 1972). Consequently, the incidence of vitiligo, pernicious anemia, celiac disease, juvenile-onset diabetes mellitus, and thyroid disease is higher than normally expected in patients with LEMS. In a study performed by Lennon *et al.* (1982), 48% of LEMS patients without a tumor were found to have gastric and/or thyroid antibodies. Furthermore, a number of LEMS patients without a detectable tumor appear to possess specific polymorphisms of genes representing the different classes of human leukocyte antigens (HLA). The HLA represent a complex of genes encoding polymorphic molecules. These molecules are divided into three classes and are found on almost every nucleated cell in the human body. Moreover, they are involved in processing and presenting foreign antigens to T-cells of the immune system and in the development of T-cells within the thymus (McCusker and Singal, 1990; Rammensee *et al.*, 1993). Slightly different forms (alleles) of the genes encoding each HLA class exist between individuals and each allele within a given class is named differently. On the basis of HLA-typing there appears to be a strong association with HLA-B8 in regards to class I, and a strong association for HLA-DR3 and HLA-DR2 among class II with LEMS in patients without a detectable tumor (Parsons *et al.*, 2000; Willcox *et al.*, 1985; Wirtz *et al.*, 2001). While the etiology of LEMS antibodies is even more elusive in patients without a detectable tumor, susceptibility to LEMS is increased in patients possessing specific HLA alleles.

VOLTAGE-DEPENDENT Ca^{2+} CHANNEL SPECIFICITY

Immunoprecipitation assays have been developed which not only extend clinical findings regarding LEMS, but also offer insight into the specificity of antibodies directed against VDCC subtypes in LEMS. The various immunoprecipitation techniques used all basically relied upon solubilization of cell membranes, extraction of crude fractions of membrane proteins including VDCC, purification of VDCC in the presence of radiolabeled anti-VDCC ligand, and incubation of extracted radiolabeled-ligand-VDCC complexes with LEMS or control patient sera. Following corrections for nonspecificity, levels of radioactivity were correlated with specific antibody binding.

The original description of this assay used the human neuroblastoma cell line, IMR32 (Sher *et al.*, 1989), which exhibits excitability and secretory properties similar to those of sympathetic neurons and also express N- and L-, and T-type Ca^{2+} channels (Grassi *et al.*, 1994). VDCC were partially purified from IMR32 cells using ^{125}I-ω-conotoxin GVIA (^{125}I-ω-Cg Tx), a toxin which binds to N-type channels. Approximately 92% of LEMS patients' sera bound to ^{125}I-ω-Cg Tx-VDCC complexes. Although there was no significant difference between sera from LEMS patients with and without SCLC, false positive results were observed in 9% of control patient sera (including patients with neurological disorders other than LEMS) and 43% of patients with SCLC but without LEMS.

A study performed at the same time but using human derived SCLC or colon adenocarcinoma tissue cultures instead, found 52% of LEMS patients had positive antibodies for N-type VDCC (Lennon and Lambert, 1989). None of the patients with other neurological diseases (myasthenia gravis, amyotrophic lateral sclerosis, parkinson's disease, or chronic inflammatory demyelinating peripheral neuropathy) exhibited positive antibodies, whereas 10% of SCLC patients without LEMS were seropositive. Unlike the study performed by Sher *et al.* (1989), seropositivity was found more frequently in patients with LEMS who possessed SCLC than in LEMS patients with evidence or nonlung tumors or those without evidence of any cancer.

Similar findings were observed among all LEMS patients' sera tested against VDCC isolated from the human neuroblastoma cell line, SKN-SH in comparison to the results using human derived SCLC cultures (Leys *et al.*, 1989, 1991). Although both SKN-SH and SCLC cells used in this assay possess N-type Ca^{2+} channels as determined by ^{125}I-ω-CgTx GVIA binding, SKN-SH cells appear to have a greater number of ^{125}I-ω-CgTx GVIA binding sites. In contrast to earlier findings, a greater percentage of LEMS patients without SCLC were seropositive compared with LEMS patients with SCLC. The frequency of positive antibodies was found to be significantly high in sera from patients with other autoimmune disorders (rheumatoid arthritis and systemic lupus erythematosus) (Leys *et al.*, 1989).

More recently, tests with greater sensitivity and specificity in detecting antibodies in LEMS patients have been developed. Instead of extraction of solubilized N-type VDCC, these newer assays used ^{125}I-ω-conotoxin MVIIC (^{125}I-ω-CmTx), in concentrations specific for extracting P/Q-type channels. Lennon *et al.* (1995) found that 100% of LEMS patients without lung cancer and 91% with SCLC had high titers of antibodies for ^{125}I-ω-CmTx-VDCC complexes from SCLC and human cerebellar and cerebral membranes. However, lower antibody titers were found in patients with cancer and no evidence of neurologic dysfunction or in patients with amyotrophic lateral sclerosis.

Suenaga *et al.* (1996) have also described similar findings. Although none of the sera tested from patients with SCLC without LEMS had detectable P/Q-type antibodies, 40% were seropositive for N-type antibodies. Furthermore, 25% of patients with systemic lupus erythematosis (SLE) possessed antibodies against N-, but not P/Q-type channels.

In accordance with the earlier findings, Motomura *et al.*, 1997 observed that 92% of LEMS patients were seropositive for P/Q-type channel antibodies, whereas only 33% had detectable N-type channel antibodies. However, the specificity reported for this assay was higher than that of earlier reports and as such, all control, including myasthenia gravis, rheumatoid arthritis, and systematic lupus erythematosis sera tested were negative for antibodies against P/Q-type channels. Furthermore, all patient sera with antibodies against N-type channels also possessed antibodies against P/Q-type channels. Although a comparison of individual LEMS patients showed that anti-P/Q-type VDCC did not correlate with the severity of the disease, longitudinal studies within patients receiving therapy exhibited an inverse relationship between antibody titers and CMAP amplitudes.

On the basis of the findings using immunoprecipitation assays, antibodies against P/Q-and N-type VDCC appear to be involved in the pathology of LEMS. Data from the longitudinal studies further support the role of multiple VDCC subtypes in LEMS. However, antibodies against P/Q-type channels appear to affect specifically the majority of patients with LEMS, whereas antibodies to N-type channels appear to have a less prominent and definitive role but may contribute to the autonomic side effects seen in some patients with LEMS.

The specific VDCC antibodies involved in LEMS, however, have been better elucidated using functional studies. Early studies found Ca^{2+} current flow through L-, but not T-type VDCC in undifferentiated mouse neuroblastoma $\times$ glioma hybrid cells (NG 108 15) is reduced following exposure to IgG from LEMS patients in comparison to exposure to control IgG (Peers *et al.*, 1990). Similar findings were observed using thyroid C cells exposed to LEMS IgG; N- and L-, but not T-type Ca^{2+} currents were inhibited by LEMS IgG (Kim *et al.*, 1993). In contrast, human neuroblastoma IMR32 cells exposed to antibodies from two different LEMS patients not only reduced N- and L-, but T-type Ca^{2+} currents as well (Grassi *et al.*, 1994).

Although Grassi *et al.* (1994) observed an apparent nonspecificity of LEMS IgG for VDCC in IMR32 cells,

entry of Ca^{2+} was reduced to a greater extent through N- as compared to L-type channels. Similarly, LEMS IgG had a stronger inhibitory effect on Q- than L-type Ca^{2+} channels from rat insulinoma (RINm5F) cells (Magnelli *et al.*, 1996). These findings are in direct accordance with the actions of LEMS IgG on SCLC cultures, in which ω-Aga IVA and nicardipine were 38–84% and 18% less effective in reducing Ca^{2+} currents through P/Q- and L-type channels, respectively (Viglione *et al.*, 1995). Early studies have suggested that the actions of LEMS IgG at BAC cells occur via downregulation of L-type Ca^{2+} channels (Blandino and Kim, 1993; Kim and Neher, 1988), however, several lines of evidence support the findings that LEMS IgG primarily target P/Q-type VDCC in BAC cells. These include the following: (1) voltage-dependent Ca^{2+} entry into BAC cells is reduced by approximately 48% following exposure to LEMS sera by (Blandino and Kim, 1993; Kim, 1987; Kim and Neher, 1988), (2) approximately 40% of the Ca^{2+} currents in BAC cells are mediated by P/Q-type channels (Kim *et al.*, 1998), and (3) P/Q-type Ca^{2+} currents in BAC cells are reduced by approximately 80% following exposure to LEMS IgG (Kim *et al.*, 1998). More compelling evidence for the specificity of LEMS IgG for P/Q-type Ca^{2+} channels has been provided using human embryonic kidney (HEK) cells expressing transfected cDNA clones encoding human VDCC subunit proteins (Pinto *et al.*, 1998, 2002). Voltage-dependent Ca^{2+} entry into HEK cells expressing P/Q-type Ca^{2+} channels ($\alpha_{1A}, \alpha_2\delta, \beta_{4a}$) was inhibited following exposure to LEMS lgG. On the other hand, current carried through N- (α_{1B}, $\alpha_2\delta$, β_{1b}), L- ($\alpha_{1D,1C}$, $\alpha_2\delta$, $\beta_{4a,3a}$), and R-type channels ($\alpha_{1E}, \alpha_2\delta, \beta_{4a1b}$) expressed in HEK cells was not altered by LEMS IgG. Although some of the patient sera used contained antibodies that immunoprecipitated N-type Ca^{2+} channels, Ca^{2+} entry (as determined by whole-cell Ba^{2+} currents) through N-type Ca^{2+} channels transfected into HEK cells was unaltered. This discrepancy can be explained by the following: transfected N-type channels may not represent those expressed in vivo due to splice variations or the presence of different subunit combinations, the density of channels expressed following transfection may be too high and N-type channel antibody specificity too low to have an appreciable functional effect, the rate of N-type channel upregulation may outweigh channel downregulation during LEMS lgG exposure, and the epitopes on N-type channels may be inaccessible to LEMS IgG when expressed in HEK cells. A lack of an inhibitory effect of LEMS sera on N-type Ca^{2+} channels has also been observed using human H146 SCLC cells (Viglione *et al.*, 1995). Similar findings have also been shown using electric ray organ synaptosomes, which are purely cholinergic nerve terminals; LEMS IgG inhibited K^+-evoked release of ACh from electric ray organ synaptosomes by reducing P/Q-, but not N-type Ca^{2+} currents (Satoh *et al.*, 1998).

Other studies have also exhibited a preferential effect of LEMS IgG for P/Q- over N-type Ca^{2+} channels. This specificity of LEMS IgG has been shown using BAC cells (Engisch *et al.*, 1999).As such, a direct relationship between inhibition of P/Q-, but not N-type currents in BAC cells and severity of symptoms as measured by CMAP was detected (Engisch *et al.*, 1999). Furthermore, so-called high-voltage activated (HVA) Ca^{2+} currents recorded from cultures of murine neonatal motor neurons are reduced to a greater extent than are those in cultures of neonatal murine sensory neurons (Garcia *et al.*, 1996; Garcia and Beam, 1996). The differential effect of LEMS sera on voltage-dependent Ca^{2+} entry in rat sensory and motor neurons may be explained by the diversity of VDCC-subtypes on these neurons. Although both neuronal types from neonatal mammals possess P-, N-, and L-type channels, the predominant subtype of VDCC is believed to be N-, and P-type on neonatal sensory and motor neurons, respectively (Mintz *et al.*, 1992; Mynlieff and Beam, 1992). Thus, the greater reduction of Ca^{2+} entry in motor neurons may be due to the predominance of P/Q-type channels located on these cells in comparison to sensory neurons that possess N-type channels. These differences may account for the lack of evidence for sensory nerve dysfunction in patients with LEMS.

On the basis of the findings that some LEMS patient sera contain antibodies that reduce Ca^{2+} entry through N-type channels and early studies that suggested that autonomic neurons mainly utilize N-type channels to control catecholamine release (Hirning *et al.*, 1988) it was proposed that autonomic symptoms in LEMS patients resulted from down-regulation of N-type channels (Grassi *et al.*, 1994; Lang *et al.*, 1987; Sher *et al.*, 1993). However, more recent findings have shown that transmitter release from autonomic nerves relies upon Ca^{2+} entry through P/Q-, N-, R-, and sometimes L-type channels, depending upon the mode of nerve stimulation (Hong and Chang, 1995; Lemos and Nowycky, 1989; Owen *et al.*, 1989; Waterman, 1996, 1997). In contrast to earlier suggestions, autonomic symptoms in LEMS patients probably also reflect a preferential action on P/Q-type Ca^{2+} channels at autonomic nerve terminals. Houzen *et al.* (1998) found that transmitter relese from autonomic nerves subserved by P/Q-, but not N-type VDCC was reduced following exposure for 6 h to IgG from three patients with LEMS. Although transmitter release from autonomic nerves obtained from adult mice injected with one of four LEMS patient IgG for 8 days attenuated Ca^{2+} entry through N-type channels, all four LEMS patients IgG affected

P/Q-type Ca^{2+} channels (Waterman *et al.*, 1997). In addition, Houzen *et al.* (1998) found that LEMS IgG reduced transmitter release from parasympathetic, but not sympathetic nerves. This finding may reflect the VDCC subtypes subserving transmitter release from parasympathetic and sympathetic nerves used in their study (N-type channels were involved in transmitter release from both types, but P/Q-type channels controlled release only from parasymapthetic nerves).

Other more direct studies have also shown a preferential effect of LEMS sera on certain subtypes of VDCC at mammalian motor nerves. Ca^{2+} currents recorded from motor nerve terminals of mice injected with LEMS sera for 30 days are reduced in comparison to respective control recordings (Smith *et al.*, 1995; Xu *et al.*, 1998). Moreover, whereas nifedipine did not affect Ca^{2+} currents from control motor nerve terminals, it did block a component of current remaining in the animals that were treated with LEMS plasma (Smith *et al.*, 1995; Xu *et al.*, 1998). Furthermore, Xu *et al.* (1998) found that ω-Aga IVA was approximately 30% less effective in reducing the amplitude of Ca^{2+} currents recorded from LEMS motor nerve terminals in comparison to controls and the Ca^{2+} current remaining after exposure of LEMS motor nerve terminals to ω-Aga IVA is antagonized by nifedipine. These findings suggest that exposure to LEMS sera not only reduces P/Q-type Ca^{2+} currents, but induces the appearance of a novel L-type Ca^{2+} current at the motor nerve terminal as well. Similar findings have been observed using cultures of mammalian motor neurons (Garcia and Beam, 1996); while both LVA (presumptive T-type currents) and HVA currents at cultures of mammalian motor neurons were reduced following exposure to LEMS sera, L-type currents were spared. However, acute exposure of rat forebrain synaptosomes to LEMS sera does not alter the binding characteristics of nitrendipine (Xu *et al.*, 1998), thus providing evidence that the development of L-type Ca^{2+} currents requires long-term exposure to LEMS sera.

While LEMS antibodies appear to target a variety of Ca^{2+} channel subtypes, albeit with differing sensitivities, VDCC found at rodent skeletal and cardiac muscle and insect skeletal muscle are insensitive to the actions of LEMS antibodies. The inability of LEMS antibodies to affect these channels may be a function of the specific α_1 subunit comprising the VDCC on these tissues. Thus, LEMS antibodies do appear to exhibit some species and tissue specificity. Furthermore, the preferential action on P/Q-type Ca^{2+} channels most likely accounts for the observations that mature mammalian neuromuscular transmission, which is subserved by P/Q-type Ca^{2+} channels underlies the primary clinical sign in LEMS patients.

L-TYPE VOLTAGE-DEPENDENT Ca^{2+} CHANNELS IN LEMS

In corroboration with the findings of Xu *et al.* (1998), L-type Ca^{2+} currents which do not normally control release of ACh at mature mouse motor nerve terminals become involved in release of ACh following passive transfer of LEMS by injection of mice with LEMS plasma for 30 days (Flink and Atchison, 2002). Specifically, nerve-evoked release of ACh from motor nerve terminals was reduced by ∼55% in comparison to release of ACh from motor nerve terminals of mice injected with control plasma by ∼55%. In the presence of nimodipine, nerve-evoked release of ACh from LEMS motor nerve terminals was further reduced by ∼43%, whereas nimodipine had no effect on release of ACh from control preparations. Although acute exposure of motor nerves from mature mice to LEMS patient plasma for 2 and 24 h reduced the nerve-evoked release of ACh to ∼40 and 44%, respectively in comparison to motor nerves exposed to control plasma, release of ACh from any of the acutely exposed preparations was not sensitive to nimodipine.

The prolonged period of time necessary for L-type Ca^{2+} channels to become involved in ACh release following injection of LEMS plasma supports the idea that involvement of L-type channels is not due merely to unmasking of normally silent channels already present on the motor nerve terminal. This is also supported by the findings that L-type Ca^{2+} currents are not present at mature mammalian motor nerve terminals exposed to control plasma for 30 days when function of P/Q-type channels is blocked transiently by ω-Aga IVA (Xu *et al.*, 1998). Processes such as formation of new channel subunits and assembly are most likely required for functional L-type channels to become involved in ACh release during LEMS (Fig. 3). This finding is similar to those obtained at motor nerve terminals during reinnervation (Katz *et al.*, 1996) or recovery from botulinum toxin-induced poisoning (Santafe *et al.*, 2000), which reflect processes that occur during maturation of motor nerves. Thus, immature motor nerves also possess multiple subtypes of VDCC that are involved in release of ACh, but whose involvement is reduced until the mature motor nerve only has one primary subtype of VDCC involved in ACh release (Rosato-Siri *et al.*, 2002; Rosato-Siri and Uchitel, 1999; Santafe *et al.*, 2001, 2002; Sugiura and Ko, 1997). However, LEMS antibodies have not been shown to damage the nerve terminal or induce-sprouting of immature motor nerves (Fukunaga *et al.*, 1983; Tsujihata *et al.*, 1987) and thus, it is unclear if mechanisms underlying L-type Ca^{2+} channel expression are similar to those involved during motor nerve maturation.

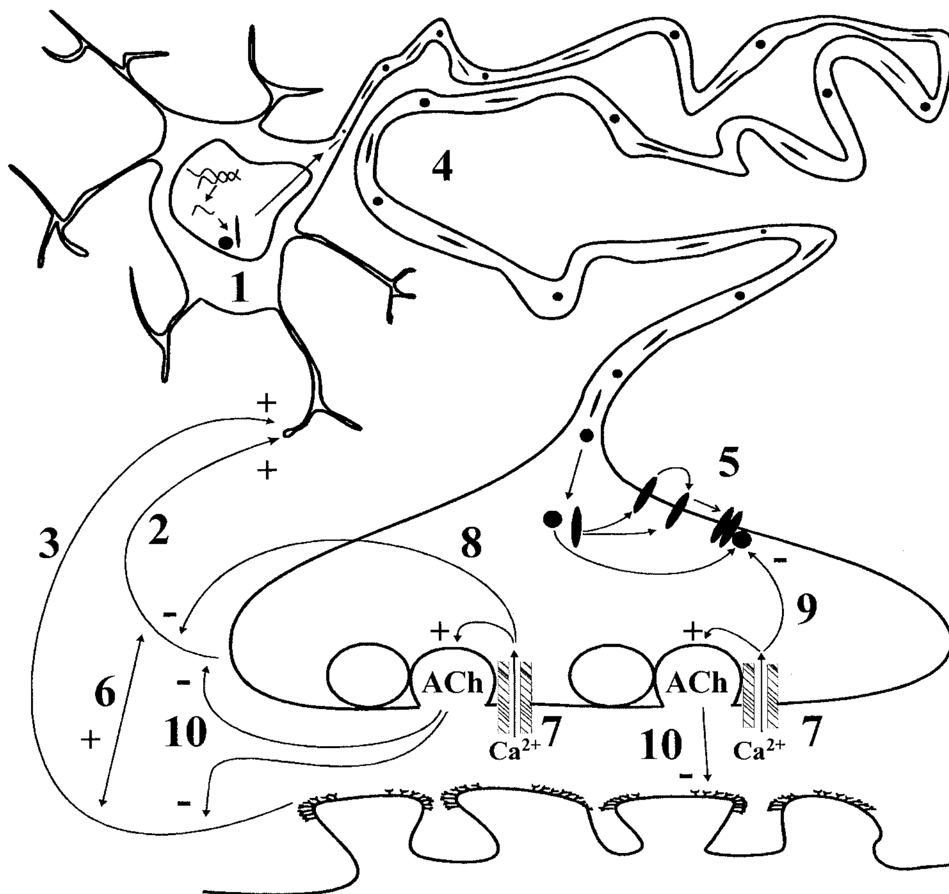

Fig. 3. Hypothetical model depicting the development of functional L-type Ca^{2+} channels at adult mouse motor nerve terminals. L-type Ca^{2+} channel subunits are produced in the motor nerve cell body (1) in response to tonic excitatory signals originating from pre- (2) and/or postsynaptic (3) structures. L-type Ca^{2+} channel subunits are then transported (4) along the axon and assembled (5) into intact channels at the motor nerve terminal. Signals from pre- and postsynaptic structures can also excite one another to enhance input signals to the cell body (6). However, Ca^{2+} entry (7) through the normal complement of P/Q-type Ca^{2+} channels inhibits both the tonic excitatory presynaptic signal (8) and function of L-type Ca^{2+} channels already present at the terminal (9). ACh released in response to Ca^{2+} entry through the normal complement of P/Q-type channels also inhibits the tonic excitatory pre- and postsynaptic signals (10). Therefore, in the presence of the normal complement of P/Q-type Ca^{2+} channels, production of L-type Ca^{2+} channel subunits is terminated. Antibodies in patients with LEMS reduce the number of functional P/Q-type Ca^{2+} channels, and subsequently reduces both entry of Ca^{2+} into the terminal and nerve-evoked release of ACh. Thus, in LEMS, inhibitory signals normally generated by both Ca^{2+} entry through P/Q-type channels and P/Q-type Ca^{2+} channel-dependent ACh release are lost, which in turn may enhance the production and function of L-type Ca^{2+} channels.

It is also possible that relocation of newly synthesized and preexisting L-type channels may occur in LEMS. It appears unlikely, however, that the only mechanism involved is that of relocation of preexisting channels. This conclusion is based on the lag time preceding involvement of L-type channels in release of ACh from motor nerve terminals exposed to LEMS plasma. Experiments involving immunostaining with α_1 subunits subtypes believed to comprise L-type channels of neuromuscular preparations obtained from mice injected with LEMS or control plasma for varying days, may clarify the role of relocation.

In addition to channel expression and relocation, other means of differential modulation must exist in order to ensure precise control of ACh release when multiple subtypes of VDCC are present on the motor nerve terminals, these may include: differences in specific accessory subunits comprising the channel, relationship with other

non-VDCC receptors or channels, and/or diverse second messenger pathways. Although the specific accessory subunit subtypes comprising VDCC at the mature mammalian motor nerve terminal are unknown, function of VDCC is influenced by the subtypes of the accessory subunits comprising the channel (Brice *et al.*, 1997; Brice and Dolphin, 1999; Chien *et al.*, 1995; Neely *et al.*, 1993; Singer *et al.*, 1991). Furthermore, differential modulation of multiple VDCC subtypes has been observed to involve various other neurotransmitter receptor subtypes, including both noradrenergic and opioid-type receptors. For instance, stimulation of α_1 and β_1 adrenergic receptors increases nerve-stimulated release of ACh from rat motor nerves, by activating L- and N-type Ca^{2+} channels, respectively (Wessler *et al.*, 1990). In contrast, activation of mu-opioid receptors on acutely dissociated dorsal root ganglion neurons reduces Ca^{2+} currents by acting through G-proteins (Moises *et al.*, 1994). Ca^{2+} entry through L-type channels also appears to be limited by Ca^{2+}-dependent inactivation at mature mammalian motor nerve terminals. Although P/Q-type Ca^{2+} channels located in many cell types are also directly inactivated during increasing intracellular Ca^{2+} levels (Tareilus *et al.*, 1994), this inhibitory mechanism does not seem to affect P/Q-type Ca^{2+} channel-dependent release of ACh from mature mammalian motor nerve terminals during low-frequency nerve-stimulation. Furthermore, mechanisms affecting phosphorylation of VDCC have been observed to modulate channel function. Release of 5-hydroxytryptamine (5-HT) from synaptosomal preparations isolated from spinal cords of adult rats is increased by protein kinase C-dependent activation of L-type Ca^{2+} channels (Talley *et al.*, 1997). Activation of protein kinase C by stimulation of muscarinic receptors also increases L-type Ca^{2+} channel activity at major pelvic ganglia of adult rats (Sculptoreanu *et al.*, 2001). Moreover, inhibition of protein phosphatases, which normally dephosphorylate proteins, involves L-type Ca^{2+} channels in ACh release from mature mammalian motor nerves (Urbano *et al.*, 2001). Alterations in stimulus parameters have also been observed to affect channel function by acting through phosphorylation-dependent mechanisms; amplitudes of Ca^{2+} currents are enhanced during intense depolarization by increasing phosphorylation of L-type Ca^{2+} channels (Sculptoreanu *et al.*, 1995). Furthermore, prolonged depolarization of mature mammalian motor nerve terminals activates protein kinases through stimulation of A_{2A} adenosine receptors and in turn, unmasks L-type channels involved in release of ACh (Correia-de-Sa *et al.*, 2000a,b). Although the exact mechanism has not been examined, intense depolarization of adult mouse motor nerves induced by 3,4 DAP, which blocks voltage-dependent K^+ channels, enhances ACh release by activating and prolonging the activation of P/Q-and L-type VDCC (Hong and Chang,1990). On the basis of these observations, it seems likely that following synthesis of L-type Ca^{2+} channel subunit proteins, and formation and insertion of newly assembled functional channels into the motor nerve terminal membrane during exposure to LEMS plasma, more immediate mechanisms also exist, which enhance or permit involvement of these newly expressed channels in ACh release.

While the exact secondary mechanism is not certain, it is possible that mechanisms which aid in prolongation of membrane depolarization and thus prolongation of channel openings, enhance L-type channel involvement in release of ACh from LEMS motor nerve terminals. More specifically, loss of activation of Ca^{2+}-activated potassium channels (K_{Ca}) may enhance involvement of L-type Ca^{2+} channel-dependent release of ACh during LEMS. This idea is based upon multiple observations. K_{Ca} channels are colocalized with P/Q-and N-type channels at adult mouse and frog motor nerve terminals, respectively, and directly influence the openings of VDCC by aiding membrane repolarization (Protti and Uchitel, 1997; Robitaille *et al.*, 1993; Robitaille and Charlton, 1992; Xu and Atchison, 1996). Furthermore, intense and prolonged membrane depolarization by electrical stimulation or block of voltage-dependent K^+ channels with 3,4 DAP involves L-type VDCC in release of ACh from mature mammalian motor nerve terminals. It is possible that in LEMS, the decreased number of P/Q-type VDCC leads to a reduced Ca^{2+} entry and hence a decrease in openings of colocalized K_{Ca} channels which prolongs depolarization of the motor nerve terminal membrane. The overall effect of prolonged membrane depolarization could potentially be to enhance the involvement of L-type VDCC in release of ACh by increasing the duration of channel openings and thus, increasing Ca^{2+} entry through these channels.

We have found (Flink and Atchison, 2003) that at mammalian motor nerver terminals in the presence of K_{Ca} channel block, a significant number of normally silent L-type channels become involved in ACh release during electrical or K^+-induced (at KCI concentrations of 20 mM) stimulation. Thus, it is possible that in LEMS as the number of functional P/Q-type Ca^{2+} channels decreases, activation of K_{Ca} channels also decreases, which in turn, enhances involvement of newly synthesized and/or normally silent L-type VDCC already present at the motor nerve terminal in release of ACh. In corroboration with this hypothesis, it has been observed that exposure of BAC cells to LEMS plasma not only reduces the amplitude of P/Q-type Ca^{2+} currents, but that of K_{Ca} currents as well (Kim *et al.*, 1998). Although it is possible that in LEMS functional K_{Ca} channels are downregulated in manner

similar to that of P/Q-type Ca^{2+} channels, many studies have shown that K_{Ca} channel densities are not affected.

L-type Ca^{2+} currents involved in release of ACh during LEMS may be influenced directly or indirectly by K_{Ca} channels. As such, loss of P/Q-type channels may decrease activation of colocalized K_{Ca} channels and in turn, enhance the extent and duration of global depolarization of the motor nerve terminal membrane. Alternatively, expressed L-type VDCC at the motor nerve terminal during exposure to LEMS plasma may not be influenced by K_{Ca} channels because these channels may not be colocalized. In support of the latter argument, differential colocalization of VDCC subtypes and K_{Ca} channel has been observed at chick parasympathetic and sympathetic nerves (Wisgirda and Dryer, 1994). L- and N-type channels are both found on chick parasympathetic and sympathetic nerves, however, N-type channels are colocalized with K_{Ca} channels on chick sympathetic nerves, whereas L-type channels are colocalized with K_{Ca} on parasympathetic nerves. Furthermore, based upon pharmacological sensitivities, K_{Ca} channels found on mature mammalian motor nerve terminals are most likely of the BK ("Big" conductance) type and thus, are not only sensitive to intracellular Ca^{2+}, but depolarization as well. However, the effect of depolarization on these K_{Ca} channels is enhanced greatly by the presence of Ca^{2+}; activation of K_{Ca} channels in the presence of reduced Ca^{2+} entry as in LEMS still occurs via direct depolarization, but to a lesser extent than would normally occur. Thus in LEMS, loss of P/Q-type Ca^{2+} channels most likely leads to reduced, but not complete loss of K_{Ca} channel activation. It would therefore seem more likely that in LEMS, lack of colocalization of K_{Ca} with newly synthesized L-type Ca^{2+} channels would have a greater effect on L-type Ca^{2+} channel involvement in release of ACh than would reduced activation of K_{Ca} channels due to decreased Ca^{2+} entry through P/Q-type channels. This hypothetical model is represented in Fig. 4 and most likely acts in concert with the hypothetical model depicted in Fig. 3 to maximize involvement of L-type Ca^{2+} channels in release of ACh in LEMS.

Although expression and involvement of L-type Ca^{2+} channels in release of ACh occurs following passive transfer of LEMS to mice, other VDCC channel subtypes may also become involved in release of ACh. Nerve-stimulated release of ACh from motor nerves obtained from mice genetically altered to lack P/Q-type channels is controlled by Ca^{2+} entry through multiple subtypes of VDCC, including the N-type. However, based upon antibody specificities in LEMS, expression of other VDCC subtypes, particularly N-type channels, may not become apparent during LEMS; synthesis of newly formed N-type channels may also occur, but due to a stronger preference of LEMS antibodies for not only P/Q-, but N- as compared to L-type Ca^{2+} channels, the turnover of N-type channels may not occur fast enough for them to become involved in release of ACh. Additional work is necessary in order to test this hypothesis.

Involvement of L-type channels in release of ACh from motor nerves following passive transfer of LEMS to mice most likely occurs in humans as well. Although direct evidence for a similar findings in humans has not been reported, indirect evidence supports a role of L-type Ca^{2+} channel development at motor nerve terminals in LEMS. For example, a relationship between the development of LEMS and serum levels of the L-type Ca^{2+} channel antagonist, diltiazem has been reported in a patient with myocardial ischemia (Ueno and Hara, 1992). Additionally, use of the L-type Ca^{2+} channel antagonist, verapamil has been implicated to induce respiratory failure in patient with LEMS (Krendel and Hopking, 1986). It is quite possible, therefore, that patients with LEMS may benefit from therapies that specifically target and increase currents through newly formed L-type Ca^{2+} channels. However, more work will need to be done, specifically using tissues from patients with LEMS in order to clarify this issue.

The distinct localization of individual subtypes of VDCC at motor nerve terminals during LEMS and the resulting temporal overlap of multiple Ca^{2+} domains may act as a means to maintain a short-term plasticity until more complex mechanisms can occur (Rosato-Siri *et al.*, 2002) Thus, in LEMS, L-type channels may assume the role of lost P/Q-type channels in order to maintain a given level of ACh.

SPECIFIC ANTIGENIC SITE ON THE VOLTAGE-DEPENDENT Ca^{2+} CHANNEL

The β subunit was found to be antigenic when screened against plasma of LEMS patients with high titer levels of IgG, however, it is not considered to be the actual target in LEMS based upon several observations. The location of the subunit on the cytoplasmic side of the membrane (Catterall, 2000) makes them normally inaccessible to circulating antibodies and injection of β subunit proteins into mice induces high antibody titer levels against the β subunit without inducing any neurological dysfunctions (el Far *et al.*, 1995; Rosenfeld *et al.*, 1993a,b; Verschuuren *et al.*, 1998). The high frequency and preferential sensitivity of antibodies against P/Q-type Ca^{2+} channels in functional and immunoprecipitation assays suggested that antigenic sites reside within the primary subunit of the channel; namely, the α_{1A} subunit, which

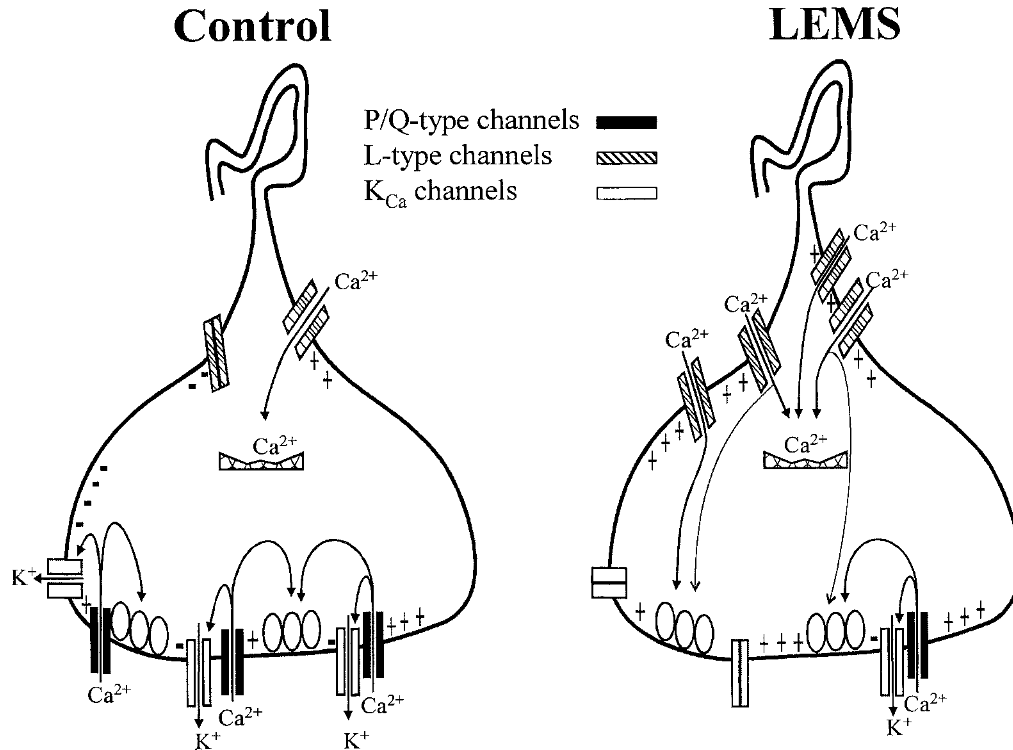

Fig. 4. Hypothetical model representing the role of K_{Ca} channels in LEMS at the mature motor nerve terminal of mice. In *Control* environments, P/Q-type Ca^{2+} channels are the primary channel subtype controlling ACh release from motor nerve terminals of mature mice. Although Ca^{2+} entry through few L-type Ca^{2+} channels located at sites distinct from the release apparatus occurs during membrane depolarization, the Ca^{2+} is buffered by intracellular mechanisms prior to becoming involved in ACh release. Activation of K_{Ca} channels during membrane depolarization and increasing intracellular $[Ca^{2+}]$ allows efflux of K^+, which aids in membrane repolarization. In turn, the probability of L-type VDCC openings and release of ACh are decreased. In addition to K^+ efflux through K_{Ca} channels, some L-type channels present at the motor nerve terminal remain closed during membrane depolarization due to the influence of other secondary mechanisms. In *LEMS*, the number of functional P/Q-type channels is reduced, while expression of L-type Ca^{2+} channels at sites distinct from the release apparatus is increased. Furthermore, newly expressed L-type Ca^{2+} channels may not be colocalized with K_{Ca} channels. During membrane depolarization, entry of Ca^{2+} through P/Q-type channels and thus, subsequent activation of K_{Ca} channels are reduced. In turn, the membrane remains depolarized for longer periods of time, which increases the duration of VDCC openings. In conjunction with increased expression of L-type channels, entry of Ca^{2+} overwhelms buffering mechanisms and participates in ACh release. However, due to buffering mechanisms and channel localization, not all of the Ca^{2+} entering through L-type channels is involved in ACh release. Adapted and modified from Smith *et al.* (1995).

contains regions that are potentially exposed to circulating antibodies. Synthetic peptides representing extracellular S5-S6 linker segments in each of the four domains of the α_{1A} subunit were used to test immunoreactivity to LEMS patient sera. It was found that 30% of LEMS patient sera showed specific antibody binding to the S5-S6 region of Domain IV and 20% of patient sera used was specific for the S5-S6 region of Domain II (Takamori *et al.*, 1997), whereas none of the sera showed reactivity for Domains I or III. However, when longer synthetic peptide regions of S5-S6 were used, 50% of the LEMS patient sera had antibodies for the S5-S6 regions of Domain III (Iwasa *et al.*, 2000). This finding implicated the conformational necessity of the S5-S6 linker region of Domain III to elicit an immunoglobulin response. Immunoreactive linear epitopes representing regions of the S5-S6 linker in Domains II and IV were observed in greater than 50% of patient sera tested (Parsons and Kwok, 2002) using a different assay than that used by Takamori *et al.* (1997).

Further support for the S5-S6 linker regions of Domains I–IV as potential antigenic epitopes was provided by immunization of rats with the synthetic peptides. Rats immunized with synthetic peptide regions

representing S5-S6 linker regions of Domains II (40%) and III (60%) induced similar electrophysiological characteristics to those observed in LEMS (Komai *et al.*, 1999; Takamori *et al.*, 2000b). Although, not every rat developed neurological deficiencies, these sites may represent potential epitopes involved in eliciting antibody responses to VDCC in LEMS. One of the problems associated with using synthetic peptides as antigens is based upon the conformational shapes of antigens required to elicit an immune response. The S5-S6 linker regions are all anchored to transmembrane regions for each domain, and thus synthetic peptides of the linker regions may not accurately reflect the conformation states of these regions. This may explain any differences that arise between studies involving the passive transfer LEMS and those involving use of synthetic peptides. However, these epitopes clearly represent prospective sites for VDCC antibody development.

OTHER NEUROLOGICAL DISORDERS RELATED TO VOLTAGE-DEPENDENT Ca^{2+} CHANNELS

While LEMS is one of the most extensively studied neurological disorders thought to result from Ca^{2+} channel dysfunction, it is not the only example of such a disorder. Alterations in functional VDCC have been implicated in other neurological disorders as well. However, unlike the situation in LEMS, these other diseases result from naturally-occurring mutations in specific Ca^{2+} channel subunits. Mutations in the gene encoding the α_{1A} subunit in humans are associated with episodic ataxia type 2 (EA2), familial hemiplegic migraine (FHM), and spinocerebellar ataxia type 6 (SCA6) (Ophoff *et al.*, 1996; Zhuchenko *et al.*, 1997). Although these diseases result from different mutations of the same gene, the symptoms associated with each condition sometimes overlap. EA2 is provoked by stress, exercise, and fatigue, characterized by ataxia, nystagmus, dysarthria, diplopia and vertigo, and begins in childhood to late adolescence. This phenotype is the result of frame-shifts and splice-site mutations that lead to truncated proteins. Mice exhibiting the *leaner* phenotype have similar changes in the gene encoding α_{1A} subunits, however, these animals exhibit severe ataxia, *absence* seizures, and premature death (Dove *et al.*, 1998; Doyle *et al.*, 1997; Herrup and Wilczynski, 1982).

Similarly, FHM presents in childhood, but represents a form of unilateral migraine that is accompanied with an aura and includes hemiparesis, aphasia, or hemianopia. Symptoms in these patients can last from a few hours up to days and are identical to attacks described in first-degree relative(s). The disease is due to missense mutations in the gene encoding the α_{1A} subunit (the CACNL1A4 gene found on chromosome 19p13.1). Similar mutations in mice also occur, producing the *tottering* phenotype (Doyle *et al.*, 1997; Fletcher *et al.*, 1996). Unlike the phenotype displayed in humans, *tottering* mice also exhibit motor and *absence* seizures, in addition to ataxia. Although the *tottering* phenotype in mice appears analogous to the *leaner* phenotype, the characteristics are much less severe. Another mutation in mice at the same gene locus as the *tottering* and *leaner* phenotypes has been observed to cause severe ataxia without seizures. This phenotype is known as the *rolling Nagoya* mouse (Mori *et al.*, 2000).

SCA6 usually begins in late adulthood and causes nystagmus, ataxia, dysarthria, and impaired vibration and position sense. Furthermore, it causes isolated atrophy of the cerebellum and is believed to be due to an expanded CAG repeat in the gene encoding the α_{1A} subunit.

In addition to cerebellar changes, *absence* seizures have been linked to a mutation in the gene encoding the α_{1A} subunit (Jouvenceau *et al.*, 2001). Alterations in other subunit proteins comprising the VDCC have also been associated with *absence* seizures. As such, reduced expression of β_4 subunits in mice leads to the *lethargic* phenotype, which not only exhibit instability of gait and convulsions, but absence seizures as well (Burgess *et al.*, 1997; McEnery *et al.*, 1998). Recently, mutations in the β_4 subunit have been suggested to underlie seizures in some human disorders (Escayg *et al.*, 2000). Although it has yet to be characterized in humans, mice with mutations of the $\alpha_2\delta_2$ protein (*ducky* mutation) exhibit ataxia and paroxysmal dyskinesia (Barclay *et al.*, 2001).

ACKNOWLEDGMENTS

The authors acknowledge the excellent secretarial assistance of Mallory Koglin and Kelly O'Brien. Supported by NIH grant R01ES05822 and a grant from the Muscular Dystrophy Association. Submitted by MTF in partial fulfillment of the requirements of the PhD degree in Pharmacology/Toxicology as part of the Medical Scientist Training Program in the College of Osteopathic Medicine at Michigan State University.

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