THE ROLE OF L-TYPE Ca^{2+} CHANNELS IN RELEASE OF ACETYLCHOLINE FROM MOTOR NERVE TERMINALS FOLLOWING PASSIVE TRANSFER OF LAMBERT-EATON MYASTHENIC SYNDROME TO MATURE MICE

BY

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ABSTRACT

THE ROLE OF L-TYPE Ca^{2+} CHANNELS IN RELEASE OF ACETYLCHOLINE FROM MOTOR NERVE TERMINALS FOLLOWING PASSIVE TRANSFER OF LAMBERT-EATON MYASTHENIC SYNDROME TO MATURE MICE

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Lambert-Eaton myasthenic syndrome (LEMS) is neuromuscular disorder in which the release of acetylcholine (ACh) from motor nerves is reduced. LEMS is believed to be due to circulating antibodies directed against voltage-dependent Ca^{2+} channels (VDCC) involved in release of ACh. Passive transfer of LEMS to mature mice by daily injections for 30 days with plasma from LEMS patients reduces the amplitude of Ca^{2+} current through P/Q-type channels and exposes an L-type Ca^{2+} current not normally found at the motor nerve terminal. The overall goal of this dissertation was to test the hypothesis that the novel L-type Ca^{2+} current expressed following passive transfer of LEMS to mice participate in release of ACh from motor nerve terminals.

Passive transfer of LEMS to mice following plasma injections for 1-30 days reduced the release of ACh in comparison to controls and induced facilitation of release of ACh during high frequency nerve-stimulation. Nimodipine, a dihydropyridine L-type Ca^{2+} channel antagonist, further reduced the nerve-stimulated release of ACh observed from LEMS, but not control motor nerve terminals following passive transfer for 20 and 30 days. However, nimodipine did not affect the nerve-stimulated release of ACh following injection of LEMS plasma to mice for 1-15 days.

Incubation of motor nerve terminals with the chelator, DM-BAPTA, which rapidly

binds to free Ca^{2+} in the cytosol did not affect release of ACh following injection of mice with control plasma. On the other hand, DM-BAPTA abolished L-type Ca^{2+} channel involvement in release of ACh following injection of mice with LEMS plasma. Based upon these findings, it appears that L-type Ca^{2+}channels involved in release of ACh from LEMS motor nerve terminals are not tightly coupled with the release apparatus.

The ability of calcium-activated potassium (K_{Ca}) channels to induce L-type Ca^{2+} channel involvement in release of ACh from motor nerve terminals was also examined. The K_{Ca} channel antagonist, iberiotoxin, increased the release of ACh from motor nerve terminals obtained from naïve mice only when the membrane was depolarized to potentials more positive than ~ -42 mV. Furthermore, nimodipine significantly reduced this enhanced release of ACh induced by iberiotoxin. Thus, it is possible that in LEMS, reduced Ca^{2+} entry into the motor nerve terminal may attenuate activation of K_{Ca} channels, which increases the extent and duration of VDCC openings. In turn, this allows L-type Ca^{2+} channels, which may be located at sites distant from the release apparatus to become involved in release of ACh.

The studies in this dissertation provide evidence for: 1) L-type Ca^{2+} channel involvement in release of ACh from motor nerve terminals occurring only after prolonged passive transfer of LEMS to mice, 2) the participation of long-term processes, such as synthesis and assembly of new channel components for L-type Ca^{2+} channel involvement in release of ACh, 3) lack of tight association of L-type Ca^{2+} channels expressed in LEMS with the release apparatus, and 4) modulation of L-type Ca^{2+} channel involvement in release of ACh by K_{Ca} channels.

For Gussie Zinner

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PREFACE

Some the data contained in this dissertation has been published previously. Chapter Two appeared as Flink and Atchison (2002) and Chapter Four appeared as Flink and Atchison (2003).

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ACh-	acetylcholine
AChE-	acetylcholinesterase
Aga-IVA-	ω-agatoxin IVA
ANOVA-	analysis of variance
AP-	action potential
ATP-	adenosine triphosphate
AZP-	active zone particles
BAC-	bovine adrenal chromaffin
bay K 8644-	1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester
BK-	big conductance calcium-activated potassium
BSA-	bovine serum albumin
Ca^{2+}-	calcium
$[Ca^{2+}]_e$-	extracellular calcium concentration
$[Ca^{2+}]_i$-	intracellular calcium concentration
cAMP-	cyclic adenosine monophosphate
CgTx GVIA-	ω-conotoxin GVIA
CHAT-	choline-o-acetyltransferase
CMAP-	compound muscle action potential
CmTx MVIIC-	ω-conotoxin MVIIC

CREB-	Ca^{2+}/cAMP response element binding protein
3,4 DAP-	3,4 diaminopyridine
DHP-	dihydropyridine
DMSO-	dimethyl sulfoxide
DRG-	dorsal root ganglion
EA2-	episodic ataxia type 2
EMG-	electromyography
EPP-	end-plate potential
EtOH-	ethanol
$F(ab)'_{2}$-	divalent antigen binding fragment of IgG
F(ab)-	monovalent antigen binding fragment of IgG
FHM-	familial hemiplegic migraine
g-	grams
GTP-	guanosine triphophate
HEK-	human embryonic kidney
HEPES-	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HLA-	human lymphocyte antigen
hr-	hour
5-HT-	5-hydroxytryptamine
HVA-	high voltage activated
i.v.-	intravenous
IBTx-	iberiotoxin

Ig-	immunoglobin
IgG-	immunoglobin G
IK-	intermediate conductance calcium-activated potassium
K^+-	potassium
K_{Ca}-	calcium-activated potassium
KCl-	potassium chloride
K_d-	equilibrium dissociation constant for ligand binding
kDa-	kilodalton
kg-	kilogram
L-	liters
LEMS-	Lambert-Eaton myasthenic syndrome
LVA-	low voltage activated
m-	quantal content
MEPP-	miniature end-plate potential
M-	mega
mg-	milligram
Mg^{2+}-	magnesium
$MgCl_2$-	magnesium chloride
ml-	milliliter
mM-	millimolar
msec-	millisec
Na^+-	sodium

NaCl-	sodium chloride
NaOH-	sodium hydroxide
NCAM-	neuronal cell adhesion molecule
nimod-	nimodipine
NMJ-	neuromuscular junction
NSF-	N-ethylmaleimide-sensitive factor
o.d.-	outer diameter
PKA-	protein kinase A
PKC-	protein kinase C
RINm-	rat insulinoma
SCA6-	spinocerebellar ataxia type 6
SCLC-	small cell lung carcinoma
SDS-	sodium-dodecylsulfate
sec-	second
SEM-	standard error of the mean
SFEMG-	single fiber electromyography
SK-	small conductance calcium-activated potassium
SLE-	systemic *lupus erythematosus*
SNAP-25-	snaptosome-associated protein of 25 kDa
SNARE-	soluble N-sensitive factor attachment protein receptor
TEA-	tetraethylammonium
v-	volume

VAMP- synaptobrevin

VDCC- voltage dependent calcium channel

CHAPTER ONE

INTRODUCTION

A. *General Introduction*

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

Although the symptoms and signs of LEMS could represent alterations of post-synaptic structures similar to those 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, vesicle size and contents and intraterminal processes involved in release of ACh in the motor nerve terminal also are unaltered (Molenaar *et al.*, 1982; Lang and Vincent, 1984). On the other hand, active zone particles, which are believed to represent voltage-dependent Ca^{2+} channels (VDCC) are disorganized and fewer in number at motor nerve terminals from patients with LEMS compared to control terminals. 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 (Katz and Miledi, 1970; Llinas *et al.*, 1976; Augustine *et al.*, 1987). Although multiple VDCC subtypes are known to 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 VDCC subtypes (Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001). However, mature motor nerve terminals primarily contain one VDCC subtype involved in release of ACh. As such, mature mammalian motor nerve terminals utilize P/Q- (Katz, 1995; Protti *et al.*, 1996), whereas amphibians (Sano *et al.*, 1987) and birds (De Luca *et al.*, 1991) mainly rely on N-type Ca^{2+} channels to control release of ACh.

Among the multiple VDCC subtypes, antibodies associated with LEMS preferentially target 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 among patients with LEMS appears to be due a decrease in the number of functional VDCC, particularly those of the P/Q-type.

B. *Neuromuscular transmission*

The neuromuscular junction represents the synaptic effector junction of the alpha motor neuron on striated muscle. It is comprised of a specialized cleft or synapse between motor nerve terminals and a highly defined region of muscle, known as the end-plate. This

specialized space couples release of chemical neurotransmitters from motor nerve terminals to electrical alterations in the end-plate region, ultimately leading to muscle contraction. The neuromuscular junction is not just an empty space, but it is composed of a unique form of basal lamina (Stephens *et al.*, 1985) containing agrin (Gautam *et al.*, 1996), a specialized enzyme involved in the degradation of the chemical neurotransmitter (Rotundo *et al.*, 1998), laminin (Martin *et al.*, 1995) and other proteins involved in mediating the structural and functional connections between the motor nerve terminal and muscle end-plate region.

The motor nerve terminal contains the chemical neurotransmitter, ACh. It may contain other neurotransmitters, as well, although the significance of these to normal physiological function, or distinct pathophysiology is as yet unclear. For example, there is evidence of release of adenosine from motor nerve terminals (Silinsky, 1984; Redman and Silinsky, 1994, 1995). ACh is packaged into specialized structures known as synaptic vesicles and is synthesized in the terminal from acetyl-coenzyme A and choline by the enzymatic reaction involving choline-O-acetyl-transferase (Browning and Schulman, 1968; Hebb, 1972; Tucek, 1982). Once synthesized, ACh is stored in synaptic vesicles via an ATPase -dependent transport system (Breer *et al.*, 1977; Parsons and Koenigsberger, 1980; Anderson *et al.*, 1982) whose function is impaired by the vesicle pump inhibitor vesamicol. Each synaptic vesicle contains approximately 7,000 -12,000 molecules of ACh (Kuffler and Yoshikami, 1975). Based on structural and functional studies, these synaptic vesicles have been categorized as two distinct populations--readily releasable and reserve pool (Heinemann *et al.*, 1993; Richards *et al.*, 2000). The readily releasable pool represents those vesicles that are immediately available for synaptic transmission, whereas the reserve pool only serves to replenish the readily releasable

pool. Ultrastructural studies have demonstrated that synaptic vesicles representing the readily releasable pool are located in physical proximity with the nerve terminal membrane in highly specialized regions known as active zones (Dreyer *et al.*, 1973; Heuser *et al.*, 1974; Rash *et al.*, 1974; Heuser *et al.*, 1979; Ceccarelli *et al.*, 1979; Heuser and Reese, 1981). The active zones are believed to be sites associated with the process of fast transmitter release (Heuser *et al.*, 1974). This assumption is based on the finding that at acutely denervated neuromuscular junctions, loss of active zones coincides with failure of evoked transmission (Ko, 1981), exocytosis captured by quick freezing occurs at the active zone (Heuser *et al.*, 1979), and active zones directly align with densities representing ACh receptors in the end-plate region of the muscle (Dreyer *et al.*, 1973; Heuser *et al.*, 1979; Heuser and Reese, 1981; Robitaille *et al.*, 1990).

Movement of vesicles from reserve to readily releasable pools represents a phenomenon known as "mobilization" and is presumably important in sustaining transmitter release during periods of intense activity (Delgado *et al.*, 2000; Richards *et al.*, 2000; Kuromi and Kidokoro, 2002). The specific processes governing mobilization remain unclear, but the synaptic vesicle protein, synapsin is thought to play a role in this process by means of a cyclical series of phosphorylation/dephosphorylation-dependent interactions with cytoskeletal proteins such as F-actin (Petrucci and Morrow, 1987; Llinas *et al.*, 1991; Humeau *et al.*, 2001). For cholinergic synapses, there is evidence that replenishment of the active recycling vesicular pool occurs primarily from newly-synthesized ACh as opposed to mobilization of pre-filled vesicles (Collier, 1986). Additionally, at some synapses, there is evidence for "incomplete release" in a process known euphemistically as "kiss and run" (Ceccarelli *et al.*, 1973; Kraszewski *et al.*,

1996; Klingauf *et al.*, 1998; Palfrey and Artalejo, 1998; Fesce and Meldolesi, 1999).

It has been observed using electrophysiological measurements that release of ACh occurs in defined packets known as quanta (del Castillo and Katz, 1954; Boyd and Martin, 1956; Katz and Miledi, 1967b). As such, each synaptic vesicle is believed to represent a single quantum of ACh (Heuser and Reese, 1973; Heuser *et al.*, 1979). Various mechanisms can induce the release of ACh from motor nerves, however, under physiological conditions, release arises either spontaneously or following action potential -induced depolarization of the nerve terminal membrane. Spontaneous release of ACh occurs asynchronously and represents single packets of quanta that cause small depolarizations of the muscle membrane within the end-plate region (Katz and Miledi, 1963, 1967b). These small fluctuations in the membrane potential in the end-plate region are known as miniature end-plate potentials (MEPP). Furthermore, the frequency of asynchronous release of single packets of quanta (MEPP frequency) increases during KCl-induced depolarization of the nerve terminal membrane. On the other hand, nerve action potentials induce multiple quanta to be released from motor nerve terminals in a synchronous manner leading to larger depolarization of the muscle membrane, which is known as end plate potentials (EPP) (Fatt and Katz, 1951; del Castillo and Katz, 1954; Boyd and Martin, 1956).

Release of ACh from motor nerves (Fig. 1.1) is multi-step process that begins with generation of an action potential *via* movement of Na^+ and of K^+ down their respective electrochemical gradients and propagation of the action potential along the axon (Hodgkin and Huxley, 1952). The action potential eventually ceases near the end of the axon, however, currents spread passively following their initiation by the action potential invade the non-

myelinated nerve terminal and depolarize the membrane (Mallart and Brigant, 1982; Mallart, 1985a). As a result of membrane depolarization, VDCC open, which in turn allows Ca^{2+} to move down its electrochemical gradient from the extracellular environment into the nerve terminal (Katz and Miledi, 1967a, 1970; Llinas *et al.*, 1981a; Augustine *et al.*, 1987). This rapid rise in intracellular [Ca^{2+}] occurs in distinct domains at active zones (Llinas *et al.*, 1992) and increases the likelihood that synaptic vesicles fuse with the membrane to release their contents into the synaptic cleft (Heuser *et al.*, 1974; Ellisman *et al.*, 1976; Ceccarelli *et al.*, 1979; Heuser *et al.*, 1979; Harris and Sultan, 1995).

While Ca^{2+} entry plays a vital role, it is not the only factor required for synaptic vesicle exocytosis. It has been observed that the delay between the influx of Ca^{2+} into the nerve terminal and subsequent changes at the muscle end-plate occur over 60-200 μs (Llinas *et al.*, 1981b; Sabatini and Regehr, 1996), which is faster than most enzymatic processes or chemical reactions. This observation has led to the supposition that Ca^{2+} entry induces the completion of synaptic vesicle and the plasma membrane fusion, a step that was mostly performed prior to Ca^{2+} entry into the nerve terminal (Sudhof and Scheller, 2000). This is also supported by the finding that, under conditions of high osmolarity, synaptic vesicle exocytosis can occur in the absence of Ca^{2+} or in Ca^{2+}-free solutions in the presence of α-latrotoxin (Hubbard *et al.*, 1968; Rosenmund and Stevens, 1996). As such, Ca^{2+} most likely acts as key regulator, but not an absolute requirement for exocytosis.

The steps involved in the regulated release of ACh following Ca^{2+} entry into the terminal remain highly elusive, however, some processes have been suggested. Fusion of synaptic vesicles with the plasma membrane has been shown to involve the formation of stable

complexes (core complex) of proteins known as SNAREs (Soluble NSF-Attachment Protein Receptors). SNAREs consist of two groups: nerve terminal, or t-SNAREs (SNAP-25-{synaptosomal protein of 25 kD} and syntaxin) found on plasma membranes and synaptic vesicle, or v-SNAREs (synaptobrevin/VAMP) located on vesicle membranes (Trimble and Scheller, 1988; Oyler *et al.*, 1989; Bennett *et al.*, 1992b; Bennett *et al.*, 1992a). The vital role these proteins play in synaptic vesicle fusion has been demonstrated following exposure of nerve terminals to *Clostridial* toxins: botulinum and tetanus (Schiavo *et al.*, 1992b). These toxins act as proteases to cleave specific proteins of the SNARE complex, ultimately inhibiting synaptic vesicle exocytosis (Link *et al.*, 1992; Schiavo *et al.*, 1992a; Blasi *et al.*, 1993; Schiavo *et al.*, 1993; Binz *et al.*, 1994; Schiavo *et al.*, 1994). Furthermore, SNAREs have been shown to mediate membrane trafficking and secretion in both mammalian and yeast cells (Fig. 1.2.) (Bennett *et al.*, 1992b; Bennett *et al.*, 1992a). The formation of the core complex is a highly regulated event that may involve the protein, munc18a/nsec1 (Hata *et al.*, 1993; Pevsner *et al.*, 1994). The rate limiting step in the core complex formation is believed to be the association of SNAP-25 and syntaxin (Nicholson *et al.*, 1998), however, this assembly can not take place until munc18a has dissociated from syntaxin (Pevsner *et al.*, 1994; Misura *et al.*, 2000). Once munc18a dissociates, a highly stable core complex is formed in such a manner that promotes a condition amenable to synaptic vesicle and plasma membrane fusion (Weber *et al.*, 1998). Binding of N-ethylmaleimide-sensitive factor (NSF) along with the NSF attachment protein (α-SNAP) to this tightly assembled protein structure results in the hydrolysis of ATP and dissociation of the core complex (Sollner *et al.*, 1993).

Membrane trafficking and release also appear to involve the GTP-binding protein,

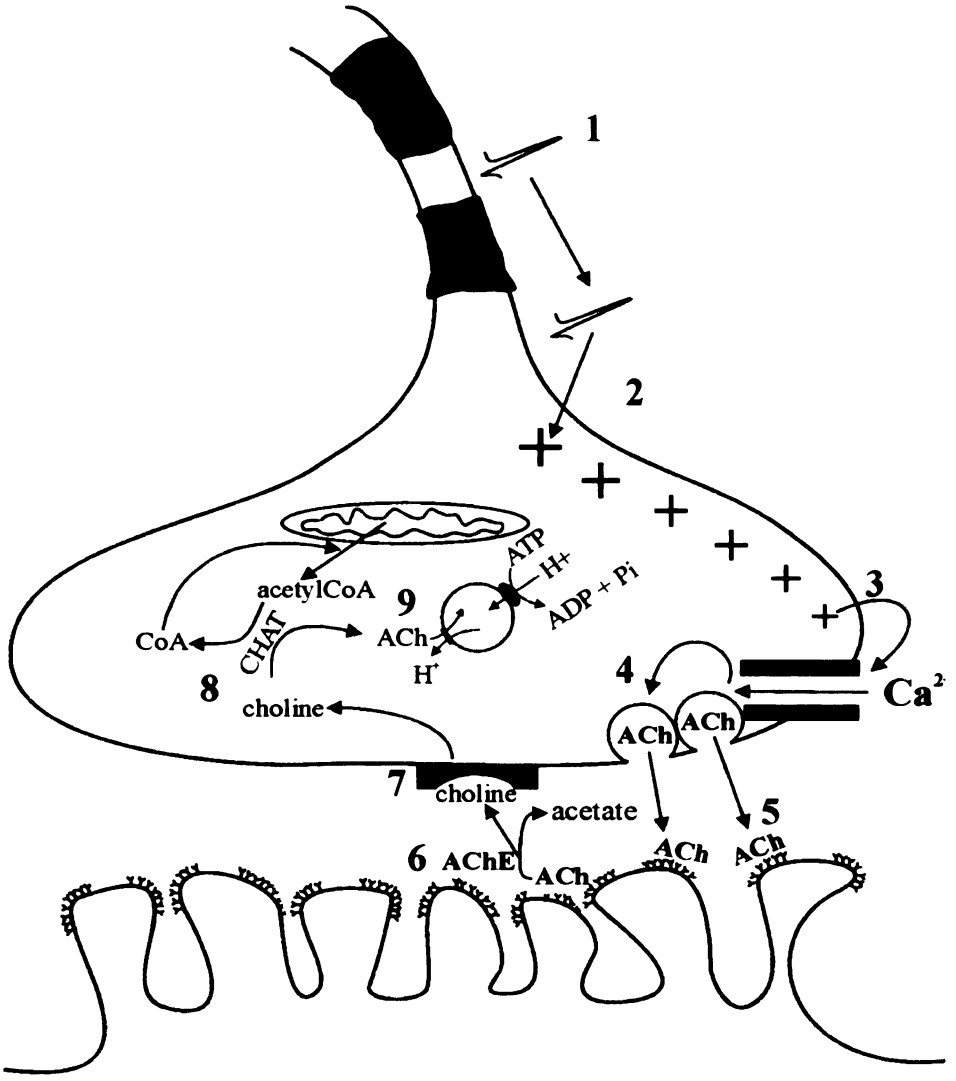

Figure 1.1. Neuromuscular transmission. Stimulation of the nerve leads to initiation and propagation of an action potential (1). Electrotonic spread of currents generated by the action potential depolarizes the nerve terminal membrane (2) and induces the opening of voltage-dependent Ca^{2+} channels, which permit rapid influx of Ca^{2+} into the terminal (3). Entry of Ca^{2+} initiates release of acetylcholine (ACh) from docked synaptic vesicles (4). Once released into the synaptic cleft, ACh diffuses across the cleft and binds to receptors located on the end-plate region of the muscle (5). The effects of bound ACh are rapidly terminated by the enzymatic processes of AChE (acetylcholinesterase), which cleaves ACh into acetate and choline (6). Choline is taken back into the cytosol of the nerve terminal by a high affinity transporter (7). Once inside the terminal, choline is combined with acetylCoA by CHAT (choline-O-acetyltransferase) to reform ACh (8). Free ACh is then taken up into synaptic vesicles by a specific ATP-dependent transport system (9).

rab3A, which associates with synaptic vesicles (Fischer von Mollard *et al.*, 1990). In the GTP-bound form, rab3A not only associates with the synaptic vesicle, but with other proteins, as well (Shirataki *et al.*, 1993; Wang *et al.*, 1997; Cao *et al.*, 1998). The association of these proteins may act to tether and target the synaptic vesicle to the membrane (Wang *et al.*, 1997; Orci *et al.*, 1998). During or after exocytosis, GTP bound to rab3A is hydrolyzed to GDP leading to the removal of rab3A from the synaptic vesicle by guanine nucleotide dissociation inhibitor (Ullrich *et al.*, 1993), thus limiting the association of the synaptic vesicle with the plasma membrane. The regulation of synaptic vesicle exocytosis by Ca^{2+} is thought to involve the vesicle protein, synaptotagmin (Geppert *et al.*, 1994; Li *et al.*, 1995a; Li *et al.*, 1995b). Most of the work implicating synaptotagmin as the key Ca^{2+} sensor is based upon indirect evidence. Mice deficient in synaptotagmin I exhibit decreased Ca^{2+}-dependent fast neurotransmitter release and unchanged Ca^{2+}-independent spontaneous release of neurotransmitter (Geppert *et al.*, 1994). Spontaneous (Ca^{2+}-independent) release of neurotransmitter, however, is increased in *Drosophila* possessing synaptotagmin mutants (DiAntonio and Schwarz, 1994) and decreased in the presence of overexpressed levels of synaptotagmin in developing spinal neurons (Morimoto *et al.*, 1998). These findings suggest that synaptotagmin acts as an inhibitor of synaptic vesicle exocytosis, but the effect can be overridden in the presence of Ca^{2+} (DiAntonio and Schwarz, 1994; Morimoto *et al.*, 1998). Structural analysis of synaptotagmin also has provided evidence for its role as a Ca^{2+} sensor. Synaptotagmin possesses two Ca^{2+} binding regions (C_2) on its cytoplasmic side that are homologous to a region within protein kinase C (Perin *et al.*, 1990; Davletov and Südhof,

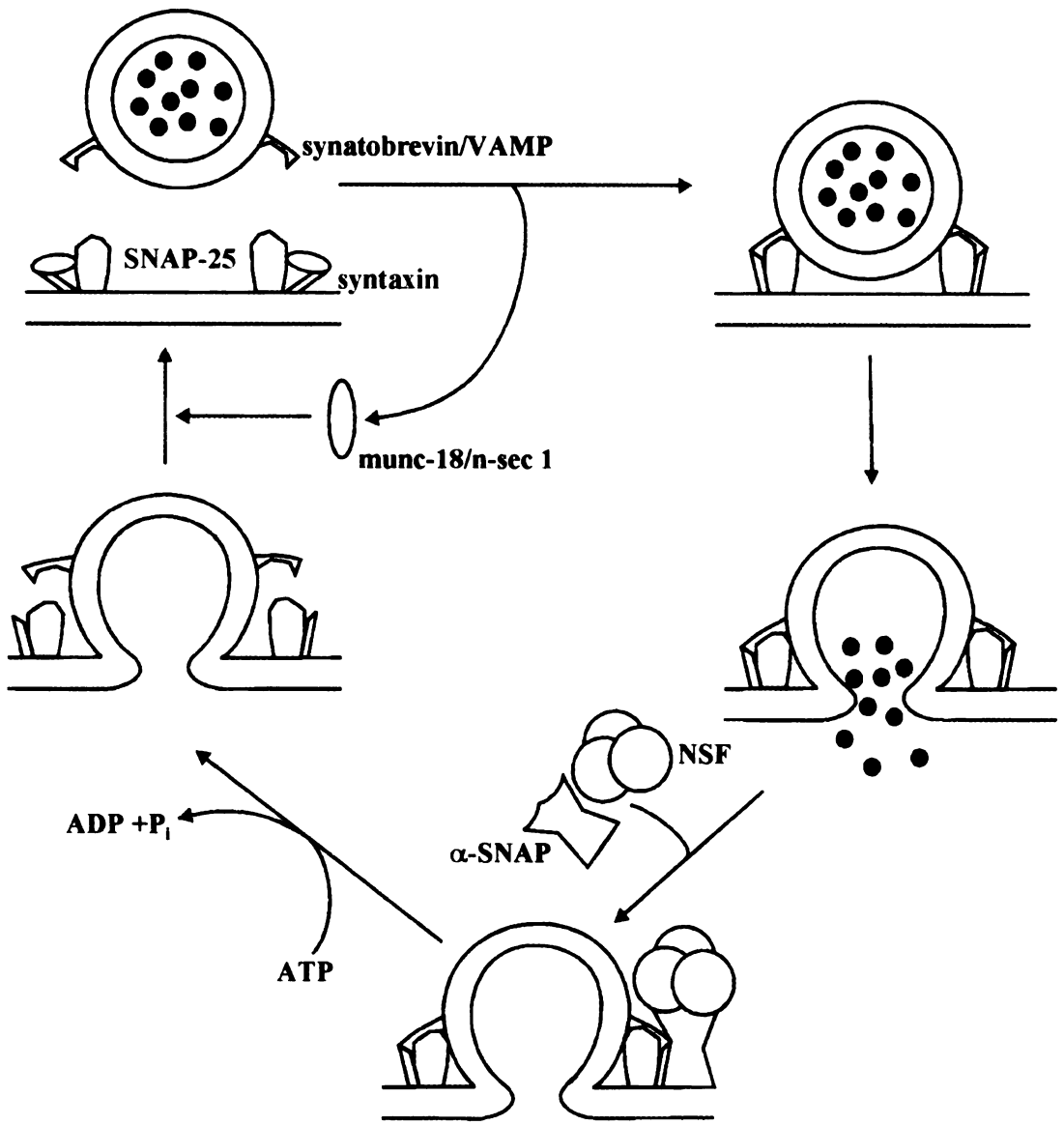

Figure 1.2. Model depicting the role of SNARE proteins in fusion of synaptic vesicle and nerve terminal membranes. Initiation of synaptic vesicle and nerve terminal membrane fusion is prevented by munc 18/n-sec 1 binding to syntaxin. Upon dissociation of munc 18/n-sec 1, syntaxin binds with SNAP-25 on the nerve terminal membrane and forms a highly stable core complex with synaptobrevin/VAMP. The core complex forces close apposition of the synaptic vesicle and nerve terminal, leading to membrane fusion and release of vesicle contents. Binding of NSF and α-SNAP results in hydrolysis of ATP, and dissociation of the core complex. The synaptic vesicle then dissociates from the nerve terminal membrane following binding of munc 18/n-sec 1 to synatxin. Adapted from Südof and Schellar 2000.

1993). It is thought that binding of Ca^{2+} induces electrostatic and not conformational changes of synaptotagmin to facilitate association with syntaxin and phospholipids leading to rapid exocytosis (Shao *et al.*, 1998; Ubach *et al.*, 1998).

Once synaptic vesicles fuse with the nerve terminal membrane, ACh is released and diffuses across the synaptic cleft where it binds specifically to post-synaptic ACh receptors localized in the end-plate region of the muscle (Landau, 1978). At physiological resting membrane potentials, binding of ACh increases the conductance of Na^+ and K^+ through ACh receptor-activated cation channels (Takeuchi, 1963), which in turn leads to a localized membrane depolarization within the end-plate region (Fatt and Katz, 1951; Boyd and Martin, 1956). This change in membrane potential is directly related to the number of ACh receptors that have opened, and thus the number ACh molecules bound to the ACh receptor. Electrotonic spread of membrane currents initiated by ACh binding to ACh receptors affect localized Na^+ channels on the muscle. At specific levels of depolarization (threshold), voltage-dependent Na^+ channels located on the muscle membrane open and Na^+ influx rapidly increases to initiate a muscle action potential. The muscle action potential then activates a cascade of events that leads to the development of muscle contraction.

Continual activation and re-activation of ACh receptors is promptly terminated by removal of ACh. This step is accomplished via the action of acetylcholinesterase, an enzyme which hydrolyzes ACh to acetate and choline (Rotundo *et al.*, 1998; Gaspersic *et al.*, 1999). Choline is then taken up into the nerve terminal via a specific transporter and reused to form new ACh (Marchbanks, 1982).

C. *Voltage-dependent calcium channels*

1. General Description

Calcium ions act as second messengers that play vital roles in cellular metabolism, excitability, contraction, and gene regulation (Augustine *et al.*, 1987; Miller, 1987). The importance of Ca^{2+} in cellular functions requires precise spatial and temporal control, and as such various mechanisms exist to control Ca^{2+} levels within the cell. One such mechanism includes gating of VDCC, which represent complex heteromeric protein structures that are sensitive to changes in membrane potential (Miller, 1987). The existence of multiple types of VDCC, each with distinct biophysical properties, localizations, and densities on cell membranes, allow precise control of Ca^{2+} entry into the cell. Based on molecular, biochemical, pharmacological, and electrophysiological techniques, the following VDCC subtypes have been identified: T, L, N, P, Q, and R (Table 1.1) (Tsien *et al.*, 1988; Snutch *et al.*, 1990; Zhang *et al.*, 1993; Randall and Tsien, 1995; Catterall, 2000), referred to now as Ca_v 1.1-1.4, Ca_v 2.1-2.3, and Ca_v 3.1-3.3.

2. Functional diversity

The existence of multiple VDCC was initially observed using voltage-clamp studies with invertebrate egg cells (Hagiwara *et al.*, 1975) in which calcium currents of different electrophysiological properties were found. Subsequently, two calcium currents of varied electrophysiological properties also were observed in mammalian neurons (Llinas and Yarom, 1981; Carbone and Lux, 1984) and given the designation of HVA (high voltage activated) or LVA (low voltage activated), based upon the size of the membrane depolarization required for

channel activation. Further classification of HVA and LVA VDCC was made using single channel and whole cell recordings in chick dorsal root ganglion (DRG) neurons (Nowycky *et al.*, 1985; Fox *et al.*, 1987a, b). VDCC subtypes of the LVA class were given the designation of T-type because of their "transient" kinetics and "tiny" conductance, whereas two HVA class VDCC were designated: L-type, due to their "long lasting" openings and "large" amplitude current in Ba^{2+} solutions and N-type, which displayed current properties intermediate to those of T and L-types ("neither L or T"). T-type Ca^{2+} channels were shown to activate at relatively negative potentials, inactive rapidly, and have small single channel conductance properties. L- and N-type Ca^{2+}channels, on the other hand, required much more positive potentials for activation, inactivated slowly or not at all, and possessed larger single channel conductances. Although, N-type channels exhibit markedly different electrophysiological properties than L-type channels in chick DRG neurons, the biophysical distinction between the two channel subtypes in other model systems is often less clear. Use of pharmacological agents, however, allows clear identification of these two channel subtypes. N-, but not L-type channels, are sensitive to functional antagonism by the *Conus* snail toxin, ω-conotoxin GVIA (McCleskey *et al.*, 1987; Tsien *et al.*, 1988; Hillyard *et al.*, 1992; Grantham *et al.*, 1994). L-type Ca^{2+} current, on the other hand is affected by the 3 groups of organic compounds known as dihydropyridines (DHP), phenylalkylamines and benzothiazepines (Catterall and Striessnig, 1992; Hofmann *et al.*, 1994). These groups are typified by the drugs nifedipine, verapamil, and diltiazem, respectively. L-type VDCC are also antagonized by the peptide toxin, calciseptine, isolated from the black mamba snake, *Dendroaspis polylepsis polylepsis*. While there have been some reports showing functional

antagonism of N-type current by DHPs, this non-selectivity appears to occur in the presence of high concentrations of antagonists (Diochot *et al.*, 1995). Calciseptine, however, is selective for L-type VDCC and does not appear to affect entry of Ca^{2+} through N-type channels (de Weille *et al.*, 1991; Kuroda *et al.*, 1992; Yasuda *et al.*, 1993) . Currently there are no specific antagonists for T-type channels, although a number of chemicals antagonize T-type VDCC (Chuang *et al.*, 1998; Ernst and Kelly, 1998; Martin *et al.*, 2000).

In addition to T, L and N-type channels, three other subtypes of VDCC have been identified, based on their pharmacological, molecular, and electrophysiological characteristics. P-type Ca^{2+} current was initially identified in cerebellum purkinje cells, and were shown to be highly sensitive to the spider toxin, ω-agatoxin IVA (Aga-IVA) (Llinas *et al.*, 1989; Mintz *et al.*, 1992). It was later found that when a cloned protein representing the primary subunit believed to comprise the P-type VDCC was expressed in a cellular model system, the Ca^{2+} current was less sensitive to the effects of Aga-IVA and the inactivation kinetics were different than originally observed in purkinje cells (Mintz *et al.*, 1992; Sather *et al.*, 1993; Zhang *et al.*, 1993). An Aga-IVA sensitive current also was found in cerebellar granule cells however, this Ca^{2+} current exhibited properties resembling those described using cloned P-like channel proteins and were named Q-type channels (Zhang *et al.*, 1993; Wheeler *et al.*, 1994; Randall and Tsien, 1995). A sixth VDCC-subtype, aptly named R-type was observed to be resistant to the actions of the previously described Ca^{2+} channel antagonists. However, in the presence of SNX-482, a toxin isolated from the tarantula, *Hysterocarates gigas,* current through expressed R-type VDCC channels in mammalian cells or at neurohypophyseal nerve terminals is reduced (Newcomb *et al.*, 1998). The existence of R-type VDCC cannot, however, be

Table 1.1

Pharmacological and Biophysical
Properties of Voltage-Dependent Ca^{2+} Channel Subtypes

	HVA	**H VA**	**LVA**
Ca^{2+} current type	L	P/Q, N, R	T
Structural nomenclature	Ca_v 1.1,1.2,1.3,1.4	Ca_v 2.1, 2.2, 2.3	Ca_v 3.1, 3.2, 3.3
Previous α_1 nomenclature	S, C, D, F	A, B, E	G, H, I
Activation range	Positive to -10 mV	Positive to -20 mV	Positive to -70 mV
Inactivation range	-60 to -10 mV	-120 to -30 mV	-100 to -60 mV
Inactivation	Very slow (τ> 500 ms)	Partial ($\tau \approx$50-80 ms)	Complete ($\tau \approx$20-50 ms)
Deactivation rate	Rapid	Slow	Rapid
Single-channel conductance	25 pS	13 pS	8 pS
Single-channel openings	Continual reopening	Long burst	Brief burst, inactivation
Relative conductance	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} = Ca^{2+}$
ω-conotoxin GVIA	Resistant	Ca_v2.2 sensitive	Resistant
Dihydropyridines	Sensitive	Resistant	Resistant
ω-agatoxin IVA	Resistant	Ca_v2.1 sensitive	Resistant
Calciseptine	Sensitive	Resistant	Resistant
SNX-482	Resistant	Ca_v2.3 sensitive	Resistant
Divalent block	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$	$Ni^{2+} > Cd^{2+}$

Adapted and modified from Hille 2000

proven based solely by pharmacological antagonism with SNX-482. Newcomb *et al.* (1998) have shown that current flow in several rat central neurons was resistant to the actions of SNX-482.

3. Structure of voltage-dependent calcium channels

Initial biochemical analysis using the transverse tubules of rabbit skeletal muscle revealed the structure and composition of L-type VDCC. These channels were comprised of 5 separate subunit proteins, each given the designation of α_1, $\alpha_2\delta$, β, and γ, respectively (Curtis and Catterall, 1984; Takahashi and Catterall, 1987). It has now been shown that not all VDCC subtypes consist of 5 subunits. Most HVA Ca^{2+} channels contain at least four subunits (α_1, $\alpha_2\delta$, β), however, the association of γ subunits with non-L type HVA channels is still unresolved (McEnery *et al.*, 1991; Martin-Moutot *et al.*, 1995b; Liu *et al.*, 1996; Letts *et al.*, 1998). LVA Ca^{2+} channels (T-type), on the other hand, are believed to contain only the α_1 subunit (Lambert *et al.*, 1998; Leuranguer *et al.*, 1998; Perez-Reyes, 1998).

The structure of the VDCC is shown in Figure 1.3 (See Catterall review, 1998 and 2000). The α_1 subunit is considered the primary subunit of VDCC and consists of amino acid sequences organized into four repeated domains (I - IV), with the carboxyl and amino termini located intracellularly. Each domain is composed of six transmembrane regions (S1 - S6) with a loop between S5 and S6 embedded in the membrane. On the cytoplasmic side of the membrane, the β subunit is found at the amino end of the α_1 subunit and contains no transmembrane spanning regions. The γ subunit, on the other hand, contains four transmembrane segments and, in L-type VDCC, is also located in close proximity to the amino

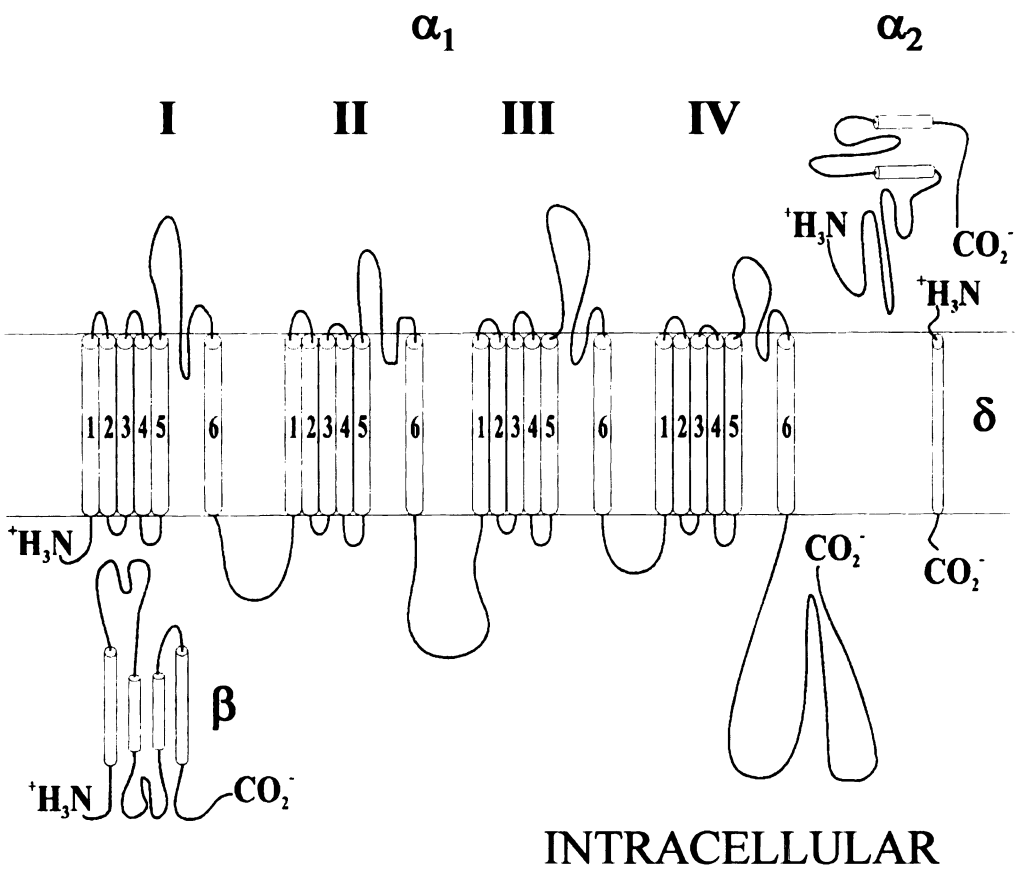

Figure 1.3. Schematic depiction of the subunit structure of a voltage-dependent Ca^{2+} channel believed to represent the P, Q, or N subtype. The α_1 subunit is comprised of 4 domains (I-IV), each with 6 transmembrane-spanning regions (numbered 1-6). The purely transmembrane-spanning δ subunit is linked to the extracelluarly located α_2 subunit by a disulfide bond. On the other hand, the β subunit is thought to be located exclusively on the intracellular side of the membrane. Note that other voltage-dependent Ca^{2+} channel subtypes may either have an additional subunit (γ), which is not shown in this schematic (L-type found on skeletal muscle) or be comprised solely of an α_1 subunit (T-type). Adapted and modified from Catterall 1998.

end of the α_1 subunit. A disulfide linkage joins the $\alpha_2\delta$ subunits, which are derived from the same gene (Jay *et al.*, 1991) and associate with the carboxyl end of the α_1 subunit. While the α_2 protein contains no transmembrane segments and is located on the extracellular face of the plasma membrane, the δ protein is comprised of 1 transmembrane segment.

4. Molecular diversity of voltage-dependent calcium channels

In conjunction with electrophysiological and biochemical analysis, molecular studies have elucidated the genes encoding the specific α_1 subunit and protein representing each known VDCC subtype. As such, α_1 subunits representing the P/Q, N, and, R, channels were named α_{1A}, α_{1B}, and α_{1E}, respectively (Snutch *et al.*, 1990; Dubel *et al.*, 1992; Sather *et al.*, 1993; Zhang *et al.*, 1993). Although, P- and Q-type channels have different biophysical properties, it is believed that they are both comprised of same α_{1A} subunit (splice variants of the same gene); antisense nucleotides directed against the α_{1A} gene or antibodies for the α_{1A} subunit reduce calcium currents through P- and Q-type channels (Williams *et al.*, 1992b; Stea *et al.*, 1994; Gillard *et al.*, 1997; Pinto *et al.*, 1998b). This is further supported by the findings of Jun *et al.* (1999), in which mice deficient in the α_{1A} gene and thus α_{1A} subunit, do not express P- or Q-type currents. Unlike other VDCC, L- and T- subtypes are compromised of different α_1 subunit types depending on the tissue in which the channel was isolated and the gene encoding the subunit; L-type VDCC were found to contain either the α_{1S}, α_{1C}, α_{1F} or α_{1D} subunit (Mikami *et al.*, 1989; Hui *et al.*, 1991; Snutch *et al.*, 1991; Williams *et al.*, 1992a; Hell *et al.*, 1993), whereas T-type VDCC were comprised of either α_{1G}, α_{1H}, or α_{1I} subunits (Perez-Reyes, 1998). This nomenclature, however, has been revised recently to clarify the structural

and functional similarities of each VDCC subtype. This led to grouping of the VDCC into three families, given the designation of: $Ca_v1,2$, and 3 (Ertel *et al.*, 2000).

In addition to the multiple α_1 subunits identified, so far, at least 4 β, 3 $\alpha_2\delta$, and distinct 5 γ subunits have also been described. For most of the subtypes (T-type excepted), the formation of fully functional channels requires the association of specific α_1 and accessory protein subunits, which is highly varied and dependent upon the species and anatomic location of the channel. To date, however, only a few specific subunit compositions of the different VDCC have been identified in their native environment.

5. Role of voltage-dependent calcium channel subunits

The α_1 subunit confers many of the properties associated with the VDCC, by acting as the Ca^{2+} ion selectivity filter and pore, possessing the channel gating sites, and containing the voltage sensor and binding sites for many specific pharmacological modulators (Catterall, 2000). The pore of the VDCC is thought to exist in the loop regions between S5- S6 segments for each domain of the α_1 subunit, and is known as the P-loop. Mutational analysis has revealed that much of the ion selectivity is determined via four glutamic acid sequences in the P-loop and, as such, loss of these regions is related to decreases in Ca^{2+} selectivity (Yang *et al.*, 1993; Yatani *et al.*, 1994; Ellinor *et al.*, 1995). Mutational analysis also has suggested that the voltage-sensing function of the α_1 subunit is confined to the S4 segment (Garcia *et al.*, 1997). However, rates of channel activation and conductance are influenced *via* other segments (Tanabe *et al.*, 1991; Dirksen *et al.*, 1997). Although, HVA channels exhibit multiple types of inactivation kinetics that differ amongst the different α_1 subtypes, it has been

observed that sequences within the S6 segment and carboxyl terminus are vital for voltage-dependent inactivation (Wei *et al.*, 1994; Zhang *et al.*, 1994).

Binding sites for pharmacological agents that modulate VDCC mainly reside on the α_1 subunit. DHPs have been shown to bind to multiple transmembrane regions of the $\alpha_{1C,S,D}$ subunits (Kalasz *et al.*, 1993; Kuniyasu *et al.*, 1998); mutagenesis of the S5 segment of domain III and S6 segment of domain IV dramatically decrease binding of DHP (Peterson *et al.*, 1997). The presence of Ca^{2+} ions also is required for binding of DHP to $\alpha_{1C,\,S,\,D}$ subunits (Schneider *et al.*, 1991). It is believed that Ca^{2+} ions keep the α_1 subunit in a conformation that facilitates DHP binding (Peterson and Catterall, 1995). Other pharmacological agents also have been observed to affect VDCC specifically by binding to the α_1 subunit. As such, Ca^{2+} current entry through α_{1A} subunits expressed without other auxiliary subunits in model cell systems are decreased in the presence of ω-Aga IVA and ω- conotoxin (CTx) MVIIC (Sather *et al.*, 1993; Zhang *et al.*, 1993). It has been shown using mutational analysis that ω-Aga IVA binds to a site at the carboxyl end of segment S3 in domain IV (Winterfield and Swartz, 2000).

Although expression of α_1 subunits alone can yield functional VDCC, many of the properties associated with the channel are affected by association with the auxiliary protein subunits ($\alpha_2\delta$, β, and γ) (Perez-Reyes *et al.*, 1989). Work by Chien *et al.* (1995) showed that expression of β_{2a} subunit proteins increase targeting of α_{1C} subunits to cell membranes without altering α_{1C} expression. Other β subunit isoforms also target α_1 subunit proteins to plasma membranes. As such, $\beta_{1b,\,2a,\,3b,\,4}$ isoforms have been observed to localize expressed α_{1A} subunit proteins to the plasma membrane of chick osteosarcoma (COS-7) cells, however, α_{1A} proteins alone or in conjunction with $\alpha_2\delta$ proteins are not found at the membrane, but

intracellularly (Brice *et al.*, 1997). While the role of the β subunit in targeting α_1 proteins to the plasma membrane appears nonspecific, other studies have shown preferential interactions of the two-subunit proteins. Expression of various isoforms of the β subunit with either α_{1A}, α_{1B}, or α_{1C} in cells in culture which exhibited membrane polarization with protein sorting mechanisms analogous to those of neurons, showed differential targeting of the α_1 subunit. This specificity was primarily dependent upon the α_1 subunit subtype, however, the destination was also differentially modified based upon the specific β subunit present (Brice and Dolphin, 1999). Thus, calcium currents in cells increase in amplitude following β subunit targeting of α_1 proteins to plasma membranes (Singer *et al.*, 1991; Neely *et al.*, 1993; Chien *et al.*, 1995; Brice *et al.*, 1997; Brice and Dolphin, 1999). Although it is possible that β subunits increase whole cell Ca^{2+} currents by increasing expression of functional α_1 subunit proteins, studies to date have shown a lack of effect of β subunits on α_1 subunit expression (Singer *et al.*, 1991; Neely *et al.*, 1993; Chien *et al.*, 1995).

Auxiliary subunits also appear to modify the gating kinetics, voltage dependence of activation and inactivation, and sensitivity of the channel protein to pharmacological agents. Co-expression of β subunits, facilitates the probability of channel pore openings of α_{1A} subunit proteins and, thus increases Ca^{2+} entry into cells (Neely *et al.*, 1993; Shistik *et al.*, 1995). In addition, voltage-dependent inactivation and activation of α_{1A} subunits is shifted to more negative potentials and rate of current activation is increased following co-expression of β subunits with α_1 subunit proteins (Singer *et al.*, 1991; De Waard and Campbell, 1995). Conversely, DRG injected with β subunit antisense oligonucleotides, which reduce the expression β subunit proteins, exhibit a positive shift in the voltage-dependent Ca^{2+} channel

activation (Berrow *et al.*, 1995). Rates of inactivation of channels containing α_{1A} subunits are increased by co-expression with β subunit isoforms, which occur with differential selectivity with the following rank order of $\beta_3 > \beta_{1b} = \beta_4 > \beta_{2a}$ (fastest to slowest) (Castellano *et al.*, 1993; De Waard and Campbell, 1995). DHP- and ω-CTx MVIIC/GVIA binding to α_{1C} and $\alpha_{1A,B}$ subunits, respectively is increased in the presence of β subunit proteins, an effect which is independent of α_1 subunit expression (Neely *et al.*, 1993; Nishimura *et al.*, 1993; De Waard and Campbell, 1995). In addition to β subunits, $\alpha_2\delta$ subunits have also been shown to shift the voltage-dependence of channel activation and inactivation in the hyperpolarizing direction, enhance current amplitudes, increase the rate of current activation and inactivation, and alter the probability of channel openings (Singer *et al.*, 1991; De Waard and Campbell, 1995; Gurnett *et al.*, 1996; Dolphin *et al.*, 1999). While the function of γ subunits in skeletal muscle is similar to that of other accessory proteins, its role in neurons *in vivo* is less clear. However, γ subunits modulate the voltage-dependence of co-expressed subunits *in vitro* and mice with a mutation in a γ subunit exhibit a unique neurological deficit, known as "stargazer" (Letts *et al.*, 1998). *Stargazer* mice show prolonged and frequent *absence* seizures, ataxic gait and a distinctive head tossing motion that is related to vestibular problems (Felix, 2000). Thus, γ subunits may play an important role in neurons as well as those observed in skeletal muscle.

6. Modulation of voltage-dependent calcium channels

a. G-protein dependent modulation

Regulation of VDCC involves multiple mechanisms that may act independently or in concert with one another. One such mechanism involves G-proteins, which have been

observed to modulate VDCC responses to various levels of membrane depolarization (Fig. 1.4.) (Hille, 1994; Dolphin, 1998; Simen and Miller, 1998). The effect of G-proteins on VDCC kinetics can occur via a direct link between G-protein activation or G-protein coupled soluble second messengers and VDCC. G-proteins are heteromeric complexes composed of three subunits designated: α, β, and γ (Hille, 1992, 1994). Upon activation of G protein-coupled receptors, GDP bound to the Gα subunit is exchanged for GTP, which in turn induces dissociation of Gβγ from the G-protein complex (Hille, 1992; Hamm, 1998). Voltage-activation or neurotransmitter binding can induce G-proteins to inhibit directly Ca^{2+} entry through N-, R- and P/Q-type channels by shifting the Ca^{2+} channel from an easily activated to a more difficult-to activate state (Herlitze *et al.*, 1996; Ikeda, 1996; Qin *et al.*, 1997; Simen and Miller, 1998; Canti *et al.*, 1999). This inhibition occurs via Gβγ and not Gα binding to modulatory sites on VDCC. Characteristics of direct G-protein induced inhibition of N-, R-, and P/Q-type Ca^{2+} channels include: positive shifts in current-voltage relationship, decrease in whole cell Ca^{2+} current amplitudes, slowed activation kinetics, and reversal of modulation by G-proteins during strong depolarization (Herlitze *et al.*, 1996; Ikeda, 1996; Qin *et al.*, 1997; Simen and Miller, 1998; Canti *et al.*, 1999).

b. Kinase-dependent modulation

L-type Ca^{2+} channels, on the other hand, do not appear to be affected by direct coupling to G-protein activation (Bourinet *et al.*, 1996; Meza and Adams, 1998; Bell *et al.*, 2001). Instead, modulation of L-type channels occurs via G-protein induced second messenger pathways. Activation of cAMP-dependent protein kinase or phosphinositide 3 kinase pathways following G-protein activation alters cardiac (Reuter *et al.*, 1983) and smooth

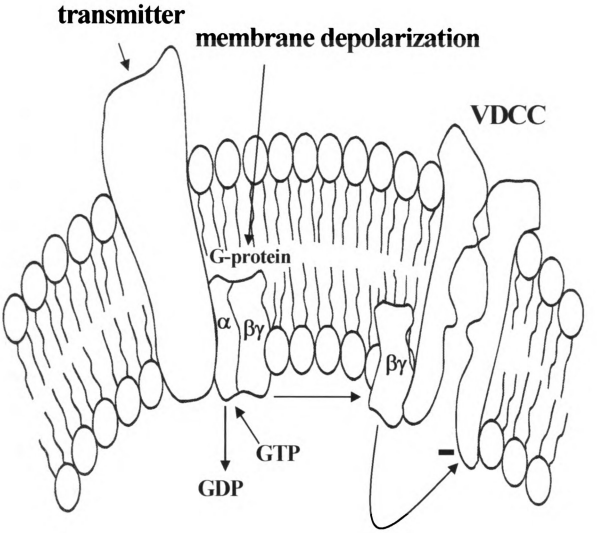

Figure 1.4. G-protein inhibition of voltage-dependent Ca^{2+} channels. Hypothetical pathway depicting direct inhibition of voltage-dependent Ca^{2+} channels (VDCC) by G-proteins. Membrane depolarization or transmitter-activation of receptors coupled to G-proteins induces the exchange of GDP bound to the α subunit of the G-protien for GTP. Binding of GTP, in turn, causes dissociation of the βγ subunit, which directly inhibits VDCC by some unknown mechanism. Adapted from Dolphin, 1998.

muscle (Viard *et al.*, 1999) L-type channel biophysical properties, respectively. As such, direct phosphorylation plays a key role in the regulation of L-type Ca^{2+} channels. During periods of intense stimulation, L-type Ca^{2+} channel current at skeletal muscle is greatly enhanced by phoshorylation of the α and β subunits of the channel by cAMP-dependent protein kinase (Arreola *et al.*, 1987; Sculptoreanu *et al.*, 1993) (Rotman *et al.*, 1995). Unlike, L-type channels from skeletal muscle, early investigations observed that phosphorylation does not change the sensitivity of cardiac muscle L-type channels to voltage, but rather increases Ca^{2+} conductance (Reuter and Scholz, 1977). More recent work has shown that gating kinetics of cardiac L-type channels is altered following phosphorylation to a state that allows more channels to open during physiological levels of stimulation (Hess *et al.*, 1984;Yue *et al.*, 1990).

In addition to L-type, P/Q- and N-type Ca^{2+} channel activity is also modulated by phoshorylation. G-protein inhibition of non-L type channels can be relieved by several mechanisms involving phosphorylation by protein kinase C (Swartz, 1993). Interaction of P/Q- and N-type channels with the SNARE complex and synaptotagmin is also disrupted following channel phosphorylation (Yokoyama *et al.*, 1997).

c. Direct role of Ca^{2+} in modulation

Increased Ca^{2+} entry also has been shown to alter directly the biophysical properties of VDCC. L-type channels found in cardiac cells are inhibited following large influxes of Ca^{2+}, an effect that appears dependent upon calmodulin (Peterson *et al.*, 1999). It has been shown that over-expression of calmodulin mutants that are insensitive to Ca^{2+} binding prevents Ca^{2+}-dependent inactivation of L-type channels (Peterson *et al.*, 1999). Binding of calmodulin to a region in the C-terminus of L-type channels is a necessary prerequisite and as such, mutations

in calmodulin binding regions prevent Ca^{2+}-dependent inactivation (Zuhlke and Reuter, 1998). In contrast, large influxes of Ca^{2+} can also have a facilitatory effect upon cardiac L-type channels that is also dependent upon calmodulin (Anderson *et al.*, 1994). However, in addition to Ca^{2+} binding to calmodulin, Ca^{2+}/calmodulin-regulated protein kinase II (Cam KII) is required for Ca^{2+}-dependent facilitation (Xiao *et al.*, 1994; Zuhlke *et al.*, 1999).

In a similar manner, Ca^{2+} entry through P/Q-type channels has a dual role. Initially large Ca^{2+} current induces facilitation with a subsequent slower rate of inactivation of P/Q-type channels via a Ca^{2+}/calmodulin-dependent pathway (Lee *et al.*, 2000). Although, this mechanism is not well understood, it is believed to occur in a different manner from that observed with L-type channels (DeMaria *et al.*, 2001).

d. Modulation of Ca^{2+} channel function by proteins involved in exocytosis

Proteins associated with the core complex of docked vesicles at active zones have been observed to regulate directly VDCCs. Over expression of the SNARE protein, syntaxin 1A decreases Ca^{2+}- dependent transmitter release (Wu *et al.*, 1999), whereas reduced expression of syntaxin enhances release (Watanabe *et al.*, 1999). N-, P/Q- and in some instances L-type Ca^{2+} channel currents have been shown to be inhibited directly by syntaxin (Bezprozvanny *et al.*, 1995; Wiser *et al.*, 1996; Wiser *et al.*, 1999). In addition to syntaxin, the SNARE protein, SNAP-25 has been also been shown to inhibit P/Q-type Ca^{2+} channel current (Zhong *et al.*, 1999). However, in the presence of the Ca^{2+} binding protein synaptotagmin, inhibition of VDCC by syntaxin and SNAP-25 is relieved (Wiser *et al.*, 1997; Wiser *et al.*, 1999; Zhong *et al.*, 1999). The effect of syntaxin modulation is not the same for each VDCC subtype. Inhibition of N-type channels by syntaxin can be partially removed by addition of SNAP-25,

whereas inhibition of L-type channels by syntaxin is unchanged following addition of SNAP-25 (Wiser *et al.*, 1996; Wiser *et al.*, 1999). It appears, therefore, that complete core complexes at active zone sites are required for physiological function of associated VDCC, which are regulated via their specific interactions.

e. Alternative methods involved in modulation

As previously described, association of auxiliary subunits modulate many of the biophysical and pharmacological properties of α_1 subunits. This may explain differences observed between P- and Q-type Ca^{2+} channel subtypes, and as such, may reflect channels containing identical α_{1A} subunits with different associated auxiliary subunit proteins. Alternatively, splice variations of α_{1A} subunits could account for dissimilarities of P- and Q-type channels. Although it is still unclear if variations between these channels represent splicing or auxiliary subunit differences, both subtypes contain α_{1A} subunits derived from the same gene (Gillard *et al.*, 1997; Pinto *et al.*, 1998a; Jun *et al.*, 1999).

D. *Voltage-dependent Ca^{2+} channels involved in neuromuscular transmission*

The small size and thus inaccessibility of most motor nerve terminals precludes direct electrophysiological correlation of presynaptic current carried by specific VDCC subtypes and concomitant release of ACh. However, recent pharmacological and molecular techniques have overcome this hindrance and as such, it has been observed that the specific VDCC subtype(s) involved in release of ACh from motor nerves is highly age- and species-dependent.

Cell bodies of motor neurons from chick embryos initially possess a dominant T-type Ca^{2+} current. However, during development, N- and L-type channels assume a major role in

controlling Ca^{2+} entry (McCobb *et al.*, 1989). Similarly, L- and N-type channels are found at developing avian motor terminals and both types are involved in release of ACh (Gray *et al.*, 1992).

The involvement of different subtypes of VDCC in Ca^{2+}-induced ACh release from motor nerve terminals of rats also displays differential age-dependence (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002; Santafe *et al.*, 2002). During embryonic stages of development, rat motor nerve terminals contain P/Q-, N-, and L-type channels that all regulate, to some extent, nerve-evoked release of ACh (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999). P/Q- and N-type channels appear to enhance release, whereas in some experimental conditions, Ca^{2+} current through L-type channels actually inhibit release of ACh (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999). As motor nerves of post-natal rats develop, the participation of each VDCC subtype in release of ACh changes, such that from day 0-4, N- and P/Q- type channels are mainly involved but, by day 5, N-type channel no longer control release of ACh (Rosato Siri and Uchitel, 1999; Rosato-Siri *et al.*, 2002). Furthermore, P/Q-type channels appear to be more efficiently coupled to release of ACh than N-type channels during post-natal days 0-4; this may be due to differential localization of each channel subtype with the release apparatus (Rosato-Siri *et al.*, 2002).

Examination using rats of various post-natal days revealed that involvement of multiple VDCC subtypes with differing effects on ACh release can be explained in part by competition between poly-innervating motor nerve terminals found at immature end-plates (Santafe *et al.*, 2001; Santafe *et al.*, 2002). Entry of Ca^{2+} through P/Q-, N-, and L-type channels at dually-

innervated immature end-plates either enhances or decreases release of ACh, depending upon the stimulus protocol and the specific terminal activated (Santafe *et al.*, 2001; Santafe *et al.*, 2002). During maturation one motor nerve will eventually be eliminated, while the other one remains in contact with the end-plate. However, the roles of VDCC at nerves during synapse elimination and at those destined to support release of ACh from adult motor nerves are different. While Ca^{2+} entry through P/Q-, N-, and L-type channels acts to suppress growth of axons that eventually will be eliminated, entry of Ca^{2+} through P/Q-, N-, and L-type channels induces excitatory processes at immature motor nerves that eventually will mono-innervate mature end-plates (Santafe *et al.*, 2001; Santafe *et al.*, 2002).

Mature motor nerves appear to possess only one VDCC-subtype principally involved in release of ACh. Whereas, N-type channels control release of ACh from amphibian (Sano *et al.*, 1987) and avian (De Luca *et al.*, 1991; Gray *et al.*, 1992) mature motor nerve terminals, P/Q-type channels control release of ACh from adult mammalian motor nerves (Uchitel and Llinas, 1992;Wessler *et al.*, 1995; Protti and Uchitel, 1996; Katz *et al.*, 1997). L-type channels, which are often found co-localized with other VDCC subtypes are not normally involved in mammalian neuromuscular transmission (Atchison and O'Leary, 1987; Atchison, 1989; Protti *et al.*, 1996; Katz *et al.*, 1997), but are involved in release of hormones from the neurohypophysis from rats (Lemos and Nowycky, 1989), noradrenaline (Owen *et al.*, 1989) from adrenal bovine chromaffin cells, and insulin from human pancreatic β cells (Davalli *et al.*, 1996).

In non-physiological settings, however, L-type Ca^{2+} channels can participate in release of ACh from even adult mammalian motor nerve terminals. Exposure of adult mammalian

motor terminals to a DHP agonist, bay K 8644, which directly modulates the response of L-type Ca^{2+} channels to potential changes, enhances release of ACh. This effect can be prevented by incubation of the preparation with DHP-type antagonists (Atchison and O'Leary, 1987; Atchison, 1989). More indirect methods have been observed to involve normally silent L-type Ca^{2+} channels in release of ACh. Rapid chelation of intracellular Ca^{2+} using DM-BAPTA unmasks L-type Ca^{2+} current at mouse motor nerve terminals that are not involved in release of ACh unless protein phosphatases are also inhibited (Urbano and Uchitel, 1999; Urbano *et al.*, 2001). A similar finding was observed at frog motor nerve terminals following an increase in intracellular levels of protein kinase C (Arenson and Evans, 2001). Alterations in stimulus conditions induce participation of L-type channels in release of ACh from rat and mouse motor nerves (Hong and Chang, 1990; Correia-de-Sa *et al.*, 2000a). This effect is thought to occur through a mechanism involving activation of adenosine A_{2A} receptors (Correia-de-Sa *et al.*, 2000b). Thus, it appears that mature motor nerve terminals possess L-type Ca^{2+} channels that are normally silent and not involved in release of ACh, but can be unmasked under certain conditions.

In a similar manner, L-type Ca^{2+} channels participate in release of ACh during certain pathological conditions. For example, release of ACh from motor nerves during reinnervation or following recovery from botulinum toxin-induced poisoning or exposure to antibodies from patients with amyotrophic lateral sclerosis is sensitive to L-type Ca^{2+} channel antagonists (Katz and Uchitel, 1996; Fratantoni *et al.*, 2000; Santafe and Uchitel, 2000). This effect may well represent a situation analagous to development of immature motor nerves.

E. *Ca^{2+}-activated K^+ channels*

1. General information

Ca^{2+}-activated K^+ (K_{Ca}) channels are found in a variety of tissues, particularly those that exhibit action potential induced-release of neurotransmitters (Knaus *et al.*, 1996; Anderson *et al.*, 1988; Vergara *et al.*, 1998). These channels not only appear to be involved in release of neurotransmitters, but in neuronal firing properties as well. Based upon pharmacological and biophysical properties, K_{Ca} channels can be separated into three families: BK, SK, and IK channels (Table 1.2.) (Sah, 1996; Vergara *et al.*, 1998). The BK ("big" conductance) channels, which can be activated by either a rise in cystolic Ca^{2+} concentration or membrane depolarization, are highly selective for K^+, and possess large single channel conductances of 100-400 pS (Marty, 1981). A number of pharmacological antagonists for this channel subtype exist and include tetraethylammonium, the scorpion toxins iberiotoxin and charybdotoxin, and the mycotoxins penitrem A and paxilline (Galvez *et al.*, 1990; Knaus *et al.*, 1994; Vergara *et al.*, 1998). The second type of K_{Ca} channel has a small single channel conductance (2-20 pS) and is thus known as SK ("small" conductance) channels (Blatz and Magleby, 1986). Elevated intracellular Ca^{2+} concentrations, but not membrane depolarization, is required for activation of these channels (Hirschberg *et al.*, 1998). In contrast to BK channels, SK channels are not sensitive to tetraethylammonium, iberiotoxin, or charybdotoxin. However, current through SK channels is antagonized by the bee venom apamin, quaternary salts of bicuculline, tubocurarine, and dequalinium (Blatz and Magleby, 1986; Johnson and Seutin, 1997). On the other hand, 1-ethyl-2-benzimidazolinone (EBIO) activates the channel by increasing sensitivity to Ca^{2+} and thus increasing the probability of channel openings (Olesen *et al.*, 1994).

Table 1.2

Pharmacological and Biophysical
Properties of Ca^{2+}-activated K^+ channels

K_{Ca} subtype	**BK**	**IK**	**SK**
Membrane depolarization	Activates	No effect	No effect
Elevated intracellular [Ca^{2+}]	Activates	Activates	Activates
Single channel conductance	100-400 pS	20-100 pS	2-20 pS
Tetraethylammonium	Sensitive	No effect	No effect
Iberiotoxin	Sensitive	No effect	No effect
Charybdotoxin	Sensitive	Sensitive	No effect
Apamin	No effect	No effect	Sensitive*

Adapted from Sah 1996 and Vergara *et al.* 1998
*Sensitivity is dependent on SK subtype and channel expression system

The third channel subtype, IK ("intermediate") is aptly named because it posses single channel conductances intermediate (20-100 pS) to those of BK and SK channels (Ishii *et al.*, 1997b; Logsdon *et al.*, 1997). In a manner similar to SK channels, IK channels are insensitive to voltage changes, but they open in response to elevated cystolic Ca^{2+} concentration. Furthermore, they are sensitive to EBIO, charybdotoxin, and clotrimazole, but not to apamin or iberiotoxin (Ishii *et al.*, 1997a; Logsdon *et al.*, 1997).

2. Ca^{2+}- activated K^+ channels and neuromuscular transmission

Ca^{2+}-activated K^+ channels have been identified at amphibian and mammalian motor nerve terminals using pharmacological, electrophysiological, and immunocytochemical staining techniques. The scorpion venom peptides, charybdotoxin and iberiotoxin both directly antagonize $K_{(Ca)}$ current during nerve-stimulation, whereas apamin has no effect. Based on pharmacological sensitivities, amphibian and mammalian motor nerve terminals appear to possess BK type K_{Ca} channels. The increase in intracellular Ca^{2+} which activates $K_{(Ca)}$ current originates from extracellular, but not intracellular sources in mice; as such, block of VDCC by Mg^{2+}, Mn^{2+}, or Co^{2+} prevents activation of $K_{(Ca)}$ current during nerve-stimulation (Mallart, 1985b).

Although the exact role of K_{Ca} channels at motor nerve terminals of mammals and amphibians is not fully understood, it is believed that they participate in nerve terminal membrane repolarization. K_{Ca} channels limit the duration of action potentials and Ca^{2+} entry at nerve terminals in frog pituitary (Obaid *et al.*, 1989) and Ca^{2+} entry and release of ACh from frog and mouse motor nerve terminals (Mallart, 1985b; Dreyer and Penner, 1987; Anderson

et al., 1988; Robitaille and Charlton, 1992; Robitaille *et al.*, 1993; Vatanpour and Harvey, 1995; Xu and Atchison, 1996; Protti and Uchitel, 1997). Furthermore, pharmacological and immunocytochemical staining techniques have shown that K_{Ca} channels are colocalized with VDCC at the nerve terminal (Robitaille *et al.*, 1993; Protti and Uchitel, 1997). This intimate localization of these channels most likely ensures a rapid activation of K_{Ca} current during Ca^{2+} entry that is triggered by an action potential.

F. *Lambert-Eaton Myasthenic Syndrome: General Description*

1. General Clinical Features

Lambert Eaton myasthenic syndrome occurs in two general populations: those with and those without detectable neoplasms (Elmqvist and Lambert, 1968; O'Neill and Newsom-Davis, 1988). Among those with neoplasms, small cell lung carcinoma (SCLC) is the most common type, with approximately 60% of all LEMS patients exhibiting a SCLC (Eaton and Lambert, 1957; Lambert *et al.*, 1961; Lambert and Rooke, 1965; Lambert and Elmqvist, 1971; O'Neill and Newsom-Davis, 1988). Evidence of LEMS almost always precedes the diagnosis of cancer (all types) by 5 months to 3.8 years. On average, the interval between onset of neuromuscular symptoms and detection of cancer in the majority of patients with LEMS occurs over 40 years of age. However LEMS has been reported to range from 17 to 79 years (O'Neill and Newsom-Davis, 1988). Males are more predominately affected than females in both cancer and non-cancer groups(Elmqvist and Lambert, 1968; O'Neill and Newsom-Davis, 1988).

2. Physical symptoms

Proximal lower extremity weakness represents the presenting complaint among most LEMS patients (Lambert *et al.*, 1961; Wise and MacDermot, 1962; Lambert and Rooke, 1965; O'Neill and Newsom-Davis, 1988). A small number of patients, however, present with generalized weakness, muscle pain and stiffness, autonomic dysfunction, or arm weakness as their primary symptom. Although arm weakness is often described, it is usually restricted to the proximal muscles and much less severe compared with that of the lower leg. Autonomic dysfunction is also a common complaint in approximately 80% of patients with LEMS. Symptoms include: dry mouth, sexual impotence, and to a lesser extent decreased sweating, constipation, difficulty with micturition, and blurred vision. (Lambert and Rooke, 1965; Henriksson *et al.*, 1977; Rubenstein *et al.*, 1979; Khurana and Mayer, 1988; O'Neill and Newsom-Davis, 1988). Cranial nerves also are affected in LEMS, and patients may exhibit diplopia and ptosis, dysarthria, dysphagia, and dysphonia. The symptoms related to cranial nerve involvement, however, are often mild and intermittent.

3. Physical Signs

The most common clinical sign observed in patients with LEMS is that of skeletal muscle weakness, mainly involving proximal and truncal muscles and most dramatically affecting the lower limbs (Lambert *et al.*, 1961; Wise and MacDermot, 1962; Lambert and Rooke, 1965; O'Neill and Newsom-Davis, 1988; Erlington and Newsom-Davis, 1994). During sustained maximal muscle contraction there is a progressive augmentation in strength followed by increasing weakness and fatigue. The augmentation occasionally makes it difficult to detect

clinical weakness.

Tendon reflexes often are depressed or absent in patients with LEMS. However, after maximal voluntary muscle contraction, tendon reflexes reappear or augment (Lambert *et al.*, 1961; Wise and MacDermot, 1962; Lambert and Rooke, 1965). Cranial nerve signs are common, including ptosis and weakness in neck flexion (O'Neill and Newsom-Davis, 1988). LEMS patients also exhibit impairments in pupillary light reflex and secretions from lacrimal and parotid glands (Rubenstein *et al.*, 1979; Heath and Cull, 1988; Khurana and Mayer, 1988; O'Neill and Newsom-Davis, 1988). Most autonomic abnormalities can be ascribed to defects of the parasympathetic (cholinergic) system, however two reports have described patients with sympathetic dysfunction (orthostatic hypotension and increased sensitivity to exogenous sympathetic agonists) (Mamdani *et al.*, 1985; Khurana and Mayer, 1988). Sympathetic dysautonomia most likely reflects an effect induced by the underlying neoplasm in the described patients and not that of LEMS; cardiovascular autonomic dysfunction is commonly found in patients with bronchial neoplasms/SCLC without LEMS (Park *et al.*, 1972; Gould *et al.*, 1986; Maier and Sommers, 1986) and breast cancer patients (Bruera *et al.*, 1986; Heath and Cull, 1988). Sensory abnromaltities are not a prominent findings in LEMS (Heath and Cull, Khurana and Mayer,O'Neil and Newsom-Davis, 1988).

LEMS patients' signs and symptoms, however, 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). Guanidine and 4-aminopyridine, which prolong the duration of action potentials and enhance Ca^{2+} entry into the nerve terminal, enhance ACh release and produce symptomatic relief, albeit frequently accompanied by serious

side effects. The actions of a similar compound, 3,4-diaminopyridine (3,4 DAP), on the other hand, are generally limited to the peripheral nervous system and 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 3,4 DAP, 20 mg 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). On the other hand, drugs that prolong the actions of ACh at motor end-plates by inhibiting acetylcholinesterase alleviate symptoms in patients with myasthenia gravis, a neuromuscular disorder in which functional ACh receptors on skeletal muscles are reduced, but offer relatively limited benefit in LEMS patients (Eaton and Lambert, 1957; O'Neill and Newsom-Davis, 1988).

4. Electrophysiological characteristics

a. Clinical

Although neuromuscular disorders (for example, myasthenia gravis) are characterized by skeletal muscle weakness, the diagnosis of LEMS can be established by electromyography (EMG). The relevant study utilizes surface electrodes to record compound muscle action potentials (CMAP) generated from muscle fibers comprising the motor unit (motor nerve + all muscle fibers innervated by that nerve) during supramaximal depolarization of the nerve (Eaton and Lambert, 1957). EMG findings associated with LEMS are depicted in Figure 1.5. In LEMS, CMAP amplitudes in response to single or low frequency (usually ≤ 5 Hz) stimulation of the motor nerve are depressed, and often can be as low as 10% of normal

(Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Jablecki, 1984; O'Neill and Newsom-Davis, 1988). During repetitive stimulation at low rates (3 Hz), CMAP also exhibit a decrementing pattern similar to that seen in myasthenia gravis. High frequency (≥10 Hz) stimulation of the motor nerve, on the other hand, induces a progressive increase in the amplitude of the CMAP. This phenomenon is known as post-activation facilitation (Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Jablecki, 1984). Similarly, CMAPs recorded immediately after maximal voluntary muscle contraction subsequently increase in comparison to that at rest. It has been postulated that post-activation facilitation reflects Ca^{2+} build-up 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 (Lambert and Rooke, 1965; Jablecki, 1984; Heath and Cull, O'Neill and Newsom-Davis, 1988). These findings suggests that mechanisms involved in nerve action potential generation and conduction are unaffected in LEMS.

Single-fiber EMG (SFEMG), a technique used to measure neuromuscular transmission in individual end-plates, is abnormal in patients with LEMS (Fig. 1.6.) (Keesey, 1989; Sanders, 1994). SFEMG is performed during minimal 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 neuromuscular "jitter." In some conditions, generation of an action potential in the second muscle fiber fails; this is known as neuromuscular "blocking." In LEMS, both jitter and blocking are dramatically increased in

comparison to controls and characteristically decrease as the rate of nerve stimulation increases (Sanders, 1994).

b. Experimental

Intracellular recordings of synaptic transmission from neuromuscular preparations isolated from patients with LEMS show electrophysiological characteristics consistent with those found using EMG: EPP amplitudes in response to single depolarizatoin of the motor nerve are smaller compared to those of control preparations; following high frequency nerve-stimulation, EPP amplitude increases progressively in size (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971; Cull-Candy *et al.*, 1980). MEPP amplitudes are not appreciatively changed at LEMS patient tissue preparations nor is their rise or decay times affected (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971; Cull-Candy *et al.*, 1980). Although spontaneous release of ACh is normally unaltered from LEMS preparations exposed to buffers containing physiological concentrations of KCl, depolarization of the nerve terminal membrane by exposure to buffers containing elevated (non-physiological) concentrations of KCl, which increases the frequency of asynchronous release of ACh in an extracellular calcium-dependent manner (seen electrophysiologically as MEPPS) is significantly lower than that observed from controls.

5. Neuromuscular junction morphology in LEMS

Histological analysis of tissue biopsies from LEMS patients does not reveal any gross abnormalities at motor nerve terminal and end-plate regions when viewed with light microscopy (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 from controls, mean vesicle diameter calculated for each biopsy was also unchanged, but 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 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 (Tsujihata *et al.*, 1987; Hesselmans *et al.*, 1992).

G. *Lambert-Eaton Myasthenic Syndrome: Autoimmunity*

1. Clinical and Experiment Findings

Early observations that an autoimmune component underlies the pathology in LEMS were based upon the findings that many patients with defects of neuromuscular transmission had associated autoimmune disorders (Norris and Panner, 1966; Takamori *et al.*, 1972;Gutmann *et al.*, 1972). More recent work has shown a higher than normal frequency of autoimmune disorders, such as vitiligo, pernicious anemia, celiac disease, juvenile-onset

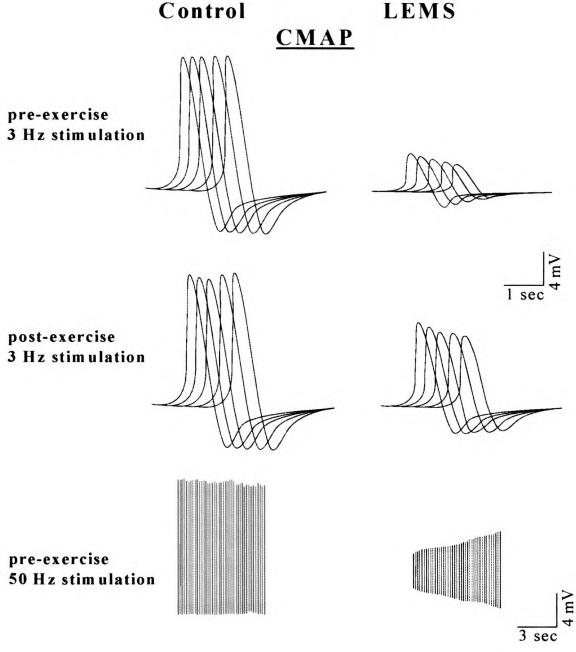

Figure 1.5. EMG recordings of CMAP from control and LEMS patients. CMAP during stimulation of the motor nerve at 3 Hz (low frequency) are smaller in amplitude from LEMS patients in comparison to controls. Following maximal voluntary muscle contractions (post-exercise), CMAP increase in amplitude in comparison to pre-exercise 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 the progressive increase in amplitudes of the CMAP from LEMS, but not control patients. Adapted from Jablecki (1984).

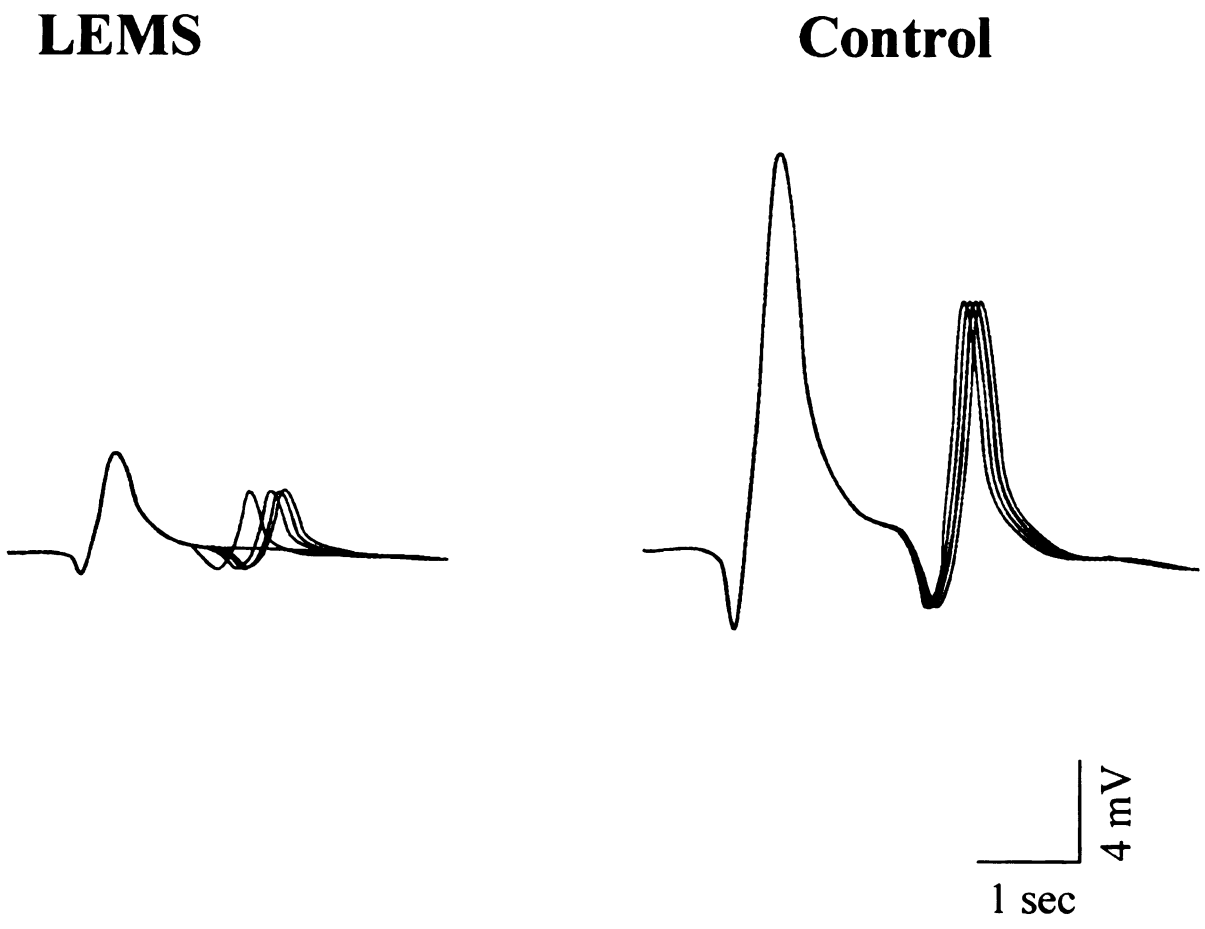

Figure 1.6. Single-fiber EMG recordings from two muscle fibers innervated by the same motor nerve. Tracings represent superimposed potentials following low frequency single shock stimulation of a motor nerve from control or LEMS patients. The duration of time between the two potentials recorded from each muscle fiber innervated by the same motor nerve varies between superimposed tracings and is known as jitter. Failure in the generation of a potential in the second muscle fiber is known as blocking. Tracings from LEMS patients exhibit a higher degree of jitter and blocking in comparison to control patients. Adapted from Sanders (1994).

diabetes and presence of other organ-specific antibodies (Lennon and Fairbanks, 1982; O'Neill and Newsom-Davis, 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 and Murray, 1981; Newsom-Davis and Murray, 1984). The autoimmune basis for LEMS was established by the findings that IgG from patients with LEMS injected into mice not only duplicated the electrophysiological characteristics observed from EMG recordings and intracellular electrophysiological recordings of muscle preparations from patients with LEMS (Lang and Murray, 1981), but ultrastructural studies showed IgG located at the motor nerve terminals as well (Fukunaga *et al.*, 1983). Passive transfer of LEMS to mice using IgG in comparison to whole plasma fractions with slightly lower IgG concentrations showed a greater reduction in release of ACh from motor nerve terminals exposed to IgG alone (Lang and Murray, 1981). These findings provide strong support for the hypothesis that LEMS is an autoantibody-mediated disorder.

2. Antigenic targets

a. Voltage-dependent calcium channels: general findings

It was originally proposed that the autoantibody identified 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 proposal was based upon the findings that repetitive nerve stimulation at high rates or increases in extracellular Ca^{2+} levels, which both increase the probability of quantal ACh release improve synaptic transmission (Lambert and Elmqvist,

1971). Although the specific antigenic target 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^+ and zero (or low) Ca^{2+} concentrations from buffer, 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 would be reduced. In the presence of high extracellular Ca^{2+} concentrations, nerve-evoked release of ACh also appeared normal, suggesting that exocytotic mechanisms that occur following Ca^{2+} entry are also unaffected in LEMS; elevated extracellular Ca^{2+} concentrations increase the amount of Ca^{2+} entering the terminal during nerve-stimulation. Thus, it appeared based upon these findings that LEMS antibodies most likely reduce Ca^{2+} entry into the nerve terminal, presumably by affecting voltage-dependent Ca^{2+} channels. Experiments involving the cardiac glycoside, ouabain, which blocks the Na^+/K^+ exchanger have provided further evidence against an intraterminal target in LEMS (Wray *et al.*, 1987). Incubation of motor nerve terminals with ouabain increases the rate of spontaneous release of ACh via a mechanism independent of Ca^{2+} entry through VDCC (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 (Molenaar *et al.*,

1982; Lang and Vincent, 1984; Meyer *et al.*, 1986).

Other possible targets in LEMS also have 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 (Prior and Newsom-Davis, 1985; Lang *et al.*, 1987). Additional observations consistent with the idea that LEMS antibodies interfere with Ca^{2+} entry through VDCC include the findings that electrical stimulation of motor nerves exposed to LEMS antibodies exhibit strikingly similar electrophysiological characteristics following exposure to heavy-metal ions, which are known to block directly Ca^{2+} channels (Jenkinson, 1957; Silinsky, 1985).

A number of studies have provided more direct support implicating VDCC as the putative target in LEMS. Incubation of SCLC tissue cultures with IgG from patients with LEMS inhibits uptake of $^{45}Ca^{2+}$ during K^+-induced depolarization (De Aizpurua *et al.*, 1988). Furthermore, this inhibition is dependent upon the concentration 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 hours reduced the voltage-dependent Ca^{2+} current by ~58% (Viglione *et al.*, 1995) in comparison to SCLC cells exposed to control IgG. In another study, exposure of SCLC cells in culture to LEMS IgG for 24 hours reduced

the voltage-dependent Ca^{2+} entry by up to 70% (Meriney *et al.*, 1996). Ca^{2+} currents in bovine adrenal chromaffin cells (BAC) also are reduced following exposure to LEMS IgG. However, direct injection of Ca^{2+} into the cell, which bypasses the need for VDCC, induced normal exocytosis (Kim, 1987; Kim and Neher, 1988; Engisch *et al.*, 1999). Similarly, ionomyocin, which forms pores in membranes 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 elucidate the antigenic target, they do not provide direct evidence that VDCC located on mammalian nerve terminals are affected by LEMS antibodies. However, K^+-induced uptake of $^{45}Ca^{2+}$ into rat nerve terminal preparations (synaptosomes) is reduced following acute exposure to sera from patients with LEMS (Hewett and Atchison, 1991, 1992a). Along these lines, Ca^{2+} currents at 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 further support the hypothesis that LEMS antibodies act on VDCC in multiple systems without altering intracellular mechanisms involved in exocytosis.

b. Voltage-dependent calcium channels: 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 all relied on 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 non-specificity, 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 sympathetic neurons and express N- and L-, and T-type Ca^{2+} channels (Grassi *et al.*, 1994). VDCC were partially purfied from IMR32 cells using ^{125}I-ω-conotoxin GVIA (^{125}I-ω-CgTx), a toxin known to bind N-type channels specifically. Approximately 92% of LEMS patients' sera bound to ^{125}I-ω-CgTx-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 (included patients with neurological disorders other than LEMS) and 43% of patients with SCLC without LEMS.

A study performed at the same time but using human derived SCLC or colon adenocarcinoma tissue cultures, 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, and 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 among patients with LEMS who possessed SCLC than in LEMS patients with non- lung tumors or those without any malignancy.

Similar findings from all LEMS patients' sera tested against VDCC isolated from the human neuroblastoma cell line, SKN-SH were observed in comparison to the results using human derived SCLC cultures (Leys *et al.*, 1989; Leys *et al.*, 1991). Although both SKN-SH

and SCLC cells used in this assay posses 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* (SLE)) (Leys *et al.*, 1989). Although it was shown that between patients, antibody titers did not correlate with severity of the disease, longitudinal studies within individual LEMS patients without SCLC exhibited an inverse relationship between CMAP amplitudes and antibody titers (Leys *et al.*, 1991).

More recently, immunological 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 among 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 erythematuous* possessed antibodies against N-, but not P-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 SLE) 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.

Based on the findings using immunoprecipitation assays, antibodies against P/Q- and N-type VDCC appear to be causally associated with 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.

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 X 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+} current as well (Grassi

et al., 1994).

Although Grassi *et al.* (1994) observed an apparent non-specificity of LEMS IgG for VDCC in IMR32 cells, entry of Ca^{2+} was reduced to a greater extent through N- in comparison 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 (Kim and Neher, 1988; Blandino and Kim, 1993), however, several lines of evidence support the findings that LEMS IgG primarily targets P/Q-type VDCC in BAC cells. These include the following: (1) voltage-dependent Ca^{2+} entry into BAC cells is reduced following exposure to LEMS sera by approximately 48% (Kim, 1987; Kim and Neher, 1988; Blandino and Kim, 1993), (2) approximately 40% of the Ca^{2+} currents in BAC cells are mediated by P/Q-type channels (Kim, 1998), and (3) P/Q-type Ca^{2+} currents in BAC cells are reduced following exposure to LEMS IgG by approximately 80% (Kim, 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.*, 1998a; Pinto *et al.*, 2002). Voltage-dependent Ca^{2+} entry into HEK cells expressing P/Q-type Ca^{2+} channels (α_{1A}, $\alpha_2\delta$, β_{4a}) was inhibited following exposure to LEMS IgG. On the other hand, current carried through N- (α_{1B}, $\alpha_2\delta$, β_{1b}), L- ($\alpha_{1D,1C}$, $\alpha_2\delta$, $\beta_{2c,3a}$), and R-type channels (α_{1E}, $\alpha_2\delta$, $\beta_{4a,1b}$) 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 maybe 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 IgG 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 also have demonstrated 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, HVA Ca^{2+} currents recorded from cultured murine neonatal motor neurons are reduced to a greater extent than cultured neonatal murine sensory neurons (Garcia and Beam, 1996; Garcia and Walrond, 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 these neuron types from neonatal mammals possess P-, N-, and L-type channels, the predominant VDCC subtype 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 posses N-type channels. These differences may account for the lack of evidence of sensory nerve dysfunction in patients with LEMS.

Based upon 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 dysautonomia in LEMS patients were due to down-regulation of N-type channels (Lang *et al.*, 1987; Sher *et al.*, 1993; Grassi *et al.*, 1994). However, more recent findings have shown that transmitter release from the same and different autonomic nerves relies on Ca^{2+} entry through P/Q-, N-, R-, and sometimes L-type channels, depending upon the mode of nerve stimulation (Lemos and Nowycky, 1989; Owen *et al.*, 1989; Hong and Chang, 1995; Waterman, 1996; 1997). In contrast to earlier suggestions, dysautonomia in LEMS patients most likely reflect a preferential action on P/Q-type Ca^{2+} channels at autonomic nerve terminals. Houzen *et al.*, 1998 found that transmitter release from autonomic nerves subserved by P/Q-, but not N-type VDCC was reduced following exposure for 6 hours to IgG from 3 patients with LEMS. Although transmitter release from autonomic nerves obtained from adult mice injected with 1 of 4 LEMS patient IgG for 8 days attenuated Ca^{2+} entry through N-type channels, all 4 LEMS patients IgG affected P/Q-type Ca^{2+} channels(Waterman

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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 nerve types, but P/Q-type channels controlled release only from parasymapthetic nerves).

Other direct studies have also shown a preferential effect of LEMS sera on VDCC subtypes 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). 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 blocked by nifedipine. On the other hand, nifedipine did not affect Ca^{2+} currents from control motor nerve terminals (Xu *et al.*, 1998). 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. 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. Similar findings have been observed using cultured mammalian motor neurons (Garcia and Beam, 1996); while both LVA and HVA currents at cultured mammalian motor neurons were reduced following exposure to LEMS sera, L-type currents were spared.

Although LEMS antibodies appear to target a heterogeneity of Ca^{2+} channel subtypes,

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albeit with differing sensitivities, VDCC found at rodent skeletal and cardiac tissue 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. In light of these observations, LEMS antibodies do exhibit some species and anatomic specificity. Furthermore, the preferential action on P/Q-type Ca^{2+} channels most likely accounts for the observations that neuromuscular transmission, which is subserved by P/Q-type Ca^{2+} channels is the primary abnormality in LEMS.

c. Voltage-dependent calcium channels: antigenic sites

The β subunit was found to be antigenic when screened against plasma of LEMS patients with high titer levels of IgG. However, the β subunit is not considered the actual target in LEMS based upon several observations. The location of these proteins on the cytoplasmic side of the membrane makes them normally inaccessible to circulating antibodies (Catterall, 2000), and injection of β subunit proteins into mice induces high antibody titer levels against the β subunit without inducing any neurological dysfunctions (Rosenfeld *et al.*, 1993b; Rosenfeld *et al.*, 1993a; el Far *et al.*, 1995; 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 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

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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 (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 Domain II and Domain 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 also provided by immunization of rats with the synthetic peptides. Rats immunized with synthetic peptide regions representing S5-S6 linker regions of Domain 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 that the conformation of the synthetic peptide may not duplicate that of the antigen, making its recognition difficult or impossible. The S5-S6 linker regions are all anchored to transmembrane regions for each domain, and thus synthetic peptides of the linkers regions may not directly represent the conformation states of these regions. This may explain any differences that arise between the passive transfer LEMS methodology and that involved using synthetic peptides. However, it is clear that these epitopes represent potential sites for VDCC antibody development.

d. Additional antigenic targets

In addition to VDCC, other antigenic targets have been proposed to exist in LEMS. In particular, the synaptic vesicle protein, synaptotagmin has been implicated. As discussed earlier, synaptotagmin is believed to act as the calcium sensor and play a vital role in the docking and fusion of vesicles during exocytosis (Perin *et al.*, 1990; Brose *et al.*, 1992). Several lines of evidence lend some support to the role of synaptotagmin in LEMS. First, it was observed that Western blots of partially purified VDCC 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 titers specific for ^{125}I-ω-CgTx GVIA- precipated VDCC labeled the 58-kDa protein on Western blots. Based on 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 (Leveque *et al.*, 1992; el Far *et al.*, 1995). The exclusion of the β subunit was shown by the finding that 6 out of 20 LEMS patients sera used reacted with synaptotagmin I expressed using *E. Coli*. (Takamori *et al.*, 1995; Takamori *et al.*, 2000a). However, only 3 out 6 of these sera reacted specifically with expressed synaptotagmin alone, whereas the other remaining patient sera exhibited cross-reactivity to purified Q- and N-type VDCC (Takamori *et al.*, 1995; Takamori *et al.*, 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 posses synaptotagmin (David *et al.*, 1993). Lastly, the EPP quantal content was significantly reduced for 50% of diaphragm preparations obtained from rats immunized with synthetic

synaptotagmin residues, and quantal content increased when these preparations were bathed in buffers 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 pronounced effect may reflect differences between synthetic peptides as immunogens, which are often only weakly recognized (Takamori *et al.*, 1990; Takamori *et al.*, 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 neither component is accessible to the actions of extracellular LEMS antibodies. During exocytosis, the N-terminus of synaptotagmin is exposed to the extracellular surface of the nerve terminal (Shoji-Kasai *et al.*, 1992) and as such, it has been postulated to represent a potential antigenic site (Leveque *et al.*, 1992; el Far *et al.*, 1995; Takamori *et al.*, 1995; Takamori *et al.*, 2000a), however this idea has to yet to be proven. Additionally, not all investigations have identified synaptotagmin positive antibodies in LEMS sera; IgG from 14 LEMS patients used to probe Western blots containing recombinant synaptotagmin,and human membrane and rat synaptosomal proteins did not label bands corresponding to synaptotagmin (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 immunoprecipitation of VDCC with anti-synaptotagmin antibodies was dependent upon the detergent used to purify partially the VDCC; in the presence of non-denaturing detergents, anti-synaptotagmin antibodies do not immunoprecipitate VDCC (Lennon *et al.*, 1995). It is also possible that LEMS sera contain other specific antibodies that only recognize proteins associated with VDCC in intact, non-denatured configurations (Leveque *et al.*, 1992). Furthermore, the specific synaptotagmin congener present in SCLC cells may not reflect the putative synaptotagmin congener believed to be affected by LEMS antibodies. Lastly, use of ionomyocin or direct injection of Ca^{2+} as 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 1988) exposed to LEMS antibodies. This finding also provides support against synaptotagmin as a putative target in LEMS. Thus, while there is some evidence consistent with a role for synaptotagmin in LEMS, the minority of patients' plasma seropositive for synaptotagmin antibodies and lack of conclusive evidence suggests that is does not play a major role.

3. 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 VDCC subtypes, including L-, N-, and P/Q-type channels that potentially contain antigenic sites responsible for antibody development in LEMS (McCann *et al.*, 1981; Roberts *et al.*, 1985;

De Aizpurua *et al.*, 1988; Pancrazio *et al.*, 1989; Sher *et al.*, 1990; Codignola *et al.*, 1993; Barry *et al.*, 1995; Viglione *et al.*, 1995; Meriney *et al.*, 1996); the VDCC- subtypes expressed on SCLC correlate with the channel subtypes affected in LEMS (De Aizpurua *et al.*, 1988; Sher *et al.*, 1990; Barry *et al.*, 1995; Viglione *et al.*, 1995); exposure of SCLCs in culture to antibodies from LEMS patients reduced evoked-Ca^{2+} influx (Roberts *et al.*, 1985; (De Aizpurua *et al.*, 1988; Lang and Newsom-Davis, 1989; 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 VDCC are found on SCLCs 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 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, however, many patients with LEMS have other autoimmune disorders or have first-blood relatives with autoimmune diseases (Norris and Panner, 1966; Gutmann *et al.*, 1972; Takamori *et al.*, 1972; O'Neill and Newsom-Davis, 1988). As such, 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 human leukocyte antigens

(HLA) represent a complex of genes encoding polymorphic molecules (divided into three classes) found on almost every nucleated cell in the human body and 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). Furthermore, slightly different forms (alleles) of the genes encoding each HLA class exist between individuals and each allele within a given class is named differently. Based upon 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 (Willcox *et al.*, 1985; Parsons *et al.*, 2000; 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.

4. Pathophysiological Mechanisms

a. Electrophysiological features

Although Ca^{2+} entry into BAC cells exposed to LEMS antibodies is reduced, single Ca^{2+} channel properties, such as activation and inactivation kinetics, ion conductance, and channel open time are unaltered (Kim and Neher, 1988; Grassi *et al.*, 1994; 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 suggestion 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 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 depolarization 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+} concentration, 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 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 VDCC in an all or none fashion.

b. Ultrastructural features

Early investigations examining ultrastructural freeze-fracture analysis of motor nerve terminals provided compelling evidence that VDCC 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). Multiple findings support the idea that VDCC 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. As such, the latency between Ca^{2+} influx and synaptic vesicle exocytosis is quite small (Llinas *et al.*, 1976) and exocytosis can be shown via quick freezing to occur near active zone particles (Heuser *et al.*, 1979). Furthermore, maximum Ca^{2+} currents in the motor nerve terminal are related to the number of active zone particles (Pumplin *et al.*, 1981). More direct support for the association of VDCC and AZPs is provided by the observation that fluorescent staining with ω-CgTx GVIA, which presumably represents labeling to VDCC labeled Ca^{2+} channels at frog motor nerve terminals in regular patterns corresponding to AZPs, precisely match fluorescent labeled ACh receptors on the post-synaptic membrane (stained with α-bungarotoxin - a high affinity ligand for the muscle type nicotinic receptor) in a manner consistent with ultrastructural findings. (Robitaille *et al.*, 1990). Furthermore, imaging of fluorescently-labelled Ca^{2+} ions in conjunction with carbon-fiber electrode techniques to map spatially 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). On the other hand, catecholamine release rarely occured at sites away from these "hotspots." These findings provide further support for the the close proximity of VDCC and transmitter release sites.

Tissue biopsies from patients with LEMS displayed a paucity and disorganization of AZPs and active zones, and clustering of AZPs in comparison to control tissues (Fukunaga, 1982). Similar findings occurred at motor nerve terminals of mice following chronic injection

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of IgG from patients with LEMS (Fukuoka *et al.*, 1987b). 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.

c. Radioligand binding

Other findings have also provided further evidence that VDCC are eventually lost in LEMS. Incubation of IMR cells for 24 hours with LEMS antibodies decreases binding of ^{125}I-ω-CgTx GVIA (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 are eliminated from the plasma membrane. However, multiple studies have shown that LEMS antibodies and ^{125}I-ω-CgTx GVIA bind to discrete sites on VDCC, thus providing evidence that LEMS antibodies induce the eventual loss of VDCC (as described below).

d. Antigenic 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.*, 1987b). Furthermore, the decrease in distance between AZPs is consistent with the width of binding regions on the IgG molecule (Fukuoka *et al.*, 1987a). These findings suggest that modulation of AZPs, and presumably VDCC, occurs by cross linking by LEMS IgG (Engel *et al.*, 1987; Fukuoka *et al.*, 1987a). This idea was tested by exposing motor nerve terminals for 24 hours 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). In light of these results, antigenic cross-linking appears to be a necessary prerequisite for functional loss of VDCC in LEMS.

e. Direct block of voltage-dependent calcium 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 rapidly (Martin-Moutot *et al.*, 1995a) 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 minutes (Meyer *et al.*, 1986) or 1 hour (Hewett and Atchison, 1991, 1992a). Similarly, exposure of SCLC or BAC cells in culture for 90 minutes or 30 minutes, respectively, to LEMS antibodies reduces voltage-dependent Ca^{2+} entry, which becomes more pronounced after 24 hours (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 hours does not consistently affect nerve-evoked release of ACh (Prior and Newsom-Davis, 1985; Kim *et al.*, 1988). Similarly, the $t_{1/2}$ of reduction in release of ACh following a single *i.v.* injection of antibodies from one patient with LEMS into mice was observed to take approximately 4-6 hours (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 have 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 VDCC elimination and thus, loss of function in part is most likely the result of direct block of the channel, which may be a first step prior to cross linking.

f. Role of complement

In addition to antibody-induced antigenic modulation, other immune components could be involved in LEMS. Complement has been observed to participate in the pathogenesis of other neuromuscular disorders. As such, complement components have been found localized at the end-plate regions of patients with myasthenia gravis and in myasthenia gravis animal model systems. 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 (Fig. 1.7.). Although both pathways share common components, they are activated differently (Goetz, 1987; Loos, 1987; Benjamini *et al.*, 1996). Whereas the classical pathway is activated by the formation of antibody and antigen complexes, 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 C3a and C3b, which are vital components that enhance

phagocytosis, immune adherence, and degranulation of mast cells and basophils (Hostetter *et al.*, 1984). C3b acts a 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 (Prior and Newsom-Davis, 1985; Lambert and Lennon, 1988). Chronic injection of LEMS antibodies into mice deficient in C5 and C3 and mice with intact complement systems produced similar impairment of nerve-evoked release of ACh from motor nerve terminals. Also, muscle biopsies of patients with LEMS examined using immuno-electron microscopy does 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 hour is dependent upon C3, but not C5 (Hewett and Atchison, 1992b). In addition, factor B was also 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

are increased in the presence of complement (Benjamini *et al.*, 1996).

H. ***Voltage-dependent calcium channels and neurological dysfunction***

Alterations in functional VDCC have been implicated in not only LEMS, but other neurological disorders as well. 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 disorders result from different mutations of the same gene, the neurologic features 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 (Herrup and Wilczynski, 1982; Doyle *et al.*, 1997; Dove *et al.*, 1998).

Similarly, FHM presents in childhood, but represents a form of migraine that is accompanied with an aura and includes hemiparesis, aphasia, or hemianopia. Symptoms and signs can last a few hours to days, and identical attacks are described in first-degree relative(s). The disorder is due to missense mutations in the gene encoding the α_{1A} subunit (the CACNL1A4 gene found on chromosome19p13.1). Similar mutations also occur in mice, producing the *tottering* phenotype (Fletcher *et al.*, 1996; Doyle *et al.*, 1997). 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 seizues. This phenotype is known as the *rolling* mouse *Nagoya* (Mori *et al.*, 2000).

SCA6 usually begins in late adulthood and causes nystagmus, ataxia, dysarthria, and impaired vibration and position sensation. It is associated isolated with atrophy of the cerebellum, and it 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. For example, 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).

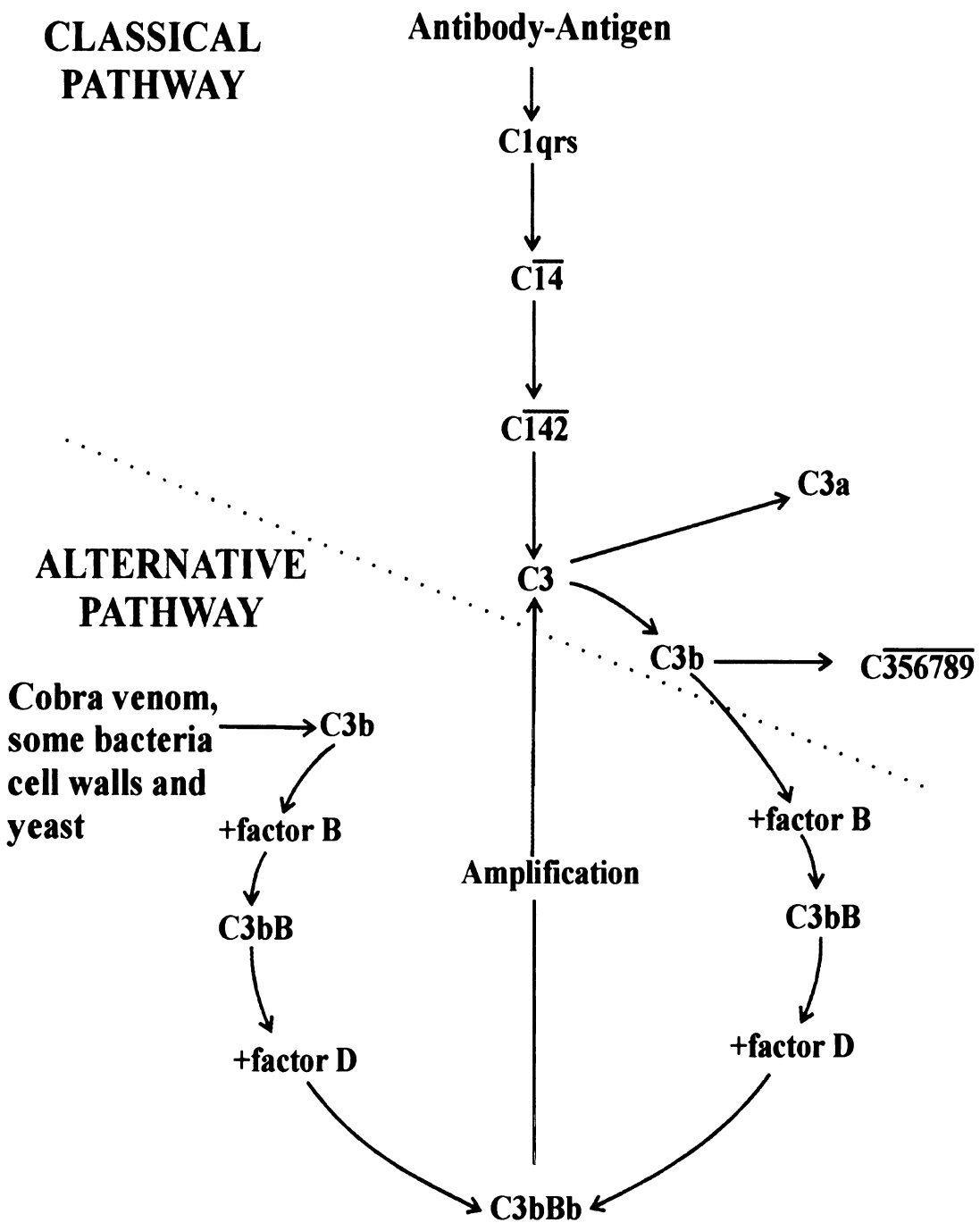

Figure 1.7. Relationship of the alternative and classical complement pathways. Adapted from Benjamini *et al.* 1996.

CHAPTER TWO

LAMBERT-EATON MYASTHENIC SYNDROME IN MICE INDUCES DIHYDROPYRIDINE SENSITIVITY OF SYNAPTIC TRANSMISSION

A. *Summary*

Lambert-Eaton Myasthenic Syndrome (LEMS) is a paraneoplastic disorder in which autoantibodies apparently target the voltage-gated Ca^{2+} channels that regulate acetylcholine (ACh) release at motor nerve terminals. P/Q-type Ca^{2+} channels are primarily involved in ACh release at mammalian neuromuscular junctions. Passive transfer of LEMS to mice by repeated administration of plasma from LEMS patients reduces the amplitude of the perineurial P/Q-type current, and unmasks a dihydropyridine (DHP)-sensitive L-type Ca^{2+} current at the motor nerve terminal. The present study sought to determine if this DHP-sensitive component contributes to ACh release. Mice were injected for 30 days with plasma from healthy human controls or patients with LEMS. For some studies, diaphragms from naïve mice were incubated with LEMS or control human plasma for 2 or 24 hr. End-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) were recorded from neuromuscular junctions of the hemidiaphragm. Injection of mice with LEMS plasma caused the characteristic electrophysiological signs of LEMS: reduced quantal content and facilitation of EPP amplitudes at high frequency stimulation. Quantal content was also reduced in muscles incubated acutely with LEMS plasma. Nimodipine, a DHP-type antagonist of L-type Ca^{2+} channels did not significantly affect quantal content of muscles incubated for 2 or 24 hr with either control or LEMS plasma or following chronic injection with control plasma. However, following 30 days injection with LEMS plasma, nimodipine significantly reduced the remaining quantal content to 57.7 ± 3.3 % of pre-nimodipine control. Thus, DHP-sensitive Ca^{2+} channels become involved in synaptic transmission at the mouse neuromuscular junction after chronic but not acute exposure to LEMS plasma. However, reductions in quantal release of ACh occur even after very short

periods of exposure to LEMS plasma. As such, development of L-type Ca^{2+} channel contribution to ACh release during passive transfer of LEMS appears to occur only after quantal release is significantly impaired for an extended duration, suggesting that an adaptive response of the ACh release apparatus occurs in LEMS.

B. *Introduction*

Lambert-Eaton Myasthenic Syndrome (LEMS) is a paraneoplastic disorder in which nerve-evoked, Ca^{2+}-dependent release of acetylcholine (ACh) from the presynaptic nerve terminal is impaired (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971). Patients with LEMS typically exhibit peripheral limb muscle weakness and fatigability, decreased reflexes, as well as various dysautonomias (Heath *et al.*, 1988; O'Neill *et al.*, 1988; Khurana *et al.*, 1988). LEMS often is associated with a small cell lung carcinoma (Lennon *et al.*, 1982). The underlying cause of LEMS is not fully understood, but the disease is thought to result from generation of antibodies against the tumor. Passive transfer of LEMS to rodents by chronic injection of plasma, serum or immunoglobulins faithfully duplicates the hallmark electrophysiological signs of LEMS- namely reduced quantal content, and facilitation of end-plate potential (EPP) amplitudes during repetitive stimulation (Lang *et al.*, 1983, 1987; Kim, 1985; Prior *et al.*, 1985).

In LEMS, circulating autoantibodies are thought to target the voltage-gated Ca^{2+} channels involved in release of ACh from the nerve terminal (Lang *et al.*, 1981, 1983; Lambert and Lennon, 1988). In several model systems, Ca^{2+} channel function is diminished after application of LEMS serum or plasma (Kim and Neher, 1988; Hewett and Atchison, 1991; Kim

et al., 1993; Garcia *et al.*, 1996; Garcia and Beam, 1996; Pinto *et al.*, 1998). More directly, in passive transfer models of LEMS, the amplitude of Ca^{2+}currents recorded from nerve terminals of murine neuromuscular preparations is reduced (Smith *et al.*, 1995; Xu *et al.*, 1998). Furthermore, active zone particles, which presumably correspond to Ca^{2+} channels at the nerve terminal, appear to be disorganized and fewer in number at motor nerve terminals in LEMS patients (Fukunaga *et al.*, 1982) or LEMS-injected mice compared with control groups (Fukunaga *et al.*, 1983; Fukuoka *et al.*, 1987). This reduction in the number of Ca^{2+} channels at the nerve terminal is likely responsible for the attenuated quantal content seen in LEMS.

Among the multiple types of voltage-gated Ca^{2+} channels that exist; several including the L-, N-, and P/Q-types have been shown to control neurotransmitter release; multiple subtypes often coexist at the same synapse to regulate transmitter release (Turner and Dunlap, 1995). At the mammalian neuromuscular junction the P/Q-type of Ca^{2+} channel is primarily involved in release of ACh (Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1996; 1997). L-type Ca^{2+} channels, which participate in release of hormones (Lemos and Nowycky, 1989) and noradrenaline from chromaffin cells of the adrenal medulla (Owen *et al.*, 1989), and N-type channels, which control ACh release at non-mammalian neuromuscular junctions (Sano *et al.*, 1987), do not appear to be involved normally in the nerve-stimulated release of ACh from mammalian motor nerve terminals (Atchison and O'Leary, 1987; Atchison, 1989; Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1996; 1997). Whereas N-type channels have been shown to be affected by LEMS serum (Peers *et al.*, 1990; Suenaga *et al.*, 1996), several recent studies suggest that following exposure to LEMS sera, function of L-type Ca^{2+} channels is spared (Garcia and Beam, 1996) or unmasked (Smith *et al.*, 1995; Xu *et al.*, 1998). Specifically,

injection of mice for 30 days with plasma from LEMS patients attenuates the amplitude of the P/Q-type Ca^{2+} current and exposes a DHP-sensitive L-type Ca^{2+} current. Inasmuch as induction of DHP-sensitive Ca^{2+} current at murine motor nerve terminals might represent an adaptive response to reduction of function of the normal complement of Ca^{2+} channels by LEMS autoimmune attack, the present study was undertaken to determine if this DHP-sensitive component contributes to the release of ACh from motor nerve terminals in the LEMS passive transfer model and whether this component develops in tandem with the onset of LEMS.

C. *Materials and Methods*

Chronic exposure studies. Animal care, handling, and experiments were performed in accordance with local university (Michigan State University Laboratory Animal Resources) and national guidelines. Experiments were performed using male ICR mice (20-22 g, Harlan Sprague-Dawley Laboratories, Madison, WI). Mice were injected once daily intraperitoneally (*i.p.*) for 30 days with 1.5 ml of plasma from patients clinically diagnosed with LEMS or plasma from healthy (control) patients. Prior to injection with plasma, mice were first injected with 300 mg/kg *i.p.* of cyclophosphamide to suppress the immune response to exogenous proteins. After 30 days of plasma injection, animals were sacrificed by decapitation following anesthesia with 80% CO_2 and 20% O_2. The diaphragm muscle with the attached phrenic nerve (Barstad and Liliheil, 1968) was then removed and prepared for electrophysiological recording.

Acute exposure studies. Naïve male ICR mice were sacrificed as described above. After removal of the diaphragm muscle and the attached phrenic nerve, the tissue was incubated for 24 hr at room temperature of 23-25°C in Dulbecco's Minimum Essential Medium with 20%

(v/v) of LEMS or control plasma. The medium was aerated continuously with 95% O_2 and 5% CO_2. The tissue was washed subsequently in buffered saline solution. Alternatively, the tissue was incubated for 2 hr at room temperature in buffered saline solution containing 20% of the appropriate plasma under continual oxygenation (100% O_2). After the specified incubation time the tissue was prepared for electrophysiological recording.

Electrophysiological measurements. Diaphragm preparations were pinned out at resting tension in a Sylgard-coated chamber and perfused continuously with buffered saline solution at a rate of approximately 1-5 ml/min. Tissues that were incubated for only 2 hr were perfused at a similar rate but contained buffered saline solution with 5% (v/v) plasma. In order to prevent muscle contraction following stimulation of the phrenic nerve, the diaphragm muscle was cut approximately 4 mm on either side of the main intramuscular nerve branch (Glavinovic, 1979; Traxinger and Atchison, 1987). This technique does not produce significant changes in the muscle cable properties (Glavinovic, 1979; Lambert *et al.*, 1981) and permits simultaneous recordings of end-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) without the complicating effects of depressing transmitter release with high Mg^{2+}/low Ca^{2+} solutions or inducing postjunctional receptor block with *d*-tubocurarine (Hubbard and Wilson, 1973). All recordings were made at room temperature of 23-25°C using conventional intracellular recording techniques. EPPs and MEPPs were recorded using borosilicate glass microelectrodes (1.0 mm- o.d., WP Instruments, Sarasota, FL) and having resistance of 5-25 MΩ when filled with 3M KCl. The phrenic nerve was stimulated supramaximally using a suction electrode attached to a stimulus isolation unit (Grass SIU, Grass Instruments, Quincy, MA) and stimulator (Grass S88). Recordings were made before and after addition of nimodipine.

Experiments performed in the presence of nimodipine were done in the dark, in order to prevent photo-oxidation of this compound. Signals were amplified using either an Axoclamp-2 (Axon Instruments, Foster City, CA) or WPI 721 (WP Instruments, Sarasota, FL) amplifier and digitized into a computer for inspection using Axoscope 8.0 (Axon Instruments) software and analyzed using MiniAnalysis 4.0 software (Synaptosoft, Decatur, GA). EPP and MEPP amplitudes were standardized to a membrane potential of –50 mV in order to correct for changes in membrane potential driving force (Katz and Thesleff, 1957). Recordings from each preparation were sampled and averaged from at least 5 different endplates before and after addition of nimodipine, yielding an *n* value of 1. Quantal content (*m*) was calculated using the ratio of the mean amplitude of the corrected EPPs to the mean amplitude of the corrected MEPPs (Hubbard *et al.*, 1969).

Solutions and Chemicals. The standard buffered saline solution contained (mM): NaCl 137.5, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 11, HEPES 14, pH adjusted to 7.4 at room temperature with NaOH and kept under continual oxygenation (100% O_2). In order to prevent depolarization-induced nerve conduction block that can occur when K^+ is released from the cut-muscle fibers, 2.5 mM KCl was used throughout the experiments (Hubbard and Wilson, 1973; Glavinovic, 1979; Traxinger and Atchison, 1987). Nimodipine was prepared as a 10 mM stock solution in 100% ethanol, which was kept at 4° C until use. The final working solution with nimodipine contained only 0.1% ethanol (v/v). Plasma samples from patients clinically diagnosed as having LEMS were graciously provided by Drs. Eva L. Feldman and James Albers (University of Michigan Health System, Ann Arbor, MI, USA), Dr. Andrew Massey (University of Kentucky Medical Center, Lexington, KY), and Dr. Shin Oh (University of Alabama Medical

Center, Birmingham, AL). Plasma was supplied with no identifiers and only patient age and gender were provided. Plasma was obtained during the course of routine plasma exchange therapy with the normal informed consent and in accordance with the respective institutions' human subject committee approval. Control human plasma was donated by healthy volunteers and obtained as outdated blood donations from the American Red Cross (Lansing, MI). Nimodipine, cyclophosphamide, and *N*-2-hydroxyethylpiperazine-*N*-*2*-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytic grade or better.

Statistical Analysis. Differences in quantal content for both acute and chronic treatment studies in animals receiving LEMS or control plasma were analyzed using a one way analysis of variance followed by Tukey's test. P values were set to < 0.05 for all statistical tests.

D. *Results*

Effect of LEMS plasma on neuromuscular transmission at low frequency stimulation. The duration of exposure to control plasma did not affect neuromuscular transmission from mouse hemidiaphragm preparations. Table 2.1 indicates that there was no significant difference between the resting membrane potentials, EPP amplitudes, or MEPP amplitudes recorded from neuromuscular preparations of mice injected chronically with control plasma or from preparations obtained from naïve mice and incubated acutely with control plasma for 2 or 24 hr ($P > 0.05$).

Conversely, Figure 2.1 demonstrates that passive transfer of LEMS markedly reduced evoked release of ACh. Quantal content of EPPs (*m*) recorded following 0.5 Hz stimulation of the phrenic nerve from hemidiaphragm preparations obtained from mice injected for 30 days with LEMS plasma was reduced significantly in comparison to mice injected for the same duration with control plasma. Similar levels of reduction in *m* were observed in preparations from mice injected with plasma from each of three LEMS patients. The composite reduction, using values obtained from all patients was 45.1 ± 3.1 % of control (Fig. 2.4).

Acute incubation of naïve murine neuromuscular preparations with LEMS plasma also caused a significant reduction in *m* in comparison to preparations exposed acutely to control plasma. 2 hr incubation of diaphragm preparations with LEMS plasma reduced *m* to 61.4 ± 5.3 % of control for the composite of three patients (Fig. 2.4). This composite reduction in *m* for all three patients was significantly different from that observed in neuromuscular preparations from mice injected chronically with LEMS plasma as well as from preparations exposed acutely to control plasma (Fig. 2.4). Plasma from only two of the individual LEMS patients significantly reduced *m* compared to preparations incubated with control plasma when applied for 2 hr (Fig. 2.3). Further incubation of naïve murine neuromuscular preparations with LEMS plasma (24 hr) also reduced *m* to 56.3 ± 5.1 % of 24 hr incubation with control plasma (Fig. 2.4). Plasma from all three of the individual LEMS patients significantly reduced *m* compared to preparations incubated with control plasma when applied for 24 hr (Fig. 2.2). However, the reduction in *m* following 24 hr incubation with LEMS plasma was not significantly different from that of neuromuscular junction preparations from mice injected chronically with LEMS plasma or from naïve murine neuromuscular preparations incubated with LEMS plasma for 2

hr (Fig. 2.4).

None of the three LEMS exposure paradigms (30 days, 2 hr, and 24 hr) significantly affected MEPP amplitudes or muscle resting membrane potentials ($P > 0.05$- results not shown).

Effect of LEMS plasma on neuromuscular transmission at high frequency stimulation. In addition to a reduced *m* at low frequency stimulation, another electrophysiological characteristic of LEMS is an increase in the amplitude of EPPs, in comparison to that of the first EPP, evoked during high frequency stimulation of the nerve (Lang *et al.*, 1983, 1987; Kim, 1985; Prior *et al.*, 1985). 40 Hz stimulation of the phrenic nerve caused an overall reduction of EPP amplitudes from all three control exposure protocols (Figs. 2.5, 2.6, and 2.7). On the other hand, LEMS plasma exposure in the 30 day injection, as well as the 2 hr, and 24 hr incubation paradigms each caused facilitation of the EPP amplitude in comparison to the initial EPP amplitude (Figs. 2.5, 2.6, and 2.7).

The involvement of DHP-sensitive Ca^{2+} channels in neuromuscular transmission from motor nerve terminals treated with LEMS plasma. Normally, DHP-sensitive L-type Ca^{2+} channels are not involved in the nerve-stimulated release of ACh from adult mammalian motor nerve terminals (Atchison and O'Leary, 1987; Atchison, 1989; Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1996; 1997). As shown in Figures 2.8, 2.9, and 2.11 the nerve-stimulated release of ACh recorded from diaphragm preparations of mice injected chronically with control plasma or from preparations incubated acutely with control plasma was not sensitive to nimodipine. In addition, quantal content from hemidiaphragm preparations incubated for 24 or 2 hr with LEMS plasma was not altered in the presence of nimodipine (Figs. 2.8 and 2.9).

Table 2.1.

Muscle resting membrane potential (RMP), MEPP amplitude, and EPP amplitude from neuromuscular junction preparations exposed for 2 hr, 24 hr, or 30 days to control plasma

Control Treatment	RMP*,# (mV)	MEPP Amplitude (mV)	EPP Amplitude (mV)
30 days	-41.4 ± 1.9	0.46 ± 0.04	9.9 ± 1.2
24 hr	-43.7 ± 1.7	0.42 ± 0.05	9.6 ± 0.84
2 hr	-42.9 ± 2.1	0.43 ± 0.05	9.3 ± 0.92

#Values are the mean ± SEM of at least 4 preparations of each control exposure.

*Preparations were "cut" to prevent muscle contraction, resulting in depolarized preparations due to the release of K^+ from cut fibers. Extracellular [K^+] was reduced to 2.5 μM; an equiosmolar increase in [NaCl] was made.

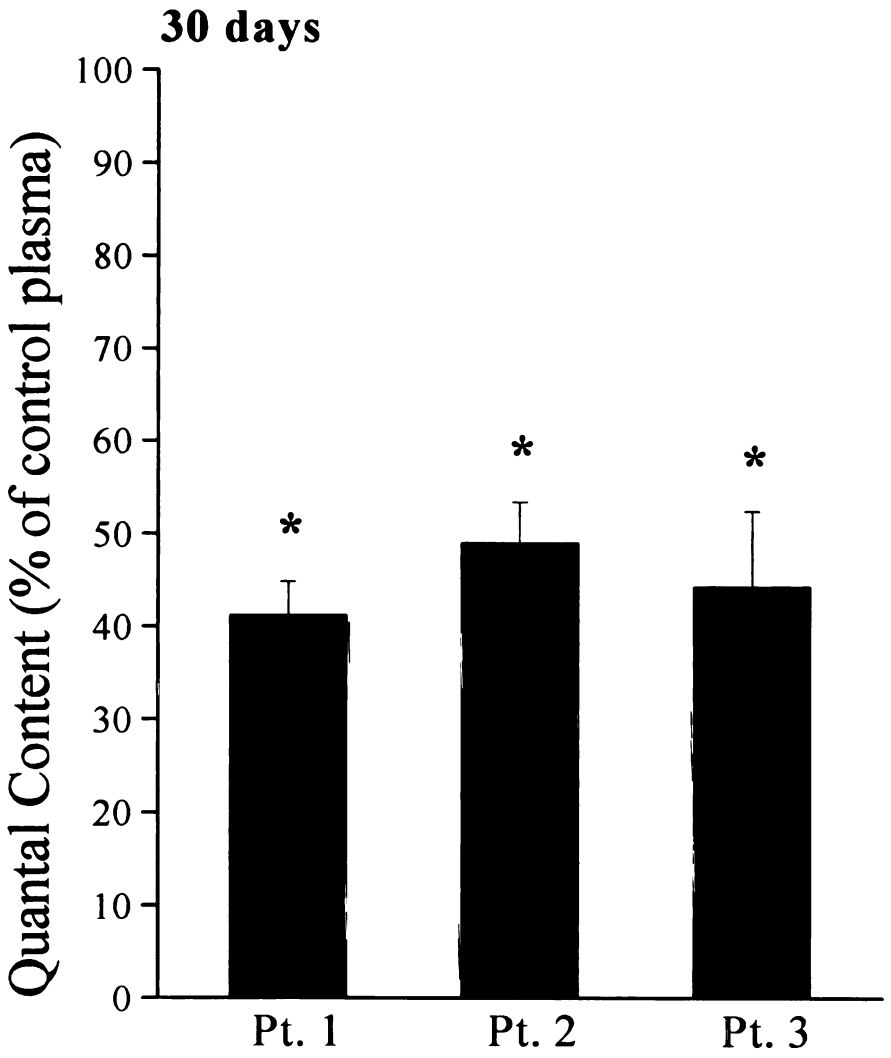

Figure 2.1. Effect of exposure to LEMS plasma for 30 days on quantal content of mouse hemidiaphragm endplates. Hemidiaphragm preparations were taken from immunosuppressed mice injected for 30 days with 1.5 ml/day (*i.p.*). MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-exposed preparations to that of control plasma-exposed preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control ($P < 0.05$).

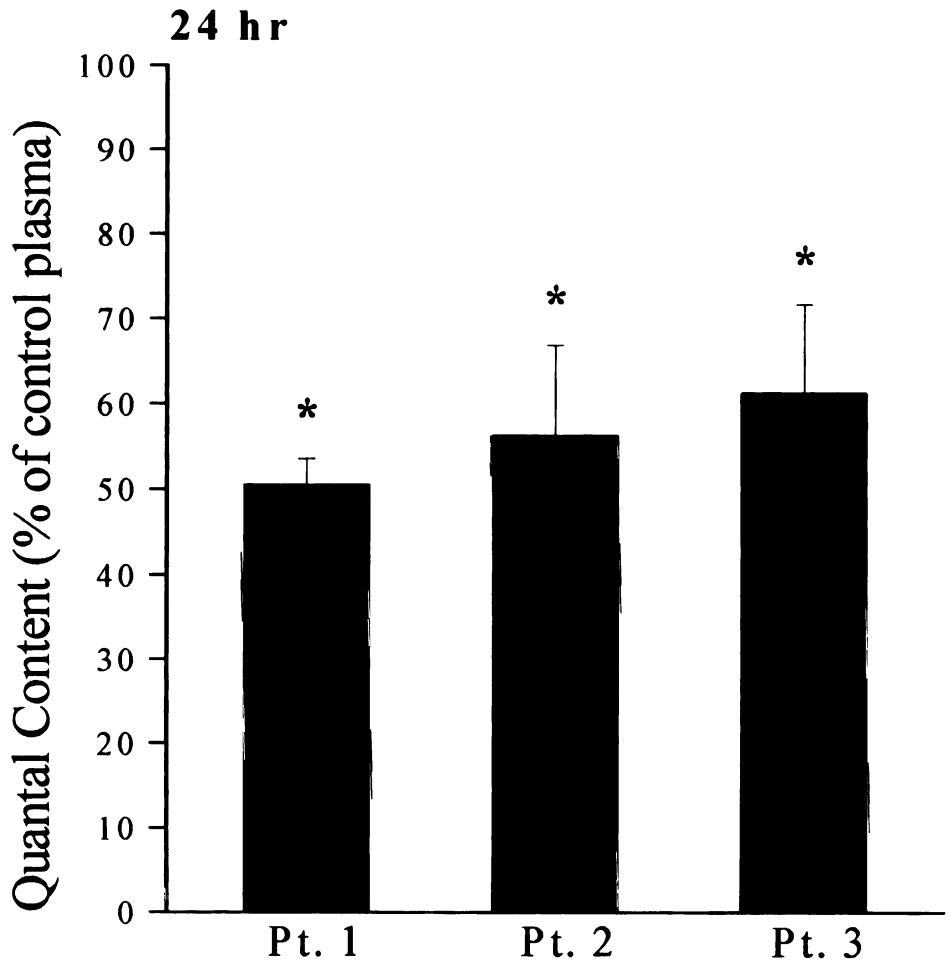

Figure 2.2. Effect of incubation with LEMS plasma for 24 hr on quantal content of mouse hemidiaphragm endplates. Hemidiaphragm preparations were taken from naïve mice and incubated for 24 hr with plasma from one of three LEMS patients (Pt.). MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-incubated preparations to that of control plasma-incubated preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control ($P < 0.05$).

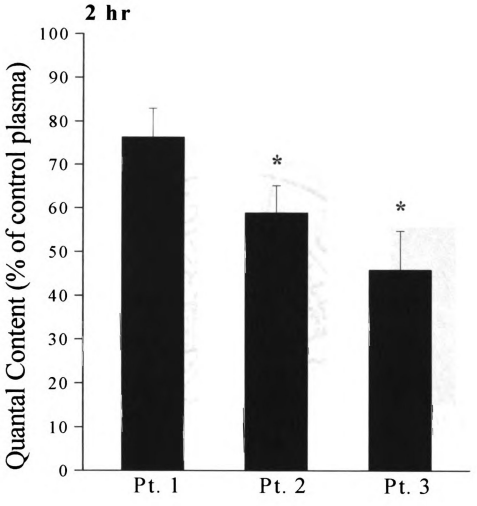

Figure 2.3. Effect of incubation with LEMS plasma for 2 hr on quantal content of mouse hemidiaphragm endplates. Hemidiaphragm preparations were taken from naïve mice and incubated for 2 hr with plasma from one of three LEMS patients (Pt.). MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-incubated preparations to that of control plasma-incubated preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control ($P < 0.05$).

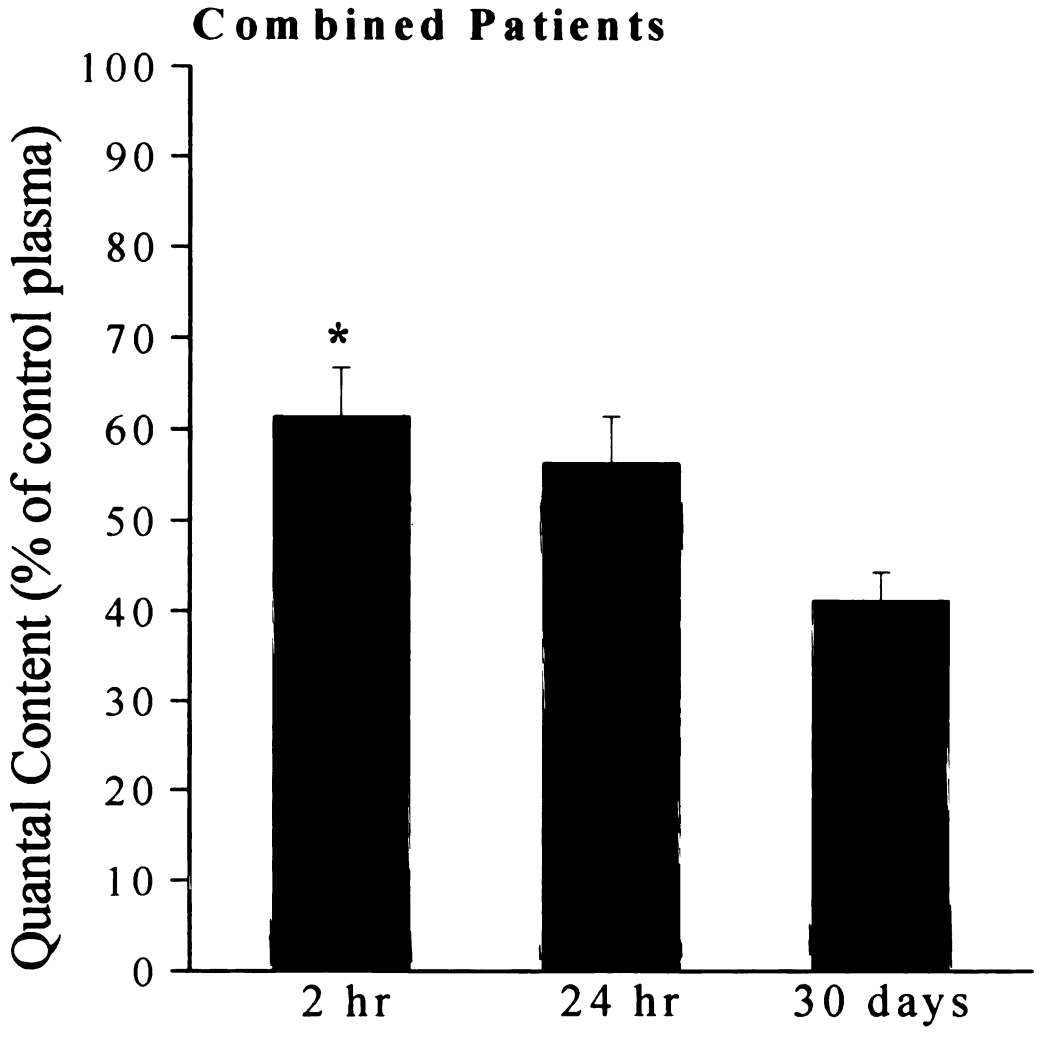

Figure 2.4. Effect of exposure to LEMS plasma on quantal content of mouse hemidiaphragm endplates. Hemidiaphragm preparations were taken from immunosuppressed mice injected for 30 days with 1.5 ml/day (*i.p.*) or from naïve mice and incubated for 24 hr or 2 hr with plasma from one of three LEMS patients. Panel represents the combined patients results for all 3 following 2 hr, 24 hr, and 30 days of exposure to LEMS plasma. MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-exposed preparations to that of control plasma-exposed preparations. Each value represents the mean ± SEM of at least 5 different preparations for each patient plasma. The asterisk (*) indicates a value significantly different from 30 days treatment ($P < 0.05$).

However, as shown for sample records in Figure 2.10 and for composite data from all preparations in Figure 2.11 , nerve-stimulated release of ACh from motor nerve terminals of mice injected chronically with LEMS plasma was sensitive to nimodipine. As shown in Figiure 2.11, *m* recorded from preparations obtained from mice injected chronically with LEMS plasma was reduced in the presence of nimodipine to 57.7 ± 3.3 % of their nimodipine-free ("control") value. Plasma from all three LEMS patients induced this nimodipine sensitivity of neuromuscular transmission.

E. *Discussion*

LEMS is associated with the loss of functional voltage-gated Ca^{2+} channels at motor nerve terminals and thus the reduction of the nerve-evoked release of ACh. Normally, P/Q-type, but not L- or N-type Ca^{2+} channels control the nerve-stimulated release of ACh from adult mammalian motor nerve terminals (Atchison and O'Leary, 1987; Atchison, 1989; Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1996; 1997). Passive transfer of LEMS to mice by 30 days of plasma injection attenuates the perineural voltage changes associated with the amplitude of the P/Q-type Ca^{2+} current and induces the appearance of a DHP-sensitive L-type Ca^{2+} current at murine motor nerve terminals (Smith *et al.*, 1995; Xu *et al.*, 1998). The primary objective of the present study, therefore, was to determine if this novel DHP-sensitive L-type Ca^{2+} current now becomes involved in synaptic transmission from adult mammalian motor nerve terminals in LEMS.

Nimodipine, a DHP-sensitive L-type Ca^{2+} channel blocker, had no effect on *m* from mice injected chronically for 30 days with control plasma, nor did it affect *m* in motor nerve terminals

incubated acutely for 2 or 24 hr with either LEMS or control plasma. This finding is consistent with those observed by Xu *et al.* (1998), in which the binding characteristics of [^{3}H]-nitrendipine to rat synaptosomal nerve terminal preparations were not affected by acute application of LEMS IgG. Nimodipine, did however, attenuate *m* from motor nerve terminals isolated from mice injected for 30 days with LEMS plasma. Thus, following chronic injection of mice with LEMS plasma DHP-sensitive L-type Ca^{2+} channels become involved in the nerve-stimulated release of ACh from mammalian nerve terminals.

Although synaptic transmission from neuromuscular preparations isolated from mice and incubated acutely with LEMS plasma for 2 and 24 hr was not sensitive to nimodipine, *m* was reduced in comparison to hemidiaphragm preparations incubated with control plasma. In addition, facilitation of EPP amplitudes occurred in neuromuscular preparations incubated for 2 or 24 hr with LEMS plasma. The observation that *m* is reduced after a brief exposure to LEMS plasma is consistent with the findings of Hewett and Atchison (1991), in which uptake of $^{45}Ca^{2+}$ uptake into rat forebrain synaptosomal preparations during KCl-induced depolarization is reduced after acute exposure to LEMS IgG. However, this is the first study that we are aware of in which a consistent reduction in *m* is observed from neuromuscular preparations exposed for such a short duration (2 hr) to LEMS plasma. This finding may be due to the nature of our treatment paradigm, which consisted of a high concentration of plasma (20%) during the acute incubation phase and the continual presence of a low concentration (0.5%) of plasma during the recording phase.

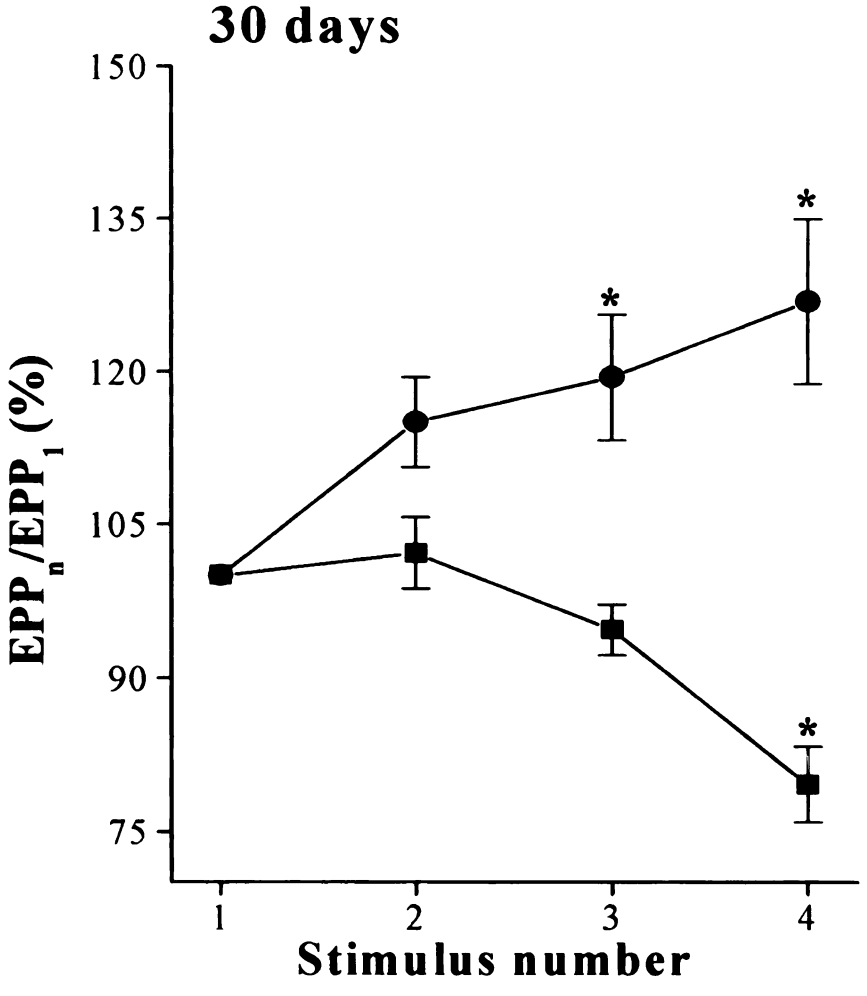

Figure 2.5 Effect of exposure to LEMS plasma for 30 days on facilitation of neuromuscular transmission. EPP amplitudes were recorded using standard electrophysiological techniques from hemidiaphragm preparations taken from mice injected for 30 days with 1.5 ml/day (*i.p.*) of LEMS or control plasma. EPPs were elicited by stimulation of the phrenic nerve at 40 Hz. The symbols, —●— and —■— represent LEMS and control plasma-injected groups, respectively. Values are expressed as the mean ratio ± SEM of the amplitude of EPPn to that of the first EPP elicited in the train (n represents the stimulus number). The asterisk (*) indicates a value significantly different from that of the first EPP ($P < 0.05$).

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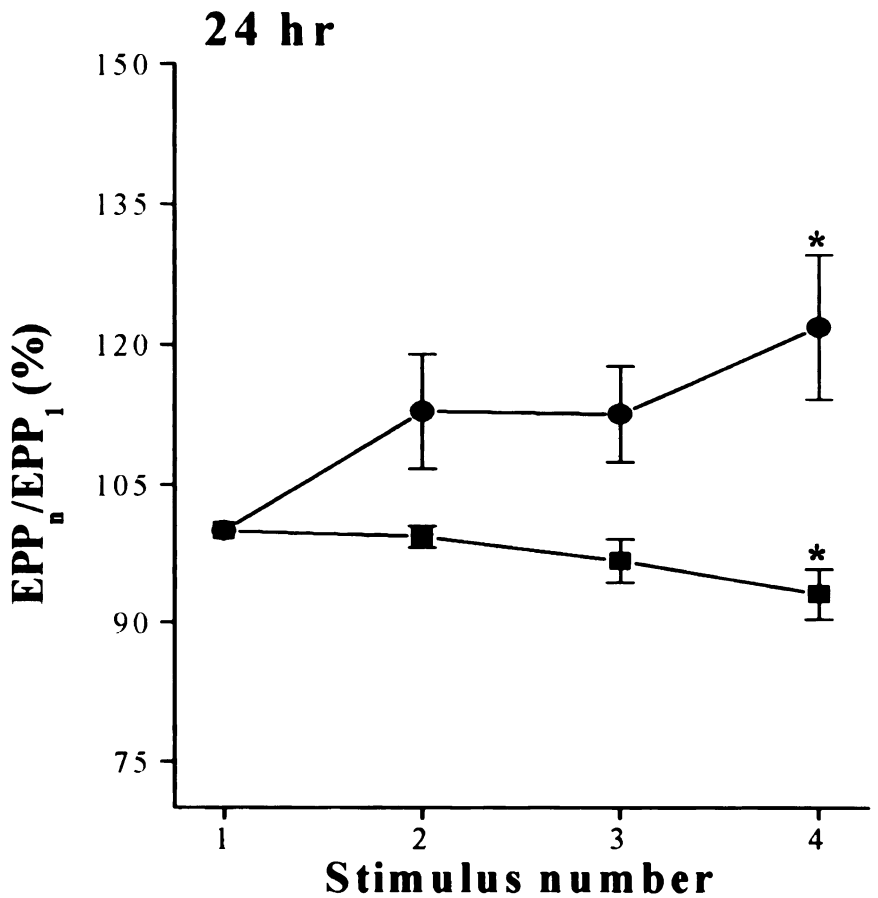

Figure 2.6. Effect of exposure to LEMS plasma for 24 hr on facilitation of neuromuscular transmission. EPP amplitudes were recorded using standard electrophysiological techniques from hemidiaphragm preparations taken from naïve mice and incubated for 24 hr with LEMS or control plasma. EPPs were elicited by stimulation of the phrenic nerve at 40 Hz. The symbols, —●— and —■— represent LEMS and control plasma-incubated groups, respectively. Values are expressed as the mean ratio ± SEM of the amplitude of EPPn to that of the first EPP elicited in the train (n represents the stimulus number). The asterisk (*) indicates a value significantly different from that of the first EPP ($P < 0.05$).

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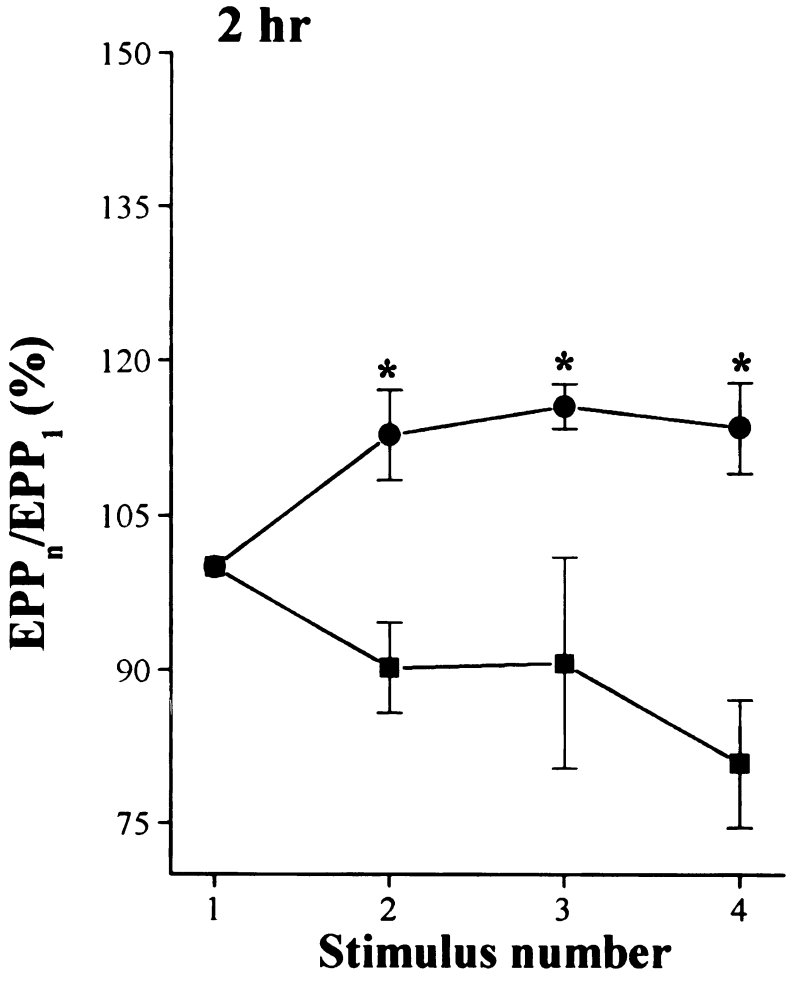

Figure 2.7. Effect of exposure to LEMS plasma for 2 hr on facilitation of neuromuscular transmission. EPP amplitudes were recorded using standard electrophysiological techniques from hemidiaphragm preparations taken from naïve mice and incubated for 2 hr with LEMS or control plasma. EPPs were elicited by stimulation of the phrenic nerve at 40 Hz. The symbols, —●— and —■— represent LEMS and control plasma-incubated groups, respectively. Values are expressed as the mean ratio ± SEM of the amplitude of EPPn to that of the first EPP elicited in the train (n represents the stimulus number). The asterisk (*) indicates a value significantly different from that of the first EPP ($P < 0.05$).

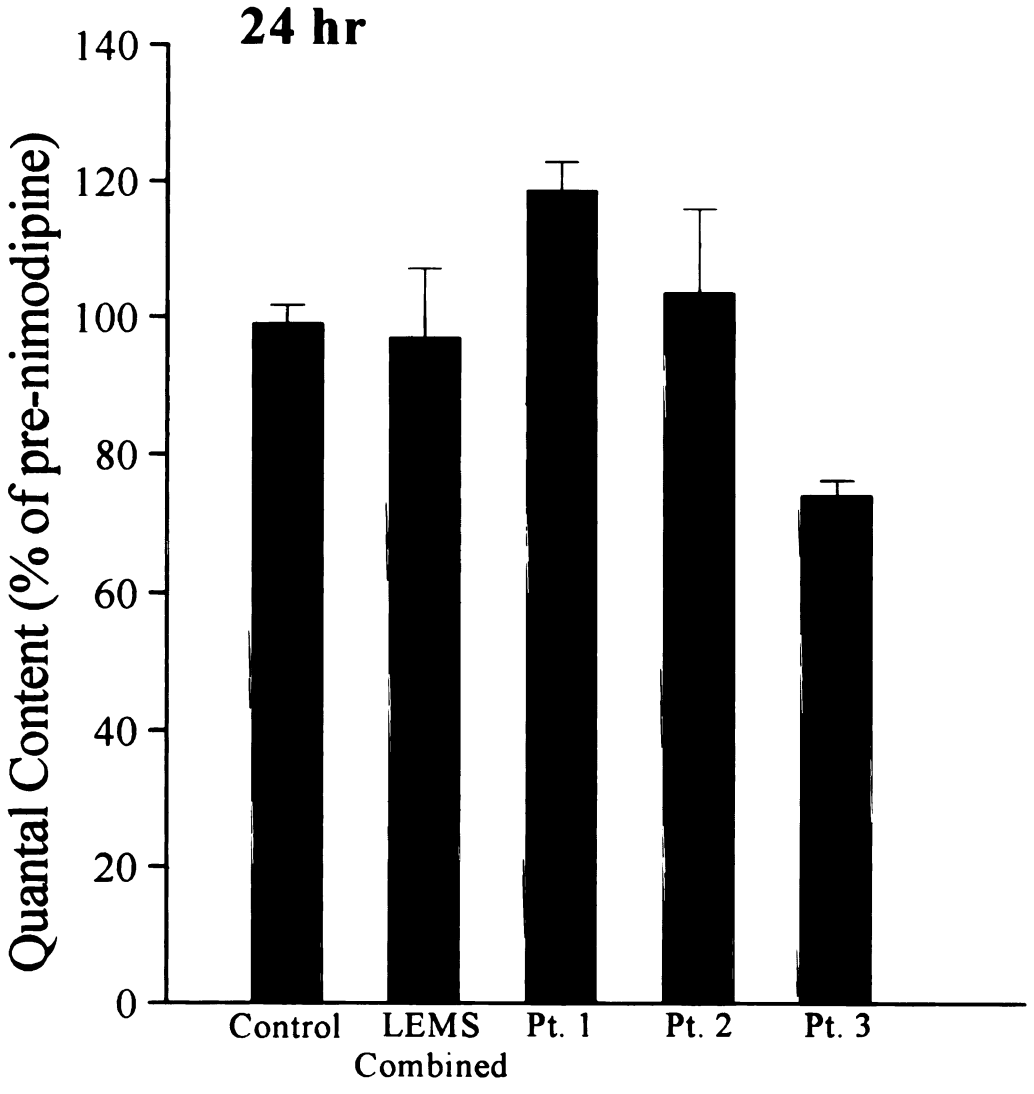

Figure 2.8. Effect of nimodipine on quantal content of mouse hemidiaphragm preparations exposed to LEMS plasma for 24 hr. Hemidiaphragm preparations were taken from naïve mice and incubated continuously for 24 hr with plasma from one of three LEMS patients (Pt.) or control plasma. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine. Each value represents the mean ± SEM of at least 5 different preparations.

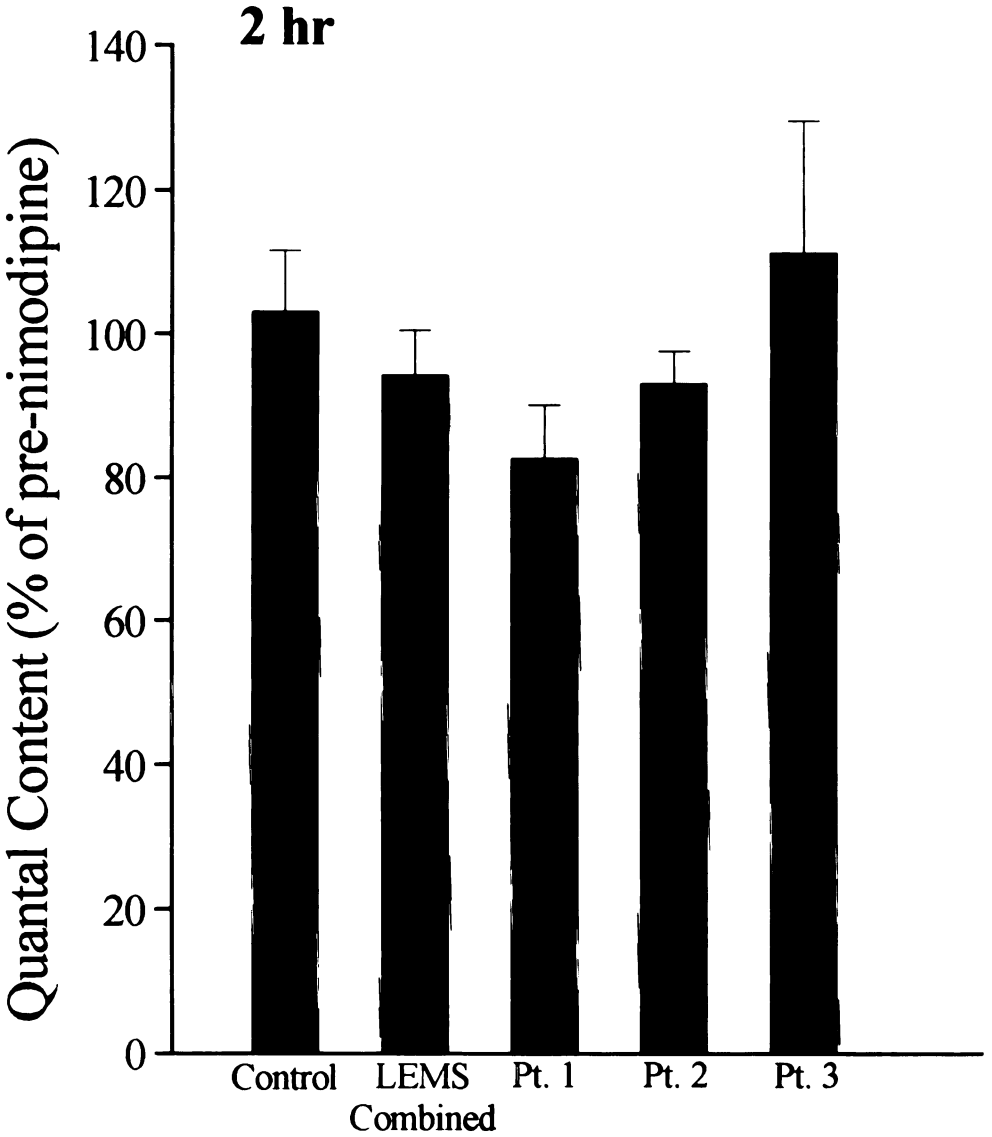

Figure 2.9. Effect of nimodipine on quantal content of mouse hemidiaphragm preparations exposed to LEMS plasma for 2 hr. Hemidiaphragm preparations were taken from naïve mice and incubated continuously for 2 hr with plasma from one of three LEMS patients (Pt.) or control plasma. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine. Each value represents the mean ± SEM of at least 5 different preparations.

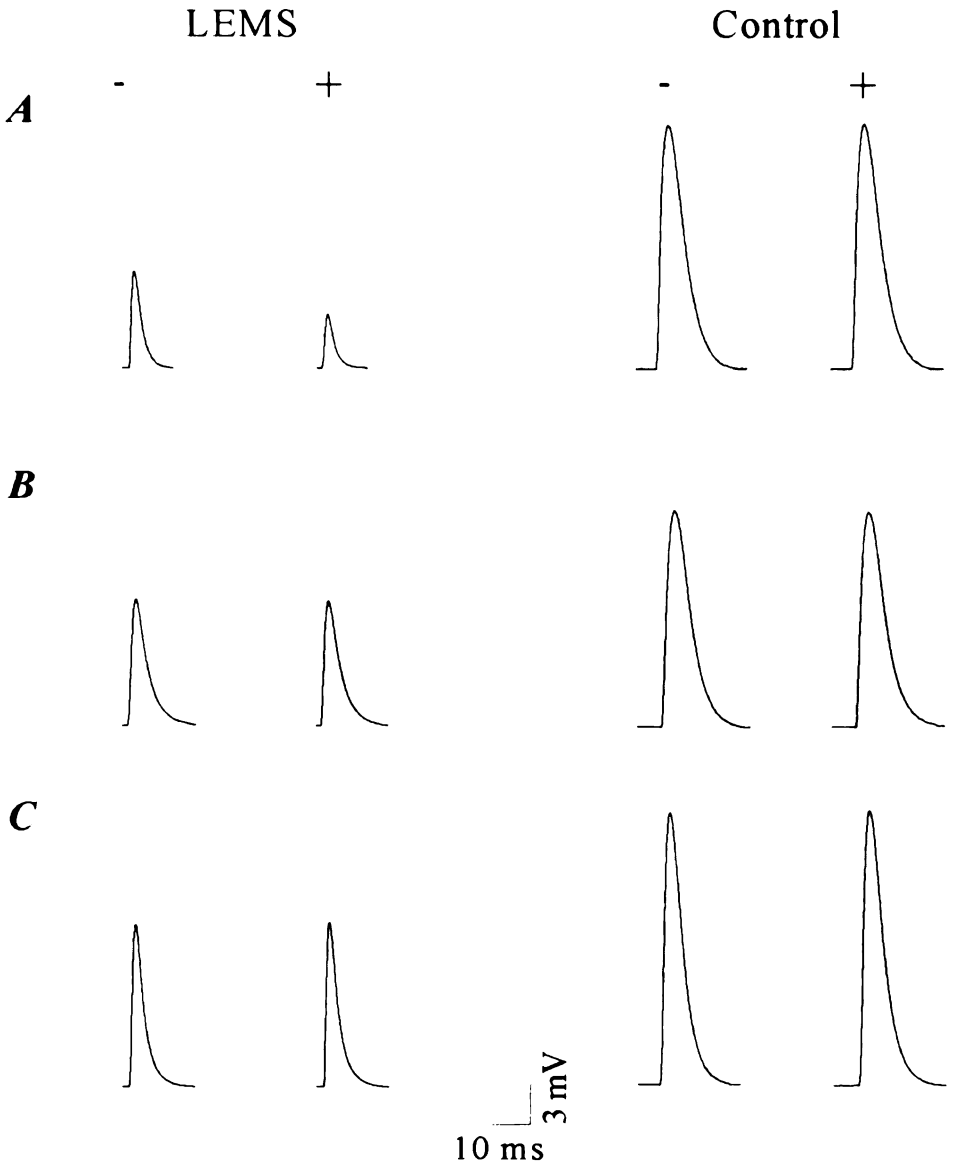

Figure 2.10. Effect of nimodipine on EPPs from LEMS and control plasma-exposed neuromuscular junction preparations. EPPs were recorded from *A*, neuromuscular junction preparations isolated from mice injected for 30 days, *B*, neuromuscular preparations isolated from naïve mice and incubated for 24 hr or *C*, neuromuscular preparations isolated from naïve mice and incubated for 2 hr with LEMS or control plasma. Recordings from the same site are depicted before (-) and after (+) application of 10 μM nimodipine. Each tracing represents the average of at least 50 EPPs at a stimulation frequency of 0.5 Hz recorded from a single representative preparation.

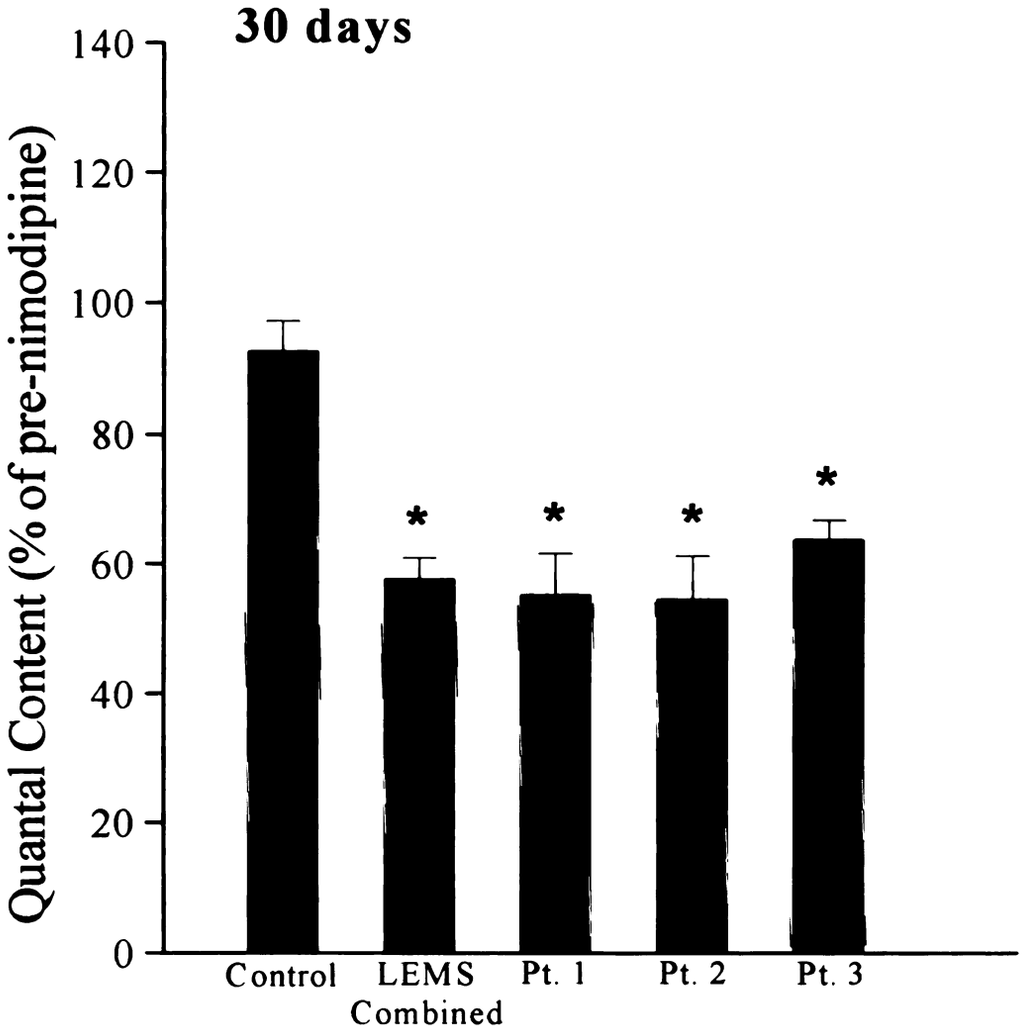

Figure 2.11. Effect of nimodipine on quantal content of mouse hemidiaphragm preparations exposed to LEMS plasma for 30 days. Hemidiaphragm preparations were taken from mice injected for 30 days with 1.5 ml/day (*i.p.*) with control plasma or plasma from one of three LEMS patients (Pt.). EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from pre-nimodipine($P < 0.05$).

Chronic passive transfer of LEMS to mice caused the typical clinical electrophysiological features seen in LEMS patients and reported in other studies: *m* was reduced in comparison to control groups and facilitation of the EPP amplitudes occurred at high frequency stimulation (Lang *et al.*, 1983, 1987; Kim, 1985; Prior *et al.*, 1985). The reduction in *m* recorded from neuromuscular preparations obtained from mice injected chronically with LEMS plasma was significantly greater than that seen in neuromuscular preparations following 2 hr LEMS incubation paradigms. This difference most likely reflects the time course required to induce LEMS after passive transfer to mice. As shown by Prior *et al.* (1985), passive transfer of LEMS to mice by daily injections of LEMS IgG does not induce a maximum reduction in *m* until approximately 10 days, and the half-maximal reduction in *m* occurs after 1.5 days of treatment.

Involvement of the DHP-sensitive L-type channels in synaptic transmission from mammalian motor nerve terminals following chronic LEMS exposure may reflect recruitment of normally silent L-type channels which are already present at the terminal. Addition of the DHP agonist, Bay K 8644 to mammalian neuromuscular preparations increases *m* (Atchison and O'Leary, 1987; Atchison, 1989) and L-type channels have been shown to be involved in the spontaneous release of ACh from mammalian motor nerve terminals in the presence of physiological concentrations of extracellular KCl (Losavio and Muchnick, 1997). In addition, pretreatment of mouse neuromuscular preparations with the intracellular Ca^{2+} buffer, DM-BAPTA-AM unmasks a nitrendipine-sensitive perineural Ca^{2+} current (Urbano and Uchitel, 1999). However, this latter study did not find evidence for involvement of this unmasked L-type channel in ACh release. This discrepancy may be due to the spatial localization of the L-

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type channels at the nerve terminal. If L-type channels are not in close proximity to the active zone release machinery (see Robitaille *et al.*, 1990) then most likely the influx of Ca^{2+} through the L-type channels would have to occur in a more diffuse manner than that observed with Ca^{2+} channels clustered about the release sites as is postulated to occur normally (Llinas *et al.*, 1992). DM-BAPTA-AM could therefore, buffer the Ca^{2+} entering through L-type channels before it reached the release apparatus.

One possible explanation for the unmasking of silent DHP-sensitive L-type channels seen after chronic LEMS-plasma exposure may be a reduction in the Ca^{2+}-activated-potassium (K_{Ca}) current at the motor nerve terminal. K_{Ca} channels are believed to be located in close proximity to the Ca^{2+} channels involved in transmitter release from motor nerve terminals (Robitaille and Charlton, 1992; Robitaille *et al.*, 1993; Xu and Atchison, 1996; Protti and Uchitel, 1997). These K_{Ca} channels respond to Ca^{2+} influx through Ca^{2+} channels localized at active zones (N-type in frog and presumably, P/Q-type in mice and rats) and may normally limit the duration and extent to which the nerve terminal remains depolarised in response to an action potential. Loss of Ca^{2+} channels due to LEMS plasma exposure may reduce activation of these K_{Ca} channels, thus prolonging the duration and extent of nerve terminal depolarization. This enhanced nerve terminal depolarization could allow silent L-type channels, which may be located at a site distant from the ACh release machinery to remain open long enough to become involved in synaptic transmission (Smith *et al.*, 1995). The DHP-sensitivity seen after chronic, but not acute LEMS exposure could reflect the difference in the extent to which *m* is reduced among the different experimental paradigms. Unlike 2 hr LEMS incubation, chronic injection of mice with LEMS plasma may reduce Ca^{2+} influx at the nerve terminal enough to decrease

the K_{Ca} current, which in turn could unmask silent L-type channels. Although, there was no significant difference in the reduction of *m* between chronic injection of mice with LEMS plasma and 24 hr LEMS incubation, this finding may be misleading. It is likely that the predominant Ca^{2+} channel involved in transmitter release, presumably the P/Q-type in our preparations, may be lost to a greater extent in the chronically exposed LEMS mice than following 24 hr LEMS incubation because *m* in the 30 day injection regimen was reduced by ~40-45 % by nimodipine, whereas at 24 hr, 0% of the quantal release was nimodipine sensitive. Thus, the apparent similar reduction in *m* may occur because L-type Ca^{2+} channels now comprise a significant portion of the nerve-evoked release of ACh in the chronically exposed LEMS preparations. The possible distinct location of L-type Ca^{2+} channels from the normal release sites may preclude involvement of these Ca^{2+} channels in activation of K_{Ca} channels. Thus, at the motor nerve terminals of mice injected chronically with LEMS plasma, activation of K_{Ca} channels may be attenuated in comparison to motor nerve terminals exposed to LEMS plasma for only 24 hr.

Alternatively, the ability of chronic LEMS exposure to induce the involvement of L-type channels in synaptic transmission may reflect more than simple unmasking of channels already present. This is supported by the inability of acute LEMS-plasma incubation to induce DHP-sensitivity of transmitter release despite reducing *m*. For example, processes that take a longer time, such as synthesis of new mRNA and then new channels, may be required. Along these lines, L-type channels become involved in transmitter release at newly forming or regenerating mammalian neuromuscular preparations (Katz *et al.*, 1996; Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2000, 2001). However, morphological data do not support

the notion that LEMS treatment induces degeneration or sprouting of new nerve terminals (Fukunaga *et al.*, 1982, 1983; Fukuoka *et al.*, 1987).

Finally, various studies support the idea that the fast nature of the transmitter release process at motor nerve terminals necessitates a close association between voltage-gated Ca^{2+} channels and transmitter release sites (Robitaille *et al.*, 1990; Sugiura *et al.*, 1995; Seagar and Takahashi, 1998). Perhaps relocalization of L-type channels to active zones may be needed before they become involved in transmitter release (Polo-Parada *et al.*, 2001).

Involvement of L-type Ca^{2+} channels in transmitter release from adult mammalian motor nerve terminals may act as a compensatory mechanism in LEMS to overcome the chronic loss of functional P/Q-type Ca^{2+} channels. Indirect evidence suggests that a similar phenomenon occurs in humans with LEMS. Respiratory failure in a patient with LEMS has been shown to be associated with the administration of verapamil (Krendel and Hopkins, 1986). In addition, the signs of LEMS developed in a patient with ischemic heart disease who received the L-type antagonist, diltiazem and disappeared as the patient's serum levels of diltiazem decreased (Ueno and Hara, 1992). Thus, it is possible that in patients with LEMS L-type Ca^{2+} channels also become involved in synaptic transmission at the neuromuscular junction. As such, these patients might benefit from therapies directed specifically at facilitating current flow through L-type Ca^{2+} channels.

In conclusion, following passive transfer of LEMS by chronic injection, the ACh secretory process assumes a considerable component of L-type Ca^{2+} channel sensitivity. While the mechanism underlying this adaptive change is unclear, it may reflect a compensatory process to sustain transmission in the face of an autoimmune attack at the nerve terminal.

CHAPTER THREE

TIME COURSE OF INVOLVEMENT OF L-TYPE Ca^{2+} CHANNELS IN RELEASE OF ACh DURING PASSIVE TRANSFER OF LEMS TO MICE

A. *Summary*

Lambert-Eaton myasthenic syndrome (LEMS) is a cholinergic nerve disorder, in which evoked-release of ACh is diminished. Antibodies directed against voltage-dependent calcium channels (VDCC) normally involved in ACh release are believed to underlie the pathology of the disease and as such, P/Q-type Ca^{2+} channels, the primary subtype involved in ACh from adult mammalian motor nerve terminals, represent the major antigenic target in LEMS. Chronic injection of sera from patients with LEMS for 30 days into mice not only reduces the amplitude of the P/Q-type current, but unmasks a L-type current that is involved in release of ACh from the motor nerve terminal. The present study sought to further elucidate the time course of development of L-type Ca^{2+} channel involvement in release of ACh. Quantal content from neuromuscular junction preparations obtained from mice injected with plasma for 1-20 days from LEMS patients 1 and 2 was significantly reduced in comparison to control plasma. However, plasma from LEMS patient 3 did not significantly affect quantal content until day 10. Facilitation of end-plate potential (EPP) amplitudes during high frequency stimulation of the nerve occurred from preparations exposed to plasma from patients 1 and 2 at all days tested, but not from controls. In accordance with the effect of plasma from patient 3 on quantal content, facilitation of EPP amplitudes was not observed at day 1 or 5, but occurred by day 10. Nimodipine, a dihydropyridine antagonist, did not affect quantal content from preparations taken from mice injected with control or LEMS patient 3 plasma for 1-20 days or LEMS patients 1 and 2 for 1-15 days. However, the remaining quantal content and facilitation of EPP amplitudes from preparations obtained from mice injected for 20 days with plasma from patients 1 and 2, or just patient 2, respectively were significantly reduced in the presence of

nimodipine. In addition, incubation of preparations exposed to plasma from patient 1 for 20 days with DM-BAPTA-AM prevented the involvement of L-type Ca^{2+} channels in release of ACh, whereas DM-BAPTA-AM did not affect ACh release from control preparations. Although reductions in quantal content can occur in a short-time following exposure to LEMS plasma, it appears to take longer for L-type Ca^{2+} channels to become involved in release. Whatever the exact mechanism that is involved, it appears as though L-type Ca^{2+} involved in release of ACh following exposure to LEMS plasma are not located in close proximity to the normal release apparatus.

B. ***Introduction***

Lambert-Eaton myasthenic syndrome (LEMS) is neuromuscular disorder in which evoked-release of acetylcholine (ACh) from nerve terminals is impaired, causing skeletal muscle weakness, decreased tendon reflexes, and autonomic dysfunction (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971; Heath and Cull, 1988; Khurana and Mayer, 1988; O'Neill and Newsom-Davis, 1988). Several lines of evidence have shown that the defect in neurotransmission is due to circulating antibodies; as such, plasma exchange and use of immunosuppressant medications transiently alleviates many of the symptoms and signs associated with LEMS (Lang and Murray, 1981; Lennon and Fairbanks, 1982; Newsom-Davis and Murray, 1984; O'Neill and Newsom-Davis, 1988). Also, injection of IgG from patients with LEMS into mice replicates the electrophysiological characteristics of the disease--namely, reduced quantal content and facilitation following high frequency nerve stimulation (Lambert

and Elmqvist, 1971; Lang *et al.*, 1983; Kim, 1985; Prior and Newsom-Davis, 1985; Lambert and Lennon, 1988).

The putative target of the antibody associated with LEMS is believed to be the VDCC, normally involved in release of ACh from nerve terminals (Lang and Murray, 1981; Lambert and Lennon, 1988). Ultrastructural studies have shown that active zone particles, which presumably correspond to VDCC are disorganized and fewer in number at nerve terminals exposed to IgG from patients with LEMS compared with nerve terminals exposed to IgG from control patients (Fukunaga, 1982; Fukuoka *et al.*, 1987). While single VDCC kinetics and amplitude are unaffected, entry of Ca^{2+} through VDCC into various cell types treated with LEMS IgG is reduced in comparison to cells treated with control IgG (Kim and Neher, 1988; Kim *et al.*, 1993; Garcia and Beam, 1996). More direct evidence has shown that Ca^{2+} currents at neuromuscular junctions treated with LEMS sera is reduced in comparison to control treatments (Hewett and Atchison, 1991; Smith and Atchison, 1995; Xu and Atchison, 1998). Storage and synthesis of ACh and postsynaptic structures, however, are unaffected at neuromuscular junctions exposed to LEMS sera (Lang and Vincent, 1984). Taken together, the effect of LEMS IgG on reducing nerve-evoked release of ACh appears to occur by decreasing the number of functional VDCC at the nerve terminal.

Entry of Ca^{2+} through VDCC into nerve terminals is a necessary step coupling the action potential to release of transmitter (Llinas *et al.*, 1976; Augustine *et al.*, 1987). Based on pharmacological, biophysical, and molecular characteristics, various VDCC have been identified: P, Q, L, N, T, and R (Nowycky *et al.*, 1985; Tsien *et al.*, 1991; Zhang *et al.*, 1993; Randall and Tsien, 1995). During development, motor nerve terminals have been shown to

possess multiple VDCC subtypes involved in ACh release, some of which become less important in the later stages of development (Sugiura and Ko, 1997; Rosato-Siri and Uchitel, 1999; Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002). Upon maturation, release of ACh from motor nerves primarily involve only one VDCC subtype. As such, P/Q-type VDCC are the main subtype involved in ACh release at mature mammalian motor nerve terminals (Uchitel and Llinas, 1992; Katz, 1995; Protti and Uchitel, 1996). In contrast, release of ACh from motor nerves of amphibians and birds is dependent on Ca^{2+} entry through N-type VDCC (Sano *et al.*, 1987; De Luca *et al.*, 1991; Gray *et al.*, 1992). L-type VDCC, which are often found colocalized on the same nerve terminal with other VDCC subtypes, can participate in the release of catecholamines and hormones (Lemos and Nowycky, 1989; Owen *et al.*, 1989; Turner *et al.*, 1993).

A smaller percentage of patients with LEMS have been shown to possess antibodies directed against multiple VDCC subtypes, however, over 90% of patients have antibodies specifically for the P/Q subtype (Suenaga *et al.*, 1996). In corroboration with these findings many studies have shown a direct action of LEMS IgG on P/Q channels while sparing or unmasking L-type VDCC (Smith and Atchison, 1995; Garcia and Beam, 1996; Xu and Atchison, 1998). Specifically, chronic treatment of mice with plasma from LEMS patients reduces the amplitude of P/Q-type Ca^{2+} currents and exposes a DHP-sensitive L-type Ca^{2+} current, which becomes involved in ACh release at mouse motor nerve terminals (Smith and Atchison, 1995; Xu and Atchison, 1998; Flink and Atchison, 2002; Giovannini *et al.*, 2002). While development of L-type channels may represent a compensatory means to overcome loss of functional VDCC at the nerve terminal, the present study sought to elucidate further the

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time course of development of L-type VDCC involved in ACh following passive transfer of LEMS to mice.

C. *Materials and Methods*

Passive transfer of LEMS. All experiments involving handling and use of animals were done in accordance with local (Michigan State University Animal Resources) and national guidelines. Male ICR mice weighing 20-22g were obtained from Harlan Sprague-Dawley Laboratories (Madison, WI) and given daily intraperitoneal (*i.p.*) injections of 1.5 ml of plasma from patients clinically diagnosed with LEMS or healthy volunteer controls for 1 to 30 days. Mice were initially injected (*i.p.*) with cyclophosphamide (300 mg/kg) in order to prevent an immune response to exogenous plasma proteins. Following the specified number of days as indicated in the results section, animals were anesthetized via inhalation of 80% CO_2 and 20% O_2 and sacrificed by decapitation. The diaphragm and attached phrenic nerves were then isolated as described by Barstad and Lilleheil (1968).

BAPTA loading. Dimethyl BAPTA (1,2-Bis(2-amino-5-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl)ester) was loaded into mouse motor nerve terminals using a modification of procedures described by Urbano and Uchitel (1999). Uncut hemidiaphragm preparations were incubated at room temperature of 23-25°C (v/v) under continual oxygenation (100%O_2) in Ca^{2+}-free physiological saline containing either 25 μM DM-BAPTA-AM or 0.0125 % DMSO (control vehicle). Following 2 hr incubation, the tissue was washed in Ca^{2+}-free buffer for 10 min and then washed in normal buffer solution for 30 min.

Electrophysiology. Neuromuscular transmission was examined using isolated phrenic nerve hemidiaphragm preparations pinned out at resting tension in a Sylgard-coated chamber and perfused at a rate of 1-5 ml/min with physiological saline containing (in mM): NaCl 137.5,

KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 11, HEPES 14, pH adjusted to 7.4 at room temperature of 23-25°C with NaOH and under continual oxygenation (100% O_2). The muscle was cut approximately 4 mm on either side of the main intramuscular nerve branch in order to prevent muscle contraction following stimulation of the phrenic nerve (Glavinovic, 1979; Traxinger and Atchison, 1987). This technique allows simultaneous recordings of end-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) without significant changes in the muscle cable properties (Hubbard and Wilson, 1973) or synaptic transmission. The cut hemidiaphragm preparation was perfused with cold oxygenated physiological saline solution for 30 min; ionic gradients that may have been disrupted by cold temperatures were then restored by perfusing the tissue in oxygenated buffer maintained at room temperature of 23-25°C for an additional 30 min. All experiments were performed using conventional intracellular recording techniques. EPPs and MEPPs were recorded using borosilicate glass microelectrodes (1.0 mm- o.d., FHC, Bowdoinham, ME, USA) and having resistance of 5-20 MΩ when filled with 3M KCl. The phrenic nerve was stimulated supramaximally using a suction electrode attached to a stimulus isolation unit (Grass SIU, Grass Instruments, Quincy, MA) and stimulator (Grass S88). Signals were amplified using a WPI 721 (WP Instruments, Sarasota, FL, USA) amplifier and digitized into a computer for inspection using Axoscope 8.0 (Axon Instruments, Foster City, CA, USA) software and analyzed using MiniAnalysis 5.0 software (Synaptosoft, Decatur, GA, USA).

Quantal content of end-plate potentials. Recordings from each preparation were sampled and averaged from at least 5 different endplates from the same preparation to determine the mean amplitude of the EPPs and MEPPs before and after addition of nimodipine, yielding an *n* value of 1. Recordings were rejected if the 10-90 % EPP rise time was greater than 1 ms. EPPs with amplitudes ≥ 10 mV were corrected for nonlinear summation (McLachlan and Martin, 1981) using the formula $V_{corr} = V/[(1-.8*V)/E]$, where V is the

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uncorrected EPP amplitude, E is the resting membrane potential, and V_{corr} is the corrected EPP amplitude. Averaged EPP and MEPP amplitudes were first standardized to a membrane potential of –50 mV using the formula, $V_{df} = V/E * -50$ in order to correct for changes in membrane potential driving force (Katz and Thesleff, 1957), where V_{df} represents the corrected EPP amplitudes due to driving force and V represents the uncorrected EPP amplitude prior to correction for nonlinear summation. Quantal content was calculated using the ratio of the mean amplitude of the corrected EPPs to the mean amplitude of the corrected MEPPs (Hubbard *et al.*, 1969).

Statistics. Statistical significance between the various treatment groups was analyzed using a one way analysis of variance followed by Tukey's test. A two way analysis of variance was used to examine statistical significance between MEPP frequencies of various treatment days from control and LEMS-treated preparations. *P* values were set to < 0.05 for all statistical tests.

Facilitation of end-plate potential amplitudes. EPP amplitudes were recorded from LEMS- and control-muscle preparations during 40 Hz stimulation of the phrenic nerve. The effect of exposure of mouse muscle preparations to LEMS or control plasma on the induction of facilitation of EPP amplitudes was determined by first normalizing the EPP_n amplitude to that first EPP amplitude (EPP_n/EPP_1), where n = the stimulus number. Before averaging across experiments, the average normalized EPP ratio for the 2nd to 20th stimulus from one tissue preparation was then calculated to yield a *Facilitation ratio* (Average $(EPP_n/EPP_1)_{2\text{-}22}$). The effect of nimodipine was also examined on the EPP amplitudes during 40 Hz stimulation of the phrenic nerve and calculated by taking the ratio of the *Facilitation ratio* after nimodipine to the *Facilitation ratio* before nimodipine. This value was denoted Inhibition of facilitation ratio to represent the effects of nimodipine EPP amplitudes during high frequency nerve-stimulation.

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Chemicals. Plasma samples from patients clinically diagnosed as having LEMS were graciously provided by Drs. Eva L. Feldman and James Albers (University of Michigan Health System, Ann Arbor, MI, USA), Dr. Andrew Massey (University of Kentucky Medical Center, Lexington, KY, USA), and Dr. Shin Oh (University of Alabama Medical Center, Birmingham, AL, USA). Plasma was obtained during the course of routine plasma exchange therapy with the normal informed consent, in accordance with the respective institutions' Human Subjects Committee or Institutional Review Board approval and was supplied without any identifiers. Only patient age and gender were provided Control human plasma was donated by healthy volunteers and obtained as outdated blood donations from the American Red Cross (Lansing, MI, USA). Nimodipine, cyclophosphamide, and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DM-BAPTA-AM (1,2-Bis(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl)ester) was obtained from Molecular probes Inc (Eugene, OR, USA). All other reagents were of analytical grade or better.

D. Results

Effect of passive transfer of LEMS to mice on release of ACh from motor nerves at low frequency stimulation. Mice were injected for 1- 20 days with plasma either from three separate patients (designated "Patient 1, 2, or 3") with LEMS or control patients. Passive transfer of LEMS to mice with plasma from patient 1 and 2 significantly reduced release of ACh following stimulation of the phrenic nerve at 0.5 Hz in comparison to control plasma treatments for 1 - 20 days (Figs. 3.1, 3.2). On the other hand, quantal release of ACh from motor nerves obtained from mice injected with plasma from patient 3 only became significantly different in comparison to control plasma after 10 days (Fig. 3.3) of treatment. Resting

membrane potentials and MEPP amplitudes were not significantly different between any of the patient plasma or control treatment groups (Table 3.1). In addition, spontaneous release of ACh was not affected by injection of LEMS or control plasma at any day tested (Figs. 3.4, 3.5).

Effect of passive transfer of LEMS on high frequency nerve-stimulated release of ACh from motor nerves. In addition to reduced quantal release of ACh from motor nerves exposed to LEMS plasma, another electrophysiological characteristic of LEMS is enhanced facilitation of ACh release following high frequency nerve-stimulation. Treatment of mice with plasma from LEMS patients 1 and 2 significantly increased EPP amplitudes following 40 Hz stimulation of the phrenic nerve to a greater extent than did control plasma. The average ratios of amplitudes of $EPP_{2\text{-}20}$ to EPP_1 recorded from muscle preparations taken from mice injected with plasma from patients 1 and 2 for 1- 20 days were significantly greater when compared to preparations treated with control plasma (Figs. 3.6-3.11). On the other hand, treatment of mice with plasma from patient 3 did not significantly affect averaged ratios of amplitudes of $EPP_{2\text{-}20}$ to EPP_1 in comparison to control plasma preparations until after 10 days of treatment (Figs. 3.6-3.9, 3.11).

Development of Dihydropyridine sensitivity of release of ACh from motor nerves exposed to LEMS plasma. Nimodipine, a DHP-type L-type antagonist, significantly reduced the evoked-release of ACh following low frequency (0.5 Hz) stimulation of the phrenic nerve from preparations taken from mice treated with plasma from patients 1 or 2 for 20 days in comparison to pre-nimodipine treatment (Fig. 3.17). However, nimodipine was ineffective at reducing release of ACh from preparations taken from mice injected with plasma from patients 1 or 2 for 1 - 15 days (Figs. 3.13-3.16) or from preparations taken from mice injected with control plasma for 1 - 20 days(Figs. 3.13-3.17). Plasma from patient 3 did not produce DHP-sensitive L-type channel involvement in ACh at any day tested (Figs. 3.13-3.17). Furthermore,

nimodipine had no effect on spontaneous ACh release (measured as changes in MEPP frequency) from preparations exposed to either LEMS or control plasma for 1-20 days (Figs. 3.4, 3.5).

The involvement of L-type Ca^{2+} channels in ACh release following high frequency nerve-stimulation was also examined from preparations exposed to plasma from patient 1 for 20 days. Nimodipine, significantly reduced the averaged amplitude ratios of EPP_{2-20} to EPP_1 from preparations exposed to patient 1 plasma, but not control plasma for 20 days in comparison to averaged amplitude ratios of EPP_{2-20} to EPP_1 before nimodipine treatment (Fig. 3.12).

Effect of DM-BAPTA loading into motor nerve terminals following passive transfer of LEMS. The proximity of L-type Ca^{2+} channels to the transmitter release apparatus was examined using the rapid chelator of Ca^{2+}, DM-BAPTA. Adler *et al.* (1991) observed a reduction in evoked-release of ACh from squid nerve terminal following incubation with BAPTA, suggesting that the location of VDCC involved in glutamate release from this terminal is not tightly coupled with the release apparatus. However, VDCC at motor nerve terminals of mice exhibit a very tight coupling with the transmitter release apparatus (Urbano and Uchitel, 1999). For these experiments, muscle preparations obtained from mice injected with control plasma for 20 days were loaded with 25 μM DM-BAPTA-AM or DM-BAPTA control vehicle (.0125%, v/v DMSO). Following low frequency stimulation of the phrenic nerve, release of ACh was unchanged from control preparations loaded with DM-BAPTA in comparison to control preparations loaded with DMSO (Fig. 3.18). Thus, the complement of VDCC involved in release of ACh from mouse motor nerves appears tightly coupled with the normal release process. Furthermore, DM-BAPTA control (DMSO) had no effect on exposing L-type

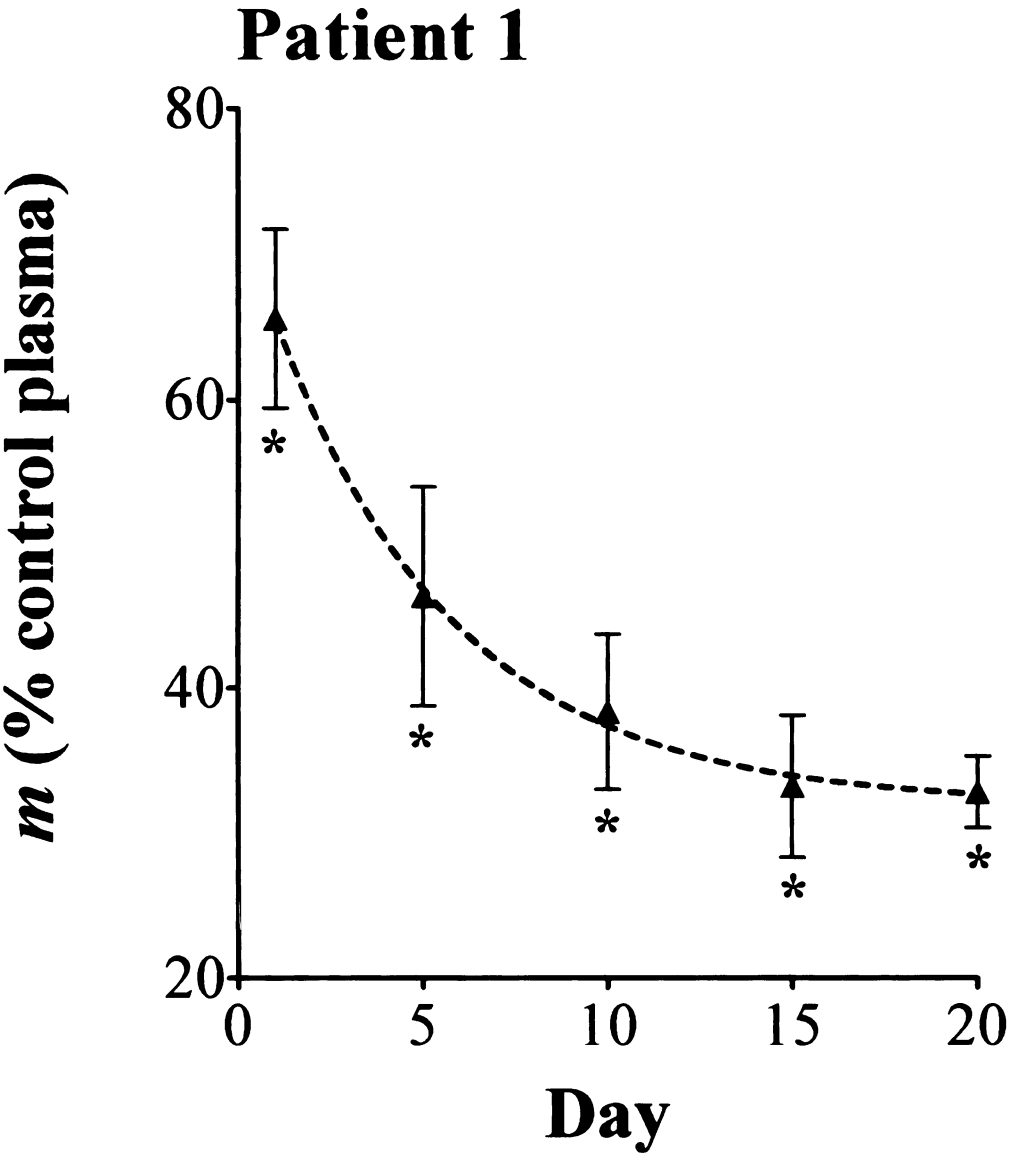

Figure 3.1. The time course of the effect of plasma from LEMS patient 1 on quantal content (*m*) at mice hemidiaphragm preparations. Hemidiaphragm preparations were obtained from mice injected with 1.5 ml/day (*i.p.*) of plasma from LEMS patient 1 for the number of days shown. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-treated preparations to that of control plasma-treated preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

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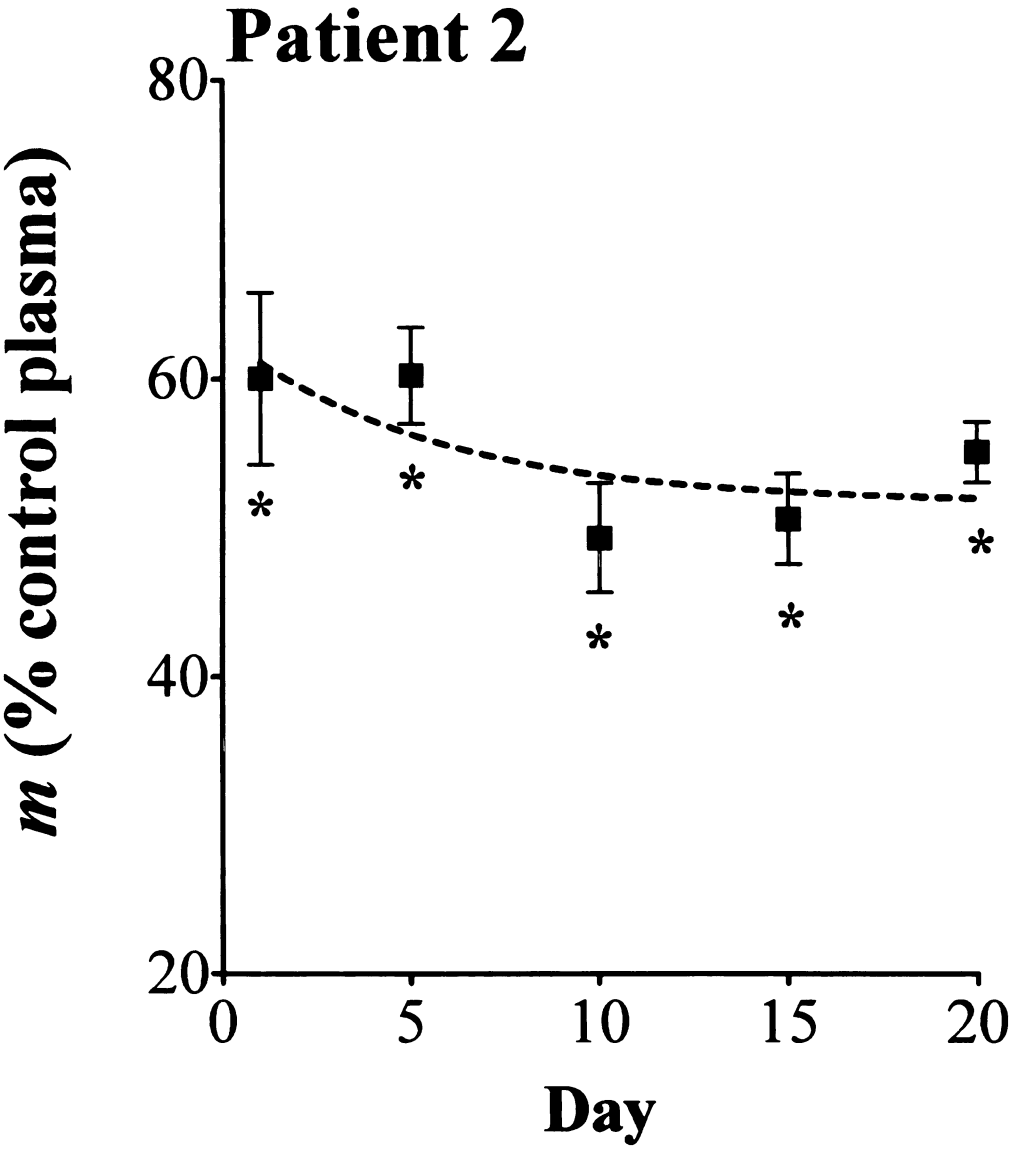

Figure 3.2. The time course of the effect of plasma from LEMS patient 2 on quantal content (*m*) at mice hemidiaphragm preparations. Hemidiaphragm preparations were obtained from mice injected with 1.5 ml/day (*i.p.*) of plasma from LEMS patient 2 for the number of days shown. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-treated preparations to that of control plasma-treated preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

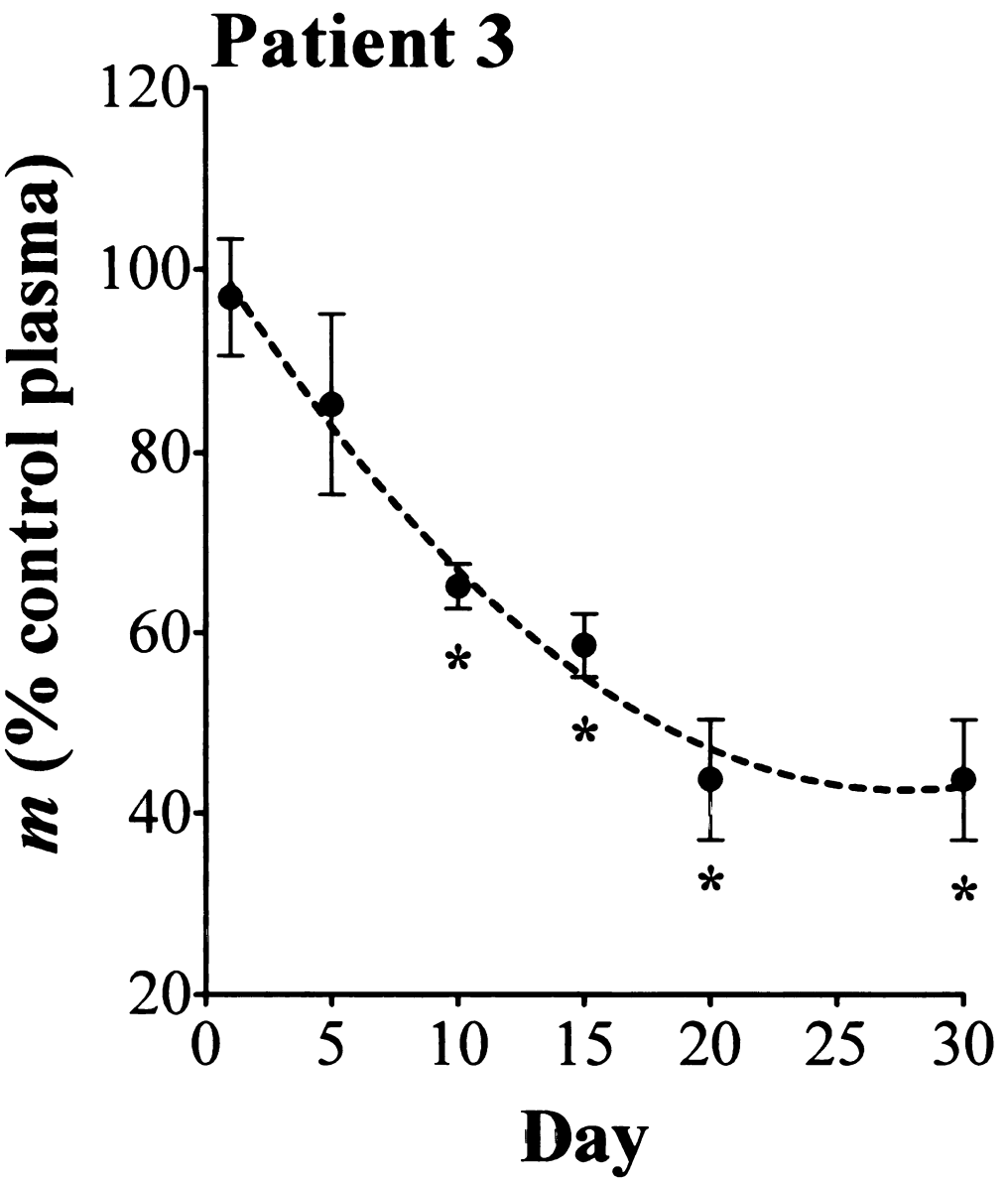

Figure 3.3. The time course of the effect of plasma from LEMS patient 3 on quantal content (*m*) at mice hemidiaphragm preparations. Hemidiaphragm preparations were obtained from mice injected with 1.5 ml/day (*i.p.*) of plasma from LEMS patient 3 for the number of days shown. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from the LEMS plasma-treated preparations to that of control plasma-treated preparations. Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

Table 3.1

Muscle resting membrane potentials (RMP)[#] and miniature end-plate potential (MEPP) amplitudes from preparations exposed to control plasma or plasma from 3 separate patients with LEMS for 1-20 days

Days	**Control**[R,A]	**Patient 1**[R,A]	**Patient 2**[R,A]	**Patient 3**[R,A]
1	-38.3 ± 2.7 0.738 ± 0.052	-41.6 ± 1.1 0.662 ± .027	-40.3 ± 1.4 0.786 ± .047	-41.5 ± 2.7 0.694 ± .057
5	-38.1 ± 1.8 0.785 ± 0.036	-41.6 ± 1.9 0.677 ± .023	-39.4 ± 2.6 0.695 ± .060	-38.7 ± 1.7 0.672 ± .024
10	-38.1 ± 7.2 0.698 ± 0.039	-39.5 ± 1.3 0.725 ± .046	40.9 ± 3.6 0.720 ± .031	-39.5 ± 1.1 0.720 ± .021
15	-39.0 ± 2.9 0.711 ± 0.037	-40.9 ± 1.9 0.746 ± .037	-41.5 ± 1.3 0.707 ± .023	-41.2 ± 2.7 0.680 ± .033
20	-38.2 ± 2.7 0.794 ± 0.064	-38.6 ± 3.4 0.694 ± .044	-40.5 ± 1.5 0.700 ± .038	-39.3 ± 2.2 0.687 ± .021

[#]Preparations were cut to prevent muscle contractions resulting in release of K^+ from the fibers and depolarization of the membrane. Values are the mean ± SEM of at least four preparations for each treatment group.

[R,A] denotes measurements of RMP (mV) and MEPP amplitude (mV), respectively.

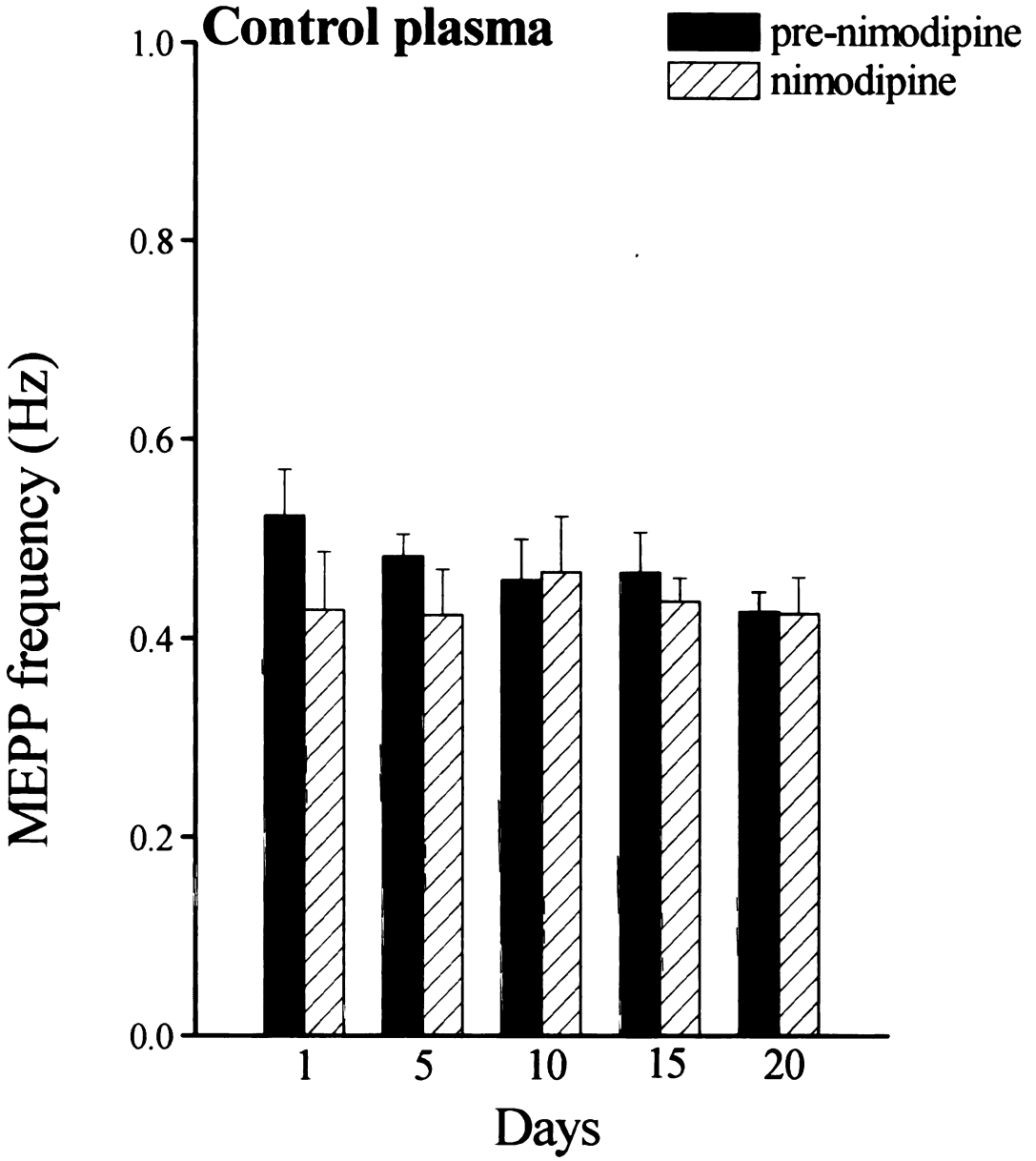

Figure 3.4. The time course of the effect of plasma from control patients on MEPP frequency at mice hemidiaphragm preparations. MEPP frequency was determined from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control patients for the time indicated. Recordings from each preparations were made in the presence or absence ("pre-nimodipine") of 10 μM nimodipine. Each value represents the mean ± SEM of at least 5 different preparations.

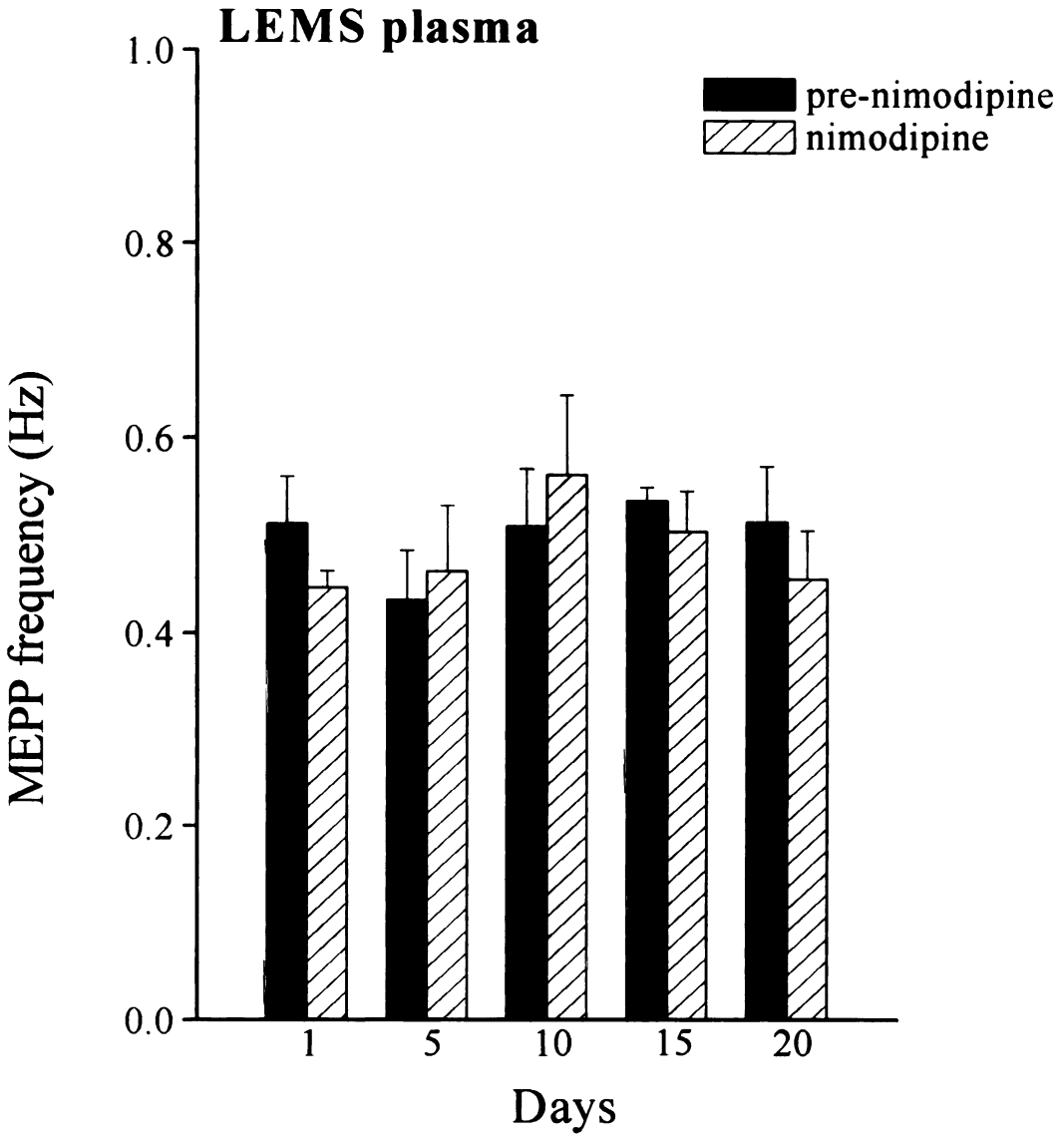

Figure 3.5. The time course of the effect of plasma from LEMS patients on MEPP frequency at mice hemidiaphragm preparations. MEPP frequency was determined from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from LEMS patients for the time indicated. Recordings from each preparations were made in the presence or absence ("pre–nimodipine") of 10 μM nimodipine. Each value represents the mean ± SEM of at least 5 different preparations.

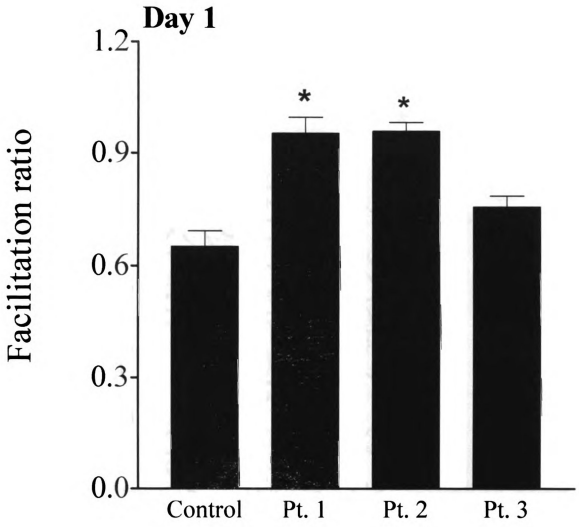

Figure 3.6. Effect of LEMS plasma injected for 1 day into mice on facilitation of transmitter release. EPP amplitudes were recorded from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients (Pt.) for 1 day. Values are expressed as the mean ± SEM of the Facilitation ratio (see Materials and Methods). The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

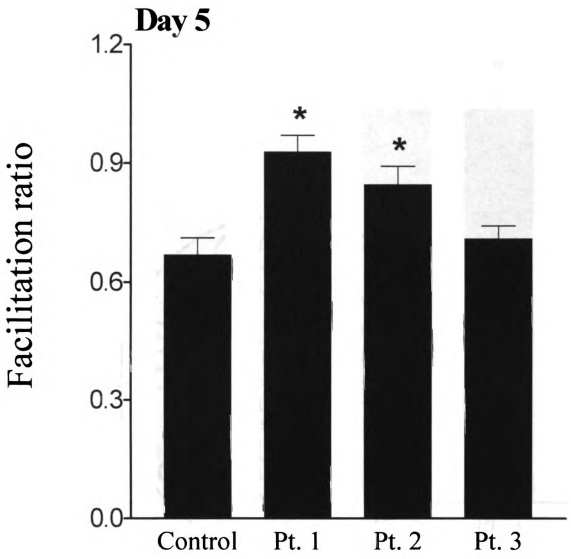

Figure 3.7. Effect of LEMS plasma injected for 5 days into mice on facilitation of transmitter release. EPP amplitudes were recorded from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients (Pt.) for 5 days. Values are expressed as the mean ± SEM of the Facilitation ratio (see Materials and Methods) The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

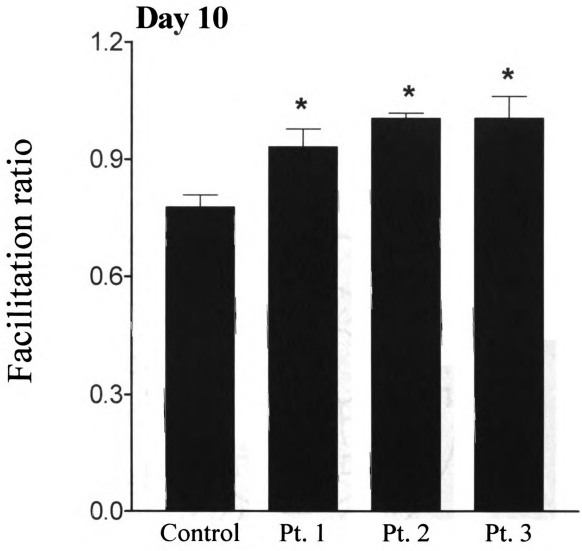

Figure 3.8. Effect of LEMS plasma injected for 10 days into mice on facilitation of transmitter release. EPP amplitudes were recorded from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients (Pt.) for 10 days. Values are expressed as the mean ± SEM of the Facilitation ratio (see Materials and Methods). The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

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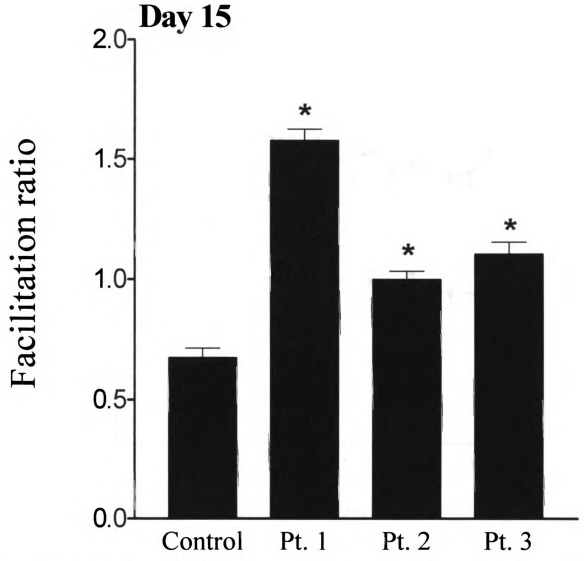

Figure 3.9. Effect of LEMS plasma injected for 15 days into mice on facilitation of transmitter release. EPP amplitudes were recorded from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients 15 days. Values are expressed as the mean ± SEM of the Facilitation ratio (see Materials and Methods). The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

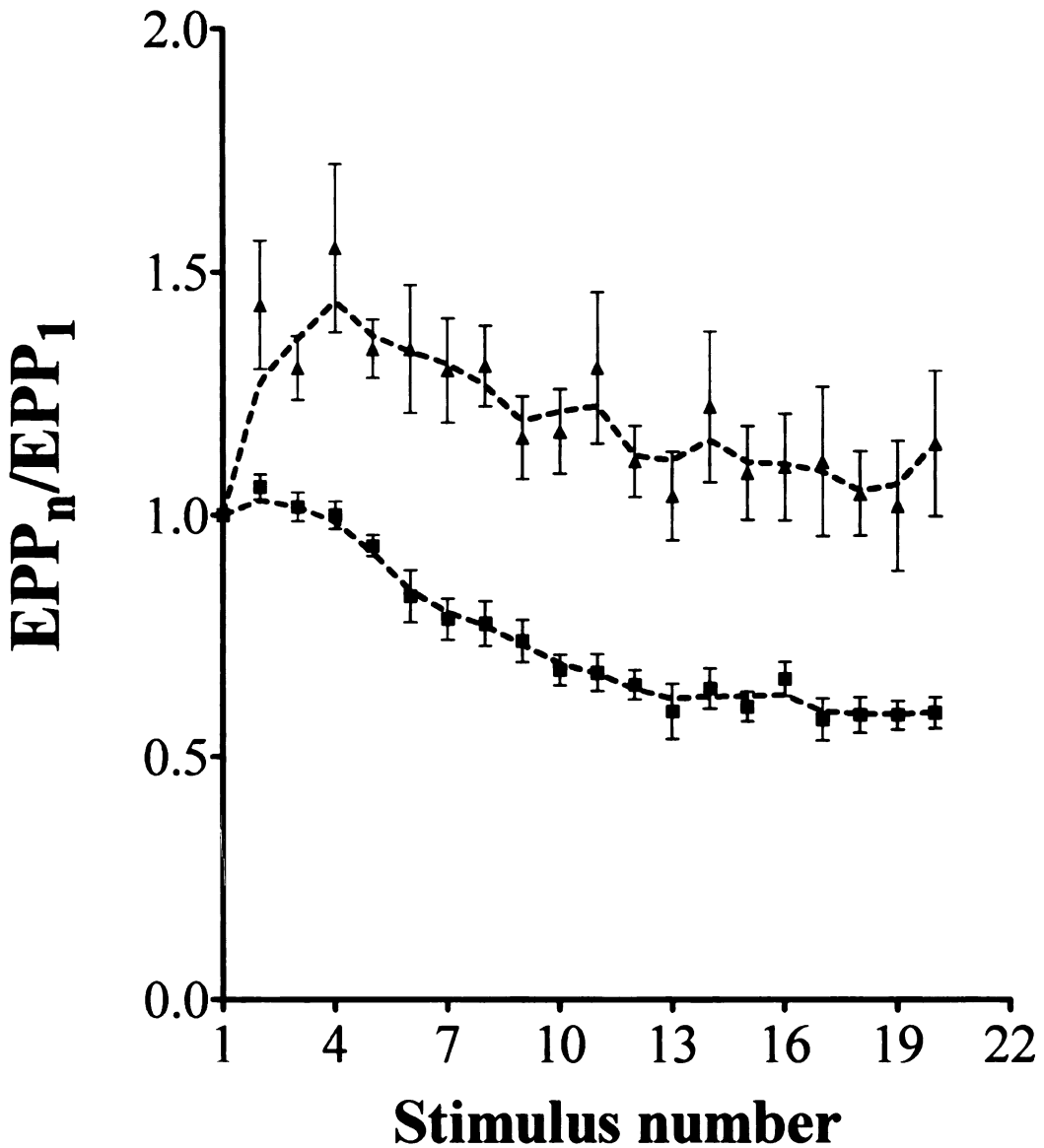

Figure 3.10. Effect of LEMS plasma injected for 20 days into mice on facilitation of transmitter release. EPP amplitudes were recorded during 40 Hz stimulation of the phrenic nerve from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patient 1 for 20 days. Graph representing the mean ± SEM of the normalized EPP plotted against a train of 20 stimuli recorded from LEMS patient 1 (–▲–) or control (–■–) plasma-exposed hemidiaphragm preparations.

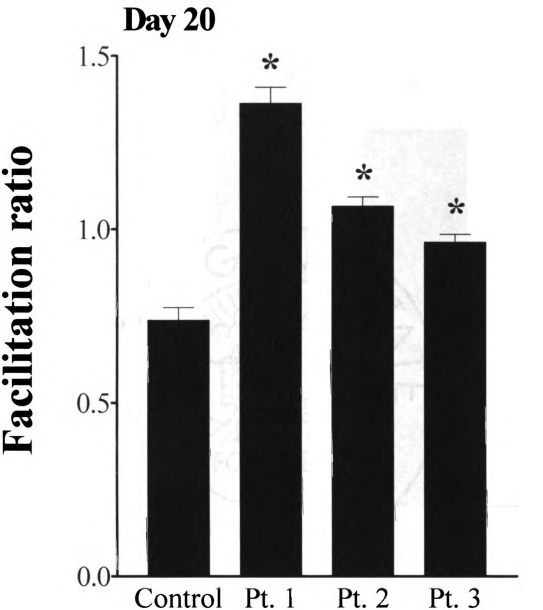

Figure 3.11. Effect of LEMS plasma injected for 20 days into mice on facilitation of transmitter release. EPP amplitudes were recorded during 40 Hz stimulation of the phrenic nerve from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients (Pt.) for 20 days. Graph representing the mean ± SEM of the Facilitation Ratio (see Material and Methods). The asterisk (*) indicates a value significantly different from control plasma ($P < 0.05$).

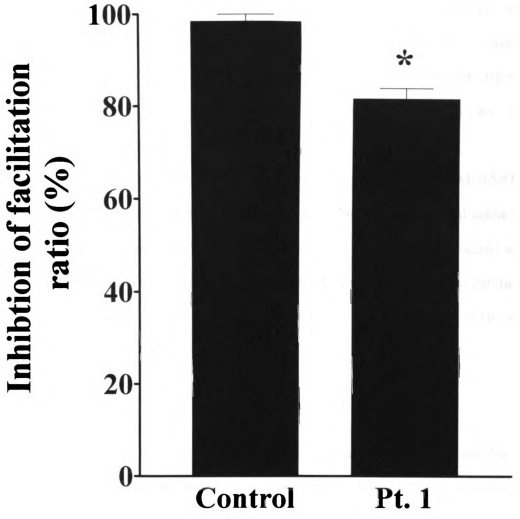

Figure 3.12. Effect of nimodipine on facilitation of transmitter release following injection of LEMS plasma into mice for 20 days. EPP amplitudes were recorded during 40 Hz stimulation of the phrenic nerve from hemidiaphragm preparations taken from mice injected with 1.5 ml/day (*i.p.*) of plasma from control or LEMS patients (Pt.) for 20 days. Graph representing the mean ± SEM of the Inhibition of facilitation ratio (see Materials and Methods). The asterisk (*) indicates a value significantly different from pre-nimodipine preparations ($P < 0.05$).

Ca^{2+} channels involved in release of ACh from control motor nerves (Fig. 3.18). In contrast, release of ACh at nerve-muscle preparations obtained from mice treated with plasma from patient 1 for 20 days and exposed to DMSO alone was sensitive to block by nimodipine, whereas preparations treated the same way but loaded with DM-BAPTA no longer exhibited DHP-sensitivity (Fig.3.19). It appears therefore, that L-type channels that are involved in release of ACh from motor nerves exposed to LEMS plasma for 20 days are not in close proximity to the normal transmitter release sites.

In order to ensure that the terminals were effectively loaded with DM-BAPTA, MEPP frequency was examined using muscle preparations bathed in physiological saline containing 12.5 mM KCl. MEPP frequency was significantly lower from preparations treated with control plasma and patient 1 plasma loaded with DM-BAPTA when compared to preparations treated identically with plasma (control plasma or patient 1) but not DM-BAPTA (exposed to DM-BAPTA control; DMSO), respectively (Figs. 3.20, 3.21).

E. *Discussion*

Immature motor nerve terminals contain multiple subtypes of VDCC involved in release of ACh, some of which become less important during maturation (Sugiura and Ko, 1997; Rosato-Siri and Uchitel, 1999; Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002). For instance, mature mammalian motor nerves contain one primary subtype of VDCC involved in ACh release; the specific mammalian subtype is species-dependent (Uchitel and Llinas, 1992; Katz, 1995; Protti and Uchitel, 1996). P/Q–, but not N– or L– type VDCC are involved in release of ACh from mature mammalian motor nerves (Sano *et al.*, 1987; De Luca *et al.*, 1991; Gray *et al.*, 1992; Uchitel and Llinas, 1992; Katz, 1995; Protti and Uchitel, 1996). Chronic exposure of adult mammalian motor nerve terminals to sera from patients with LEMS reduces P/Q-type

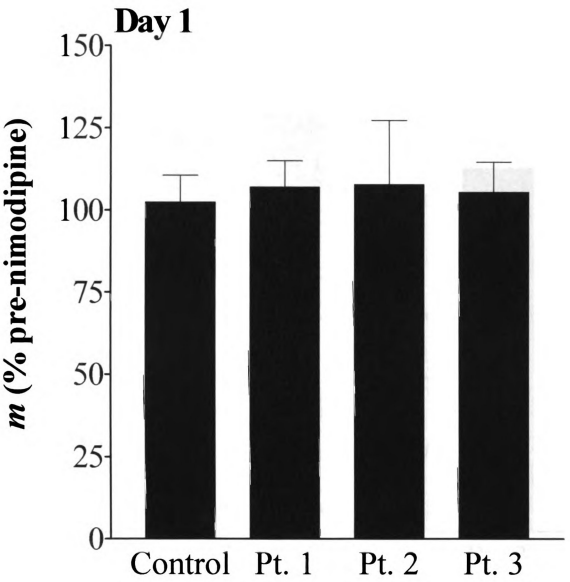

Figure 3.13. Effect of nimodipine on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 1 day. Hemidiaphragm preparations were obtained taken from mice injected with plasma from control or LEMS patients (Pt.) for 1 day. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 5 different preparations.

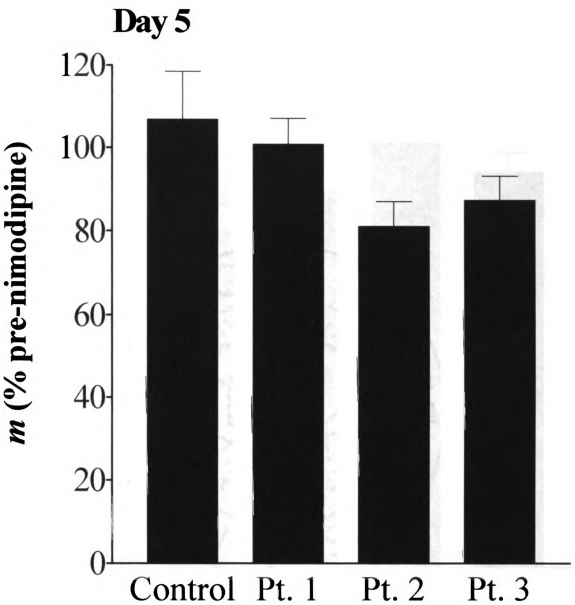

Figure 3.14. Effect of nimodipine on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 5 days. Hemidiaphragm preparations were obtained taken from mice injected with plasma from control or LEMS patients (Pt.) for 5 days. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 5 different preparations.

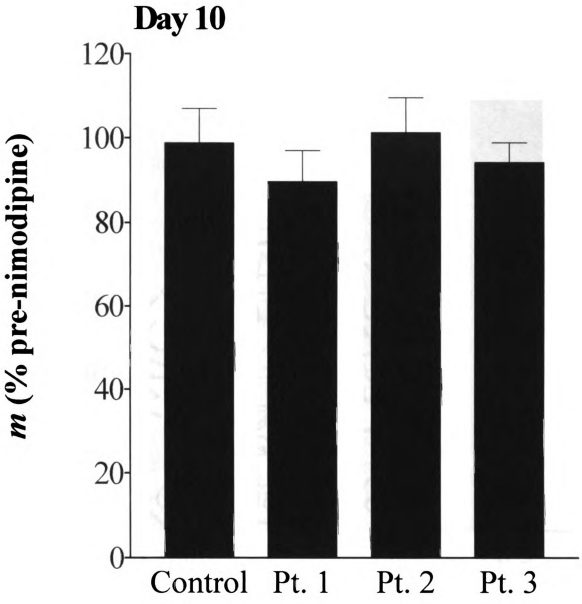

Figure 3.15. Effect of nimodipine on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 10 days. Hemidiaphragm preparations were obtained taken from mice injected with plasma from control or LEMS patients (Pt.) for 10 days. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 5 different preparations.

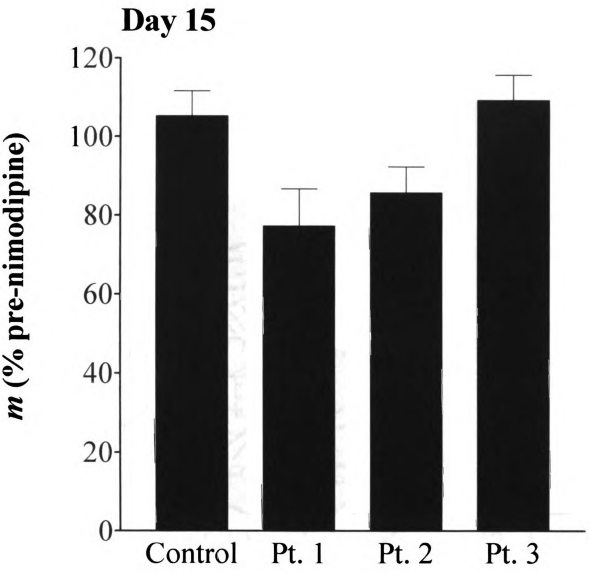

Figure 3.16. Effect of nimodipine on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 15 days. Hemidiaphragm preparations were obtained taken from mice injected with plasma from control or LEMS patients (Pt.) for 15 days. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 5 different preparations.

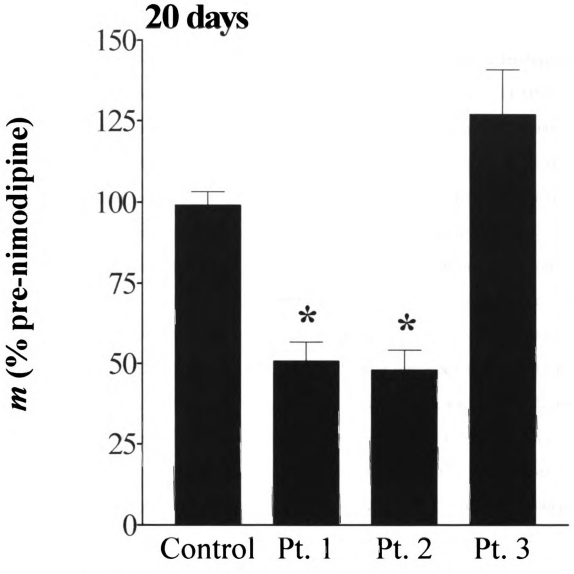

Figure 3.17. Effect of nimodipine on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 20 days. Hemidiaphragm preparations were obtained taken from mice injected with plasma from control or LEMS patients (Pt.) for 20 days. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10 μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 5 different preparations. The asterisk (*) indicates a value significantly different from pre-nimodipine preparations ($P < 0.05$).

Ca^{2+} currents and unmasks L-type Ca^{2+} currents involved in ACh release terminals (Smith and Atchison, 1995; Xu and Atchison, 1998; Flink and Atchison, 2002;Giovannini *et al.*, 2002). The primary objective of this study was to examine further the time course of development of L-type VDCC involved in ACh from motor nerves following passive transfer of LEMS to mice.

Nimodipine, a dihydropyridine-sensitive L-type Ca^{2+} channel antagonist, significantly reduced low frequency stimulated-release of ACh from motor nerve terminals from mice injected for 20 days with plasma from 2 out 3 patients with LEMS in comparison to mice injected with control patient plasma for 20 days. However, passive transfer of LEMS to mice with plasma from 3 separate patients for 1, 5, 10, and 15 days did not induce DHP-sensitive L-type Ca^{2+} channel involvement in evoked-release of ACh from motor nerves. These findings corroborate earlier work, which showed that L-type channel involvement in ACh release from motor nerves exposed to LEMS plasma occurs only after prolonged exposure (Flink and Atchison, 2002; Giovannini *et al.*, 2002). Although, MEPP frequency was unaffected by nimodipine at control and LEMS-treated preparations at all days tested, high-frequency nerve-evoked release from motor nerves exposed to LEMS patient plasma was reduced in the presence of nimodipine. Thus, it appears that strong depolarization of the nerve terminal is necessary in order to involve L-type Ca^{2+} channels in release of ACh following passive transfer of LEMS to mice by injections for 20 days. This findings correspond to those of earlier reports describing the strong voltage-dependence necessary for activation of L-type channels (Miller, 1987).

Passive transfer of LEMS to mice produced electrophysioligical characteristics similar to those of earlier reports. Evoked-release of ACh from motor nerves following exposure of mice with plasma from 2 out 3 three LEMS patients for 1 and 5 days and from all 3 patients after 10, 15 and 20 days was reduced significantly in comparison to the corresponding controls.

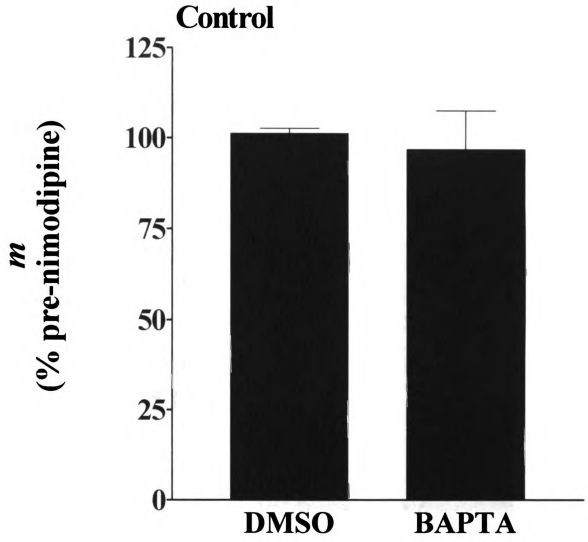

Figure 3.18. Effect of DM-BAPTA on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with control plasma for 20 days. Hemidiaphragm preparations obtained from mice injected with plasma from control patients for 20 days were incubated with either 25 μM DM-BAPTA-AM or .0125% DMSO (BAPTA vehicle) for 2 hr. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 4 different preparations.

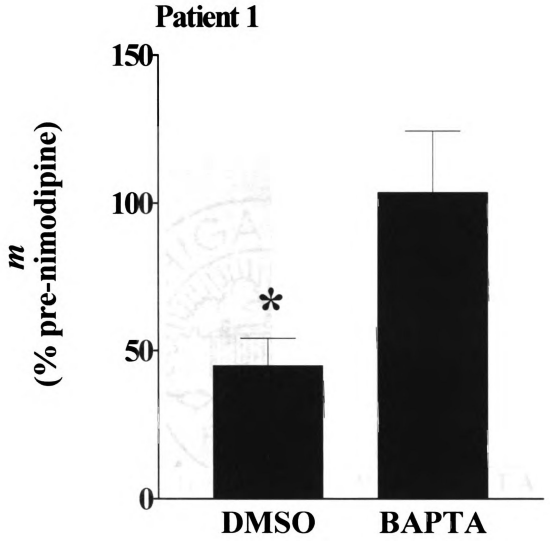

Figure 3.19. Effect of DM-BAPTA on quantal content (*m*) at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 20 days. Hemidiaphragm preparations obtained from mice injected with plasma from LEMS "patient 1" for 20 days were incubated with either 25 μM DM-BAPTA-AM or .0125% DMSO (BAPTA vehicle) for 2 hr. Quantal content was determined from each preparation using the ratio of the average EPP amplitude to the average MEPP amplitude before and after the addition of 10μM nimodipine. Values are expressed as the percentage of quantal content from preparations after the addition of nimodipine to that of the same preparation before nimodipine ("pre-nimodipine"). Each value represents the mean ± SEM of at least 4 different preparations. The asterisk (*) indicates a value significantly different from pre-nimodipine preparations ($P < 0.05$).

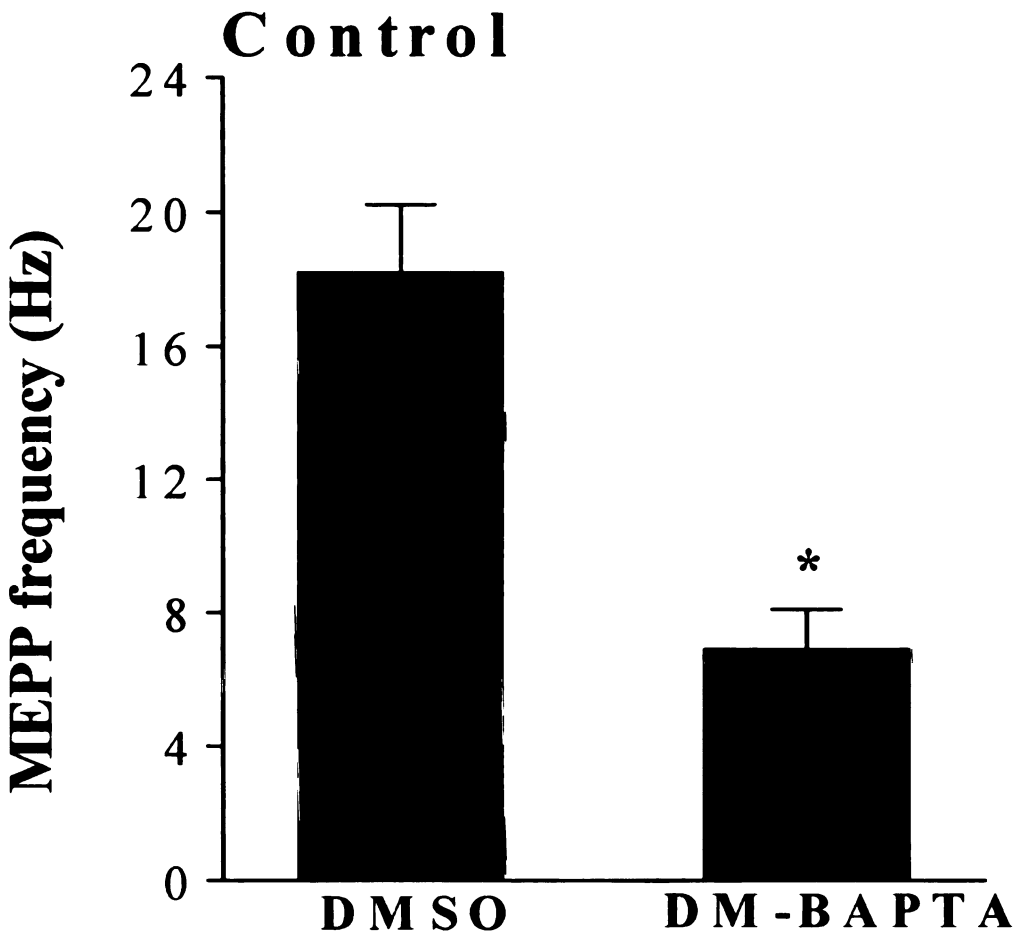

Figure 3.20. Effect of DM-BAPTA on KCl-induced MEPP frequency at hemidiaphragm preparations obtained from mice injected with control plasma for 20 days. Hemidiaphragm preparations obtained from mice injected with plasma from control patients for 20 days were incubated with either 25 μM DM-BAPTA-AM or .0125% DMSO (BAPTA vehicle) for 2 hr and subsequently exposed 12.5 KCl in the buffer. Each value represents the mean ± SEM of at least 4 different preparations. The asterisk (*) indicates a value significantly different from DMSO preparations ($P < 0.05$).

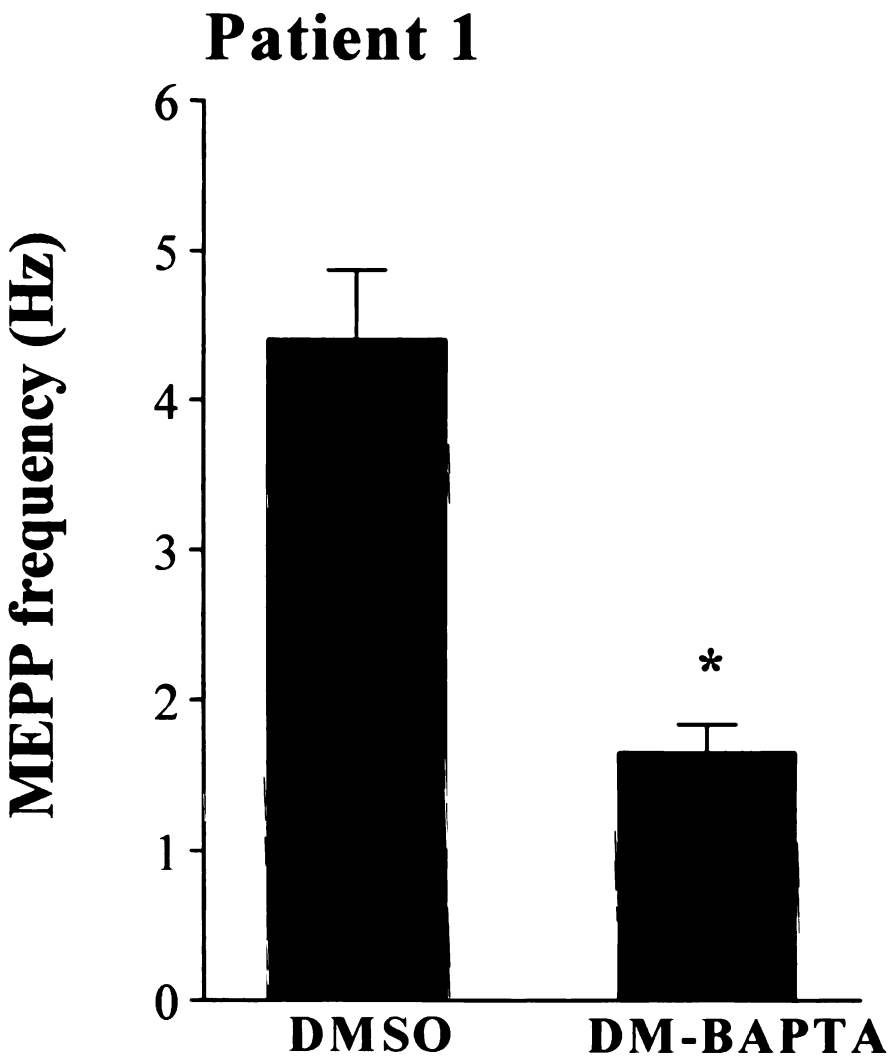

Figure 3.21. Effect of DM-BAPTA on KCl-induced MEPP frequency at hemidiaphragm preparations obtained from mice injected with LEMS plasma for 20 days. Hemidiaphragm preparations obtained from mice injected with plasma from LEMS "patient 1" for 20 days were incubated with either 25 μM DM-BAPTA-AM or .0125% DMSO (BAPTA vehicle) for 2 hr and subsequently exposed 12.5 KCl in the buffer. Each value represents the mean ± SEM of at least 4 different preparations. The asterisk (*) indicates a value significantly different from DMSO preparations ($P < 0.05$).

However, facilitation of ACh release, which is normally seen at motor nerves exposed to LEMS IgG was only observed after injection of plasma from 2 of the 3 LEMS patients at all days tested. For the third LEMS patient's plasma, facilitation of ACh release did not occur until low frequency nerve-stimulated release of ACh was already significantly reduced (day 10). This finding suggests that facilitation of ACh release following high frequency nerve-stimulation requires a significant reduction in the entry of Ca^{2+} through VDCC into the nerve terminal.

Development of L-type Ca^{2+} channel involvement in release of ACh following passive transfer of LEMS may depend on the extent to which the number of functional P/Q-type VDCC is reduced at the motor nerve terminal. Evoked-release of ACh from motor nerve terminals following treatment with LEMS plasma for 1 day was significantly greater than that seen after 20 days when L-type channel involvement became obvious. Although, reduction in ACh release appears unchanged at days 10, 15, and 20, loss of P/Q-type VDCC at motor nerve terminals most likely is greatest following the 20 day treatment regimen. L-type Ca^{2+} channels may therefore, assume the role of lost P/Q-type VDCC following the 20 day regimen. In turn, the extent of quantal content reduction appears the same at days 20, 15, and 10. This is further supported by the findings of Giovonnini *et al.*(2002), which showed that following passive transfer of LEMS for 9 days, release of ACh was sensitive to an L-type Ca^{2+} antagonist only during further blockade of P/Q-type VDCC with ω-Aga-IVA. However, when evoked-ACh release was examined without significant blockade of P/Q-type channels, L-type Ca^{2+} channels did not appear involved in release of ACh.

While involvement of L-type Ca^{2+} channels in release of ACh following passive transfer of LEMS is dependent on the extent of P/Q-type VDCC loss, other mechanisms must be involved as well. This can be explained by the following: inhibition of P/Q-dependent Ca^{2+} currents by the funnel web spider toxin, ω-Aga-IVA at motor nerve terminals exposed to

control patient sera for 30 days does not unmask L-type Ca^{2+} currents (Xu and Atchison, 1998); L-type Ca^{2+} channels do not appear to be involved in ACh release following the 10 or 15 day treatment regimens with LEMS plasma, even though the extent of nerve evoked-release of ACh appears significantly different from that observed following 1 or 5 days of treatment; motor nerve terminals exposed to plasma from patient 3 do not involve L-type VDCC in nerve evoked-release of ACh even after significant reduction in ACh release occurs.

It is possible that involvement of L-type Ca^{2+} channels in ACh release following passive transfer of LEMS is due to unmasking of normally silent channels already present on or near the motor nerve terminal. This possibility is supported by the findings that pretreatment of mammalian motor nerve terminals with bay k 8644, a DHP-type agonist, increases evoked release of ACh, which can be further reduced by addition of the DHP-sensitive antagonist nimodipine (Atchison and O'Leary, 1987; Atchison, 1989). Block of adenosine (A_2) receptors at rat motor nerve terminals also exposes L-type Ca^{2+} channel involvement in ACh release (Correia-de-Sa *et al.*, 2000a, b). Furthermore, inhibition of protein phosphatases in conjunction with rapid chelation of Ca^{2+} using BAPTA in the mammalian motor nerve terminal also unmasks L-type Ca^{2+} channels involved in ACh release (Urbano *et al.*, 2001). However, in the presence of rapid chelation (BAPTA-treatment) alone, L-type Ca^{2+} current becomes apparent, but is not involved in ACh release (Urbano and Uchitel, 1999). Interestingly, in our experiments DM-BAPTA abolished L-type Ca^{2+} channel involvement in ACh release from LEMS-treated motor nerve terminals. Thus, if inhibition of protein phosphatases occurs at LEMS-treated motor nerve terminals, then incubation with DM-BAPTA should have either enhanced or not affected L-type Ca^{2+} channel involvement in ACh release, however this was not the case.

It appears that development of L-type Ca^{2+} channels involvement in ACh from motor nerves is multifaceted. The time course describing the development of DHP-sensitivity implies that processes, which take a long time are required, such as denovo- mRNA synthesis and Ca^{2+} channel protein translation and assembly. For instance, following botulism toxin-induced poisoning, reinnervating or recovering motor nerves express L-type Ca^{2+} channel involvement in ACh release (Katz *et al.*, 1996; Santafe *et al.*, 2000). However, the motor nerve terminal does not appear to be damaged in LEMS (Fukunaga, 1982; Fukuoka *et al.*, 1987; Tsujihata *et al.*, 1987). So this involvement presumably doesn't result from reinnervation. Alternatively, L-type channel development may occur rapidly, but not be present at a location in which their involvement in ACh release is possible, perhaps during exposure LEMS IgG these channels relocate along the terminal to a point at which they can become involved in ACh release.

Loading of motor nerve terminals with the rapid onset Ca^{2+} chelator, DM-BAPTA did not affect evoked-release of ACh in solutions containing low concentrations of KCl . This suggests that the coupling between the VDCC normally involved in ACh from adult mammalian motor nerves is tightly coupled with the release machinery. Although this release corroborates the results of Urbano and Uchitel (1999), it is in contrast to that result reported by Adler *et al* (1992). However, this discrepancy most likely reflects the differences in species (squid stellate ganglion) used in their study, for which coupling of VDCC and release may not be as tight as that seen at adult mammalian motor nerve terminals. In the presence of DM-BAPTA, L-type Ca^{2+} channels do not appear involved in ACh release from motor nerve terminals exposed to LEMS plasma, suggesting that these channels are located at a site distinct from that of the normal complement of VDCC involved in ACh release. Thus, DM-BAPTA most likely binds Ca^{2+} that enters the terminal through L-type channels before the Ca^{2+} reaches ACh release sites. *It is* believed that VDCC normally involved in ACh release are clustered near active zone

release sites (Llinas *et al.*, 1992). If L-type channels are located at a distinct site, then the influx of Ca^{2+} through the L-type channels would likely occur in a more diffuse manner than what is thought to occur normally. This possibility is further supported by the findings that L-type Ca^{2+} channels are not involved in spontaneous release of ACh. At resting membrane potentials, the number of open L-type Ca^{2+} VDCC that allow entry of Ca^{2+} into the terminal may too small to induce-spontaneous release of ACh. (unstimulated MEPP frequency) Depolarization of the nerve terminal, on the other hand, would increase the probability of L-type channel openings and thus allow enough Ca^{2+} to entry the terminal be involved in ACh release.

The lack of development of L-type Ca^{2+} channel involvement in release of ACh following passive transfer with plasma from patient 3 is puzzling, but this observation may reflect differences in the specificity of the antibodies found in different patients with LEMS. Recent work has shown that over 90% of patients have antibodies directed against the P/Q-type VDCC (Suenaga *et al.*, 1996). Functional analysis has further shown that IgG from patients with LEMS reduces Ca^{2+} uptake into cells containing P/Q-, but not N-, L-, or R VDCC (Pinto, 1998; Pinto *et al.*, 2002). The specificity of LEMS IgG for P/Q-type VDCC, which are the major VDCC involved in ACh release from adult mammalian motor nerves, may explain why the primary clinical sign in LEMS is muscle weakness. However, antibodies directed against non- P/Q-type VDCC have also been detected in plasma from patients with LEMS (Lennon and Lambert, 1989; Sher *et al.*, 1989, El Far *et al.*, 1995). As such, incubation of neuroblastoma IMR32 (Grassi *et al.*, 1994) or neuroblastoma X glioma hybrid (Peers *et al.*, 1990) cells with LEMS IgG has been shown to decrease entry of Ca^{2+} through N-type and L-type VDCC. The presence of antibodies against multiple subtypes of VDCC may explain why the development of L-type Ca^{2+} channels was not observed following passive transfer of LEMS with plasma from

patient 3. Plasma from this patient may also contain antibodies specific for not only P/Q-, but L-type Ca^{2+} channels as well.

While one may question L-type Ca^{2+} channel involvement in ACh release from motor nerves of patients with LEMS, it has been shown that addition of L-type Ca^{2+} channel antagonists worsens or unmasks symptoms in LEMS patients (Krendal and Hopkins 1986, Ueno *et al.* 1992). Taken together with our findings, L-type Ca^{2+} channels assume an important role in the release of ACh in LEMS and thus may act as a compensatory mechanism to maintain a given level of transmitter release following loss of P/Q-type VDCC.

CHAPTER FOUR

IBERIOTOXIN-INDUCED BLOCK OF K_{Ca} CHANNELS REVEALS DIHYDROPYRIDINE SENSITIVITY OF ACh RELEASE FROM MAMMALIAN MOTOR NERVE TERMINALS

A. *Summary*

The role which Ca^{2+}-activated-K^+ (K_{Ca}) channels play in regulating acetylcholine (ACh) release was examined at mouse motor nerve terminals. In particular, the ability of the antagonist iberiotoxin to recruit normally silent L-type Ca^{2+} channels to participate in nerve-evoked release was examined using conventional intracellular electrophysiological techniques. Incubation of cut hemidiaphragm preparations with 10 μM nimodipine, a dihydropyridine (DHP) L-type Ca^{2+} channel antagonist, had no significant effect on quantal content of end-plate potentials following nerve-stimulation in comparison to untreated (control) preparations. However, 1 μM bay K 8644, a DHP L-type Ca^{2+} channel agonist enhanced quantal content to 134.7 ± 3.5 % of control. Iberiotoxin (150 nM) increased quantal content to 177.5 ± 9.9 % of control, whereas iberiotoxin in the presence of nimodipine increased quantal content to only 145.7 ± 10.4 % of control. Coapplication of 1 μM bay K 8644 with iberiotoxin did not significantly increase quantal content further than did treatment with iberiotoxin alone. The effects of iberiotoxin and nimodipine alone or in combination on MEPP frequency in response to KCl-induced depolarization were also examined using uncut hemi-diaphragm preparations. Nimodipine alone had no effect on MEPP frequency from preparations incubated in physiological saline containing 5-20 mM KCl. Moreover, iberiotoxin alone or combined with nimodipine also had no effect on MEPP frequency in physiological salines containing 5-15 mM KCl. However, when the [KCl] was raised to 20 mM, iberiotoxin significantly increased MEPP frequency to 125.6% of iberiotoxin-free values; combined treatment with both nimodipine and iberiotoxin prevented this increase in MEPP frequency (102.2% of iberiotoxin-free values). Thus, loss of functional K_{Ca} channels can unmask normally silent L-type Ca^{2+} channels to

participate in quantal release of ACh from motor nerve terminals particularly under conditions of intense nerve terminal depolarization.

B. *Introduction*

Release of acetylcholine (ACh) from motor nerves is a highly controlled process that requires entry of Ca^{2+} through voltage-dependent Ca^{2+} channels into the nerve terminal (Augustine *et al.*, 1987; Katz *et al.*, 1997). Multiple Ca^{2+} channel subtypes are known to exist; thus far pharmacological, electrophysiological, and molecular characteristics of L-, T-, N-, P-, Q-, and R- type channels have been described (Tsien *et al.*, 1988; Zhang *et al.*, 1993; Catterall, 1998; Newcomb *et al.*, 1998). Often more than one Ca^{2+} channel subtype coexists at the same nerve terminal to control transmitter release (Lemos and Nowycky, 1989; Turner *et al.*, 1993; Elhamdani *et al.*, 1998). However, the specific Ca^{2+} channel phenotype primarily involved in ACh release is both species- (Sano *et al.*, 1987; De Luca *et al.*, 1991; Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997) and age-dependent (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001). Release of ACh from mature mammalian motor nerves relies primarily on entry of Ca^{2+} through P/Q- (Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997), but not N-type Ca^{2+} channels, which are found on motor nerves of amphibians (Sano *et al.*, 1987) and birds (De Luca *et al.*, 1991). Developing mammalian motor nerves on the other hand, possess multiple Ca^{2+} channel subtypes involved in the release of ACh, some of which become less important during maturation (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001).

L-type Ca^{2+} channels, which can be found colocalized with other Ca^{2+} channel phenotypes, participate in release of noradrenaline from chromaffin cells of the adrenal medulla (Owen *et al.*, 1989) and in the release of oxytocin and vasopressin (Lemos and Nowycky, 1989). Pharmacological evidence has implicated a potential role of normally silent L-type Ca^{2+} channels in release of ACh from mature mammalian motor nerves. As such, bay K 8644, a dihydropyridine-sensitive L-type Ca^{2+} channel agonist, but not nimodipine, a dihydropyridine-sensitive L-type Ca^{2+} channel antagonist alters the release of ACh from mammalian motor nerves (Atchison and O'Leary, 1987; Atchison, 1989). Inhibition of protein phosphatases in conjunction with rapid chelation of intracellular Ca^{2+} in the motor nerve terminal also exposes a L-type Ca^{2+} channel component involved in release of ACh from mammalian motor nerves (Urbano *et al.*, 2001). Moreover, during certain pathological conditions, L-type Ca^{2+} channels can also participate in release of ACh as well (Katz *et al.*, 1996; Fratantoni *et al.*, 2000; Santafe *et al.*, 2000; Flink and Atchison, 2002; Giovannini *et al.*, 2002).

The control of ACh release from motor nerves requires not only precise and rapid opening of Ca^{2+} channels at the nerve terminal, but also mechanisms to close these channels as well. One such mechanism involves the opening of K^+ channels, which return the depolarized membrane to resting potential and thus alters the open state of the voltage-dependent Ca^{2+} channels (Llinas *et al.*, 1981; Augustine, 1990). Three types of K^+ currents have been identified at mammalian motor nerve terminals: a slow and fast voltage-dependent K^+-current and a Ca^{2+}-dependent-K^+ current (IK_{Ca}) (Mallart, 1985; Tabti *et al.*, 1989). The voltage-dependent K^+ currents are both sensitive to 3,4 diaminopyridine (DAP), whereas tetraethylammonium (TEA) affects only the fast component. IK_{Ca}, on the other hand is activated in the presence of Ca^{2+} and

sensitive to TEA, but not to 3,4 DAP. IK_{Ca} can also be distinguished pharmacologically from the voltage-dependent K^+ current in the presence of various scorpion toxins (Tabti *et al.*, 1989; Vatanpour and Harvey, 1995). Although the precise role which K_{Ca} channels play at the nerve terminal is unclear, they most likely participate in attenuating Ca^{2+} -dependent transmitter release by contributing to repolarization of the nerve terminal and thus altering the open state of voltage-dependent Ca^{2+} channels (Mallart, 1985; Robitaille and Charlton, 1992). Evidence exists that show K_{Ca} channels and voltage-dependent Ca^{2+} channels are not only colocalized at motor nerve terminals, but influence one another as well (Robitaille and Charlton, 1992; Robitaille *et al.*, 1993; Xu and Atchison, 1996; Protti and Uchitel, 1997).

It has been recently reported that passive transfer of Lambert-Eaton myasthenic syndrome (LEMS), a neuromuscular disorder, which causes functional loss of P/Q-type Ca^{2+} channels and thus a decrease in the depolarization-induced entry of Ca^{2+} into the motor nerve terminal (Lambert and Elmqvist, 1971; Fukunaga *et al.*, 1983; Hewett and Atchison, 1991) appears to involve L-type channels in ACh release (Flink and Atchison, 2002, Giovannini *et al.*, 2002). LEMS however, has not been shown to damage the nerve terminal nor cause sprouting of newly-formed terminals (Fukunaga *et al.*, 1983; Tsujihata *et al.*, 1987). In LEMS, reduced entry of Ca^{2+} into the nerve terminal following membrane depolarization could attenuate activation of K_{Ca} channels. This could, in turn, slow repolarization of the motor nerve terminal and thus increase the probability that colocalized or spatially-removed Ca^{2+} channels become involved in ACh release. For example, a component of transmitter release from sympathetic and motor nerves has been shown to depend upon Ca^{2+} entry through L-type channels under conditions of intense stimulation or following inhibition of voltage-dependent K^+ currents

(Hong and Chang, 1990; Somogyi *et al.*, 1997; Correia-de-Sá *et al.*, 2000a,b). The present study was designed, to determine if loss of functional K_{Ca} channels unmasks normally silent L-type Ca^{2+} channels involved in release of ACh from mammalian motor nerves. To accomplish this, iberiotoxin, a specific antagonist for K_{Ca} channels, was used to block K_{Ca} channels and the resulting sensitivity of release of ACh to DHP-type antagonists such as nimodipine was tested at murine motor nerve terminals.

C. *Materials and Methods*

Electrophysiology. Experiments were performed using male ICR mice (20-22 g, Harlan Sprague-Dawley Laboratories, Madison, WI) in accordance with local university (Michigan State University Laboratory Animal Resources) and national guidelines. Animals were sacrificed by decapitation following anesthesia with 80% CO_2 and 20% O_2. The diaphragm muscle with the attached phrenic nerve was then removed (Barstad and Lilleheil, 1968) and pinned out at resting tension in a Sylgard-coated chamber. The tissue was perfused continuously with buffered saline solution containing (mM): NaCl 137.5, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 11, HEPES 14, pH adjusted to 7.4 at room temperature of 23-25°C with NaOH and kept under continual oxygenation (100% O_2) at a rate of approximately 1-5 ml/min. The diaphragm muscle was transected into the two hemidiaphragms, and one hemidiaphragm was then cut approximately 4 mm on either side of the main intramuscular nerve branch in order to prevent muscle contraction following stimulation of the phrenic nerve (Glavinovic, 1979; Atchison, 1989). This technique does not produce significant changes in the muscle cable properties (Glavinovic, 1979). Depolarization-induced nerve conduction block that occurs

when K^+ is released from the cut-muscle fibers was prevented by the use of buffered saline solution containing 2.5 mM KCl throughout the experiments (Glavinovic, 1979; Atchison, 1989). Only one hemidiaphragm preparation per mouse was used for any given experiment.

Intact muscle preparations were used in experiments examining the effect of iberiotoxin on MEPP frequency in the presence of varying concentrations of KCl (5 - 20 mM). Osmolarity was adjusted for changes in concentrations of KCl with an equiosmolar change in the concentration of NaCl. All experiments were performed at room temperature of 23-25°C using conventional intracellular recording techniques. EPPs (end-plate potentials) and MEPPs (miniature end-plate potentials) were recorded using borosilicate glass microelectrodes (1.0 mm- o.d., WP Instruments, Sarasota, FL) and having resistance of 5-15 MΩ when filled with 3M KCl. The phrenic nerve was stimulated supramaximally at a frequency of 0.5 Hz using a suction electrode attached to a stimulus isolation unit (Grass SIU, Grass Instruments, Quincy, MA) and stimulator (Grass S88). Signals were amplified using a WPI 721 (WP Instruments, Sarasota, FL) amplifier and digitized into a computer for inspection using Axoscope 8.0 (Axon Instruments, Foster City, CA) software and analyzed using MiniAnalysis 5.0 software (Synaptosoft, Decatur, GA).

Data analysis and statistics. Control recordings were first made from untreated muscle preparations and subsequent recordings were made from the same preparation following incubation with the relevant drug treatment for the time indicted in the figure legends. Recordings from at least 5 different end-plates were used to determine the mean amplitude of the EPPs (average of 10 recordings *per* endplate) and MEPPs for each drug treatment from the same neuromuscular junction preparation, yielding an *n* value of 1. Averaged EPP and MEPP

amplitudes were first standardized to a membrane potential of –50 mV in order to correct for changes in membrane potential driving force (Katz and Thesleff, 1957). EPPs were then corrected for nonlinear summation (McLachlan and Martin, 1981) using the formula V_{corr} = V/((1-.8*V)/E), where V is the uncorrected EPP amplitude, E is the resting membrane potential, and V_{corr} is the corrected EPP amplitude. Quantal content was calculated using the ratio of the mean amplitude of the corrected EPPs to the mean amplitude of the corrected MEPPs (Hubbard *et al.*, 1969). Statistical significance between the various treatment groups was analyzed using a one way analysis of variance followed by Tukey's test. A two way analysis of variance was used to compare the effect of drug treatments on MEPP amplitudes in the presence of varying concentrations of KCl. *P* values were set to < 0.05 for all statistical tests.

Drugs and chemicals. Nimodipine, S-(–)bayK8644, and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). Iberiotoxin was obtained from Alomone labs (Jerusalem, Israel). All other reagents were of analytic grade or better. Nimodipine and S-(–) bay K 8644 were prepared as a 20 mM and 10 mM stock solution, respectively in 100% ethanol, which was kept at 4° C until use. The final working buffered saline solution with nimodipine and S-(–) bay K 8644 contained only 0.05% and 0.01% ethanol (v/v), respectively. Control experiments contained an equivalent concentration of the respective vehicle. Experiments performed in the presence of nimodipine or S-(–) bay K 8644 were done in the dark, in order to prevent photo-oxidation of these compounds. Iberiotoxin was prepared as a stock solution in deionized water containing 0.01% bovine serum albumin (w/v), and was used within a two-week period. Before incubation with

iberiotoxin, 0.01% bovine serum albumin was added to the buffered saline solution in order to prevent non-specific binding of toxin to the chamber, tubing, and glassware.

D. *Results*

Effects of K_{Ca} and L-type Ca^{2+} channels on neuromuscular transmission. Incubation of cut neuromuscular preparations with iberiotoxin increased quantal content of nerve- evoked release of ACh as shown in sample records (Fig. 4.2) and composite data (Fig. 4.1) to 177.5 ± 9.9 % of control values. Further treatment of neuromuscular preparations with nimodipine, a dihydropyridine L-type Ca^{2+} channel antagonist, in the presence of iberiotoxin reduced the enhancement of quantal content observed with iberiotoxin alone to 145.7 ± 10.4 % of control (Fig. 4.1). The final ethanol concentration (0.05%) used in physiological saline containing nimodipine and iberiotoxin had no significant effect upon the release of ACh, MEPP amplitudes or frequency, and muscle resting membrane potentials in comparison to iberiotoxin alone (data not shown). Similarly, iberiotoxin and nimodipine had no effect on resting membrane potentials (Fig. 4.3), MEPP frequency (Fig. 4.4), or MEPP amplitudes (Fig. 4.5) recorded from the cut preparations. Incubation of preparations with iberiotoxin vehicle (0.01% bovine serum albumin) alone or with nimodipine also had no effect on quantal content in comparison to untreated controls (Fig. 4.6).

Although L-type Ca^{2+} channels do not normally participate in nerve-evoked release of ACh from adult mammalian motor nerve terminals, use of the dihydropyridine L-type Ca^{2+} agonist, bay K 8644 has been shown to enhance ACh release from rat motor nerve terminals (Atchison and O'Leary, 1987; Atchison, 1989). Addition of bay K 8644 to cut neuromuscular

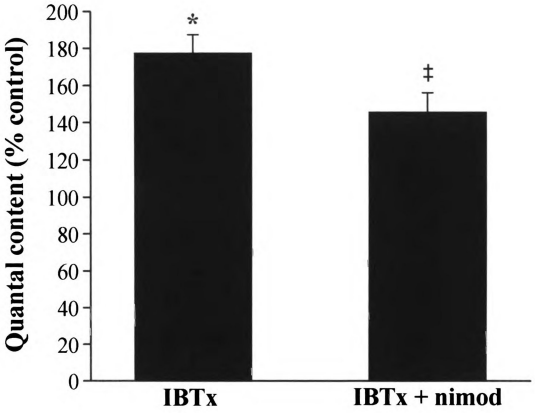

Figure 4.1. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on quantal content of mouse hemidiaphragm end-plates. Recordings were first made in the absence of any drug treatments (control), then subsequently made after 1 hr incubation with 150 nM iberiotoxin and then following further incubation with 10 μM nimodipine for 25 min in the presence of iberiotoxin. MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each hemidiaphragm preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from drug-treated preparations to that of control preparations. Each value represents the mean ± S.E.M. of at least 4 different preparations. The asterisk (*) and double dagger (‡) indicate a value significantly different from control and from control and iberiotoxin alone, respectively ($P < 0.05$).

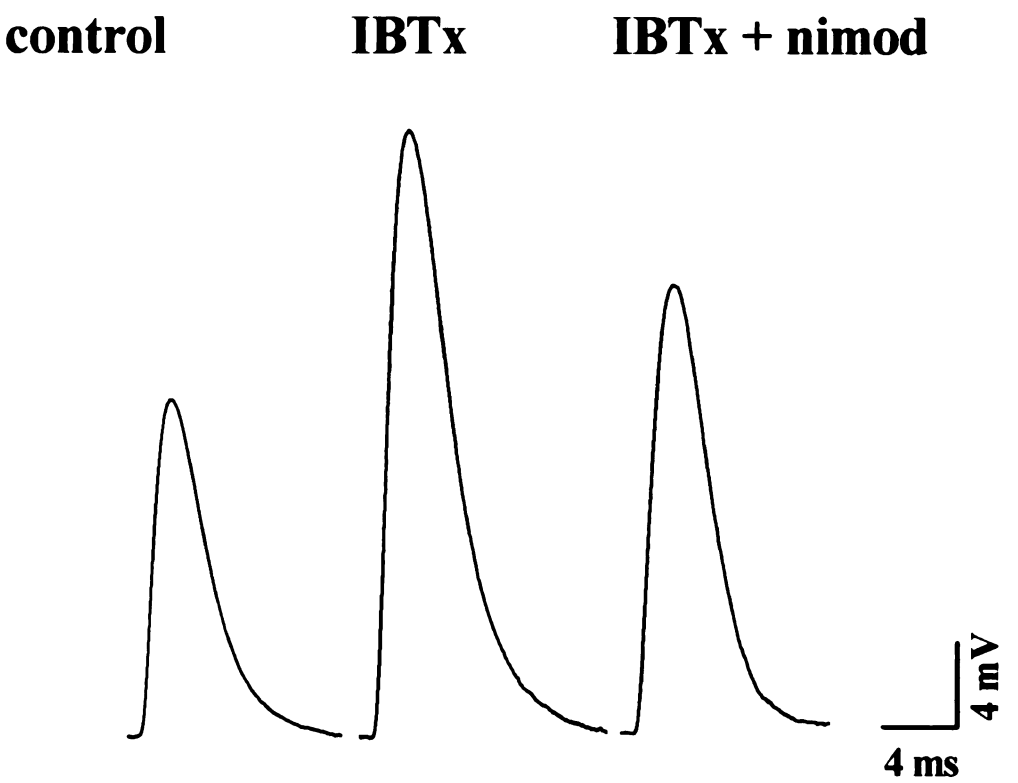

Figure 4.2. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on EPP amplitudes at mouse hemidiaphragm end-plates. Each tracing represents the average of 10 EPPs at a stimulation frequency of 0.5 Hz recorded from a single end-plate before (control), after the addition of 150 nM iberiotoxin, and following incubation with both iberiotoxin (150 nM) and nimodipine (10 μM). Each representative tracing was recorded from a different end-plate.

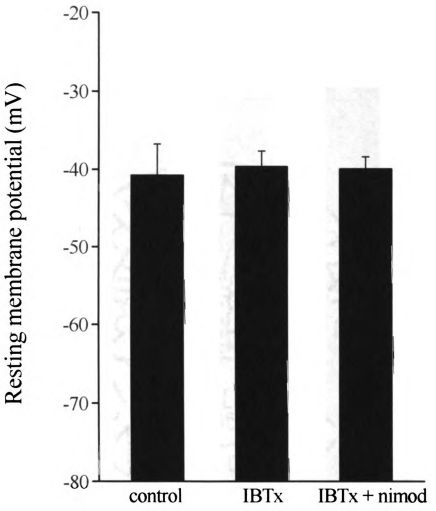

Figure 4.3. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on resting membrane potentials from cut mouse hemidiaphragm preparations. Recordings were made in the absence of any drug treatments (control), following 1 hr incubation with 150 nM iberiotoxin alone, and following further incubation with 10μM nimodipine and 150 nM iberiotoxin for an additional 25 min. Each value represents the mean ± S.E.M. of at least 4 different preparations.

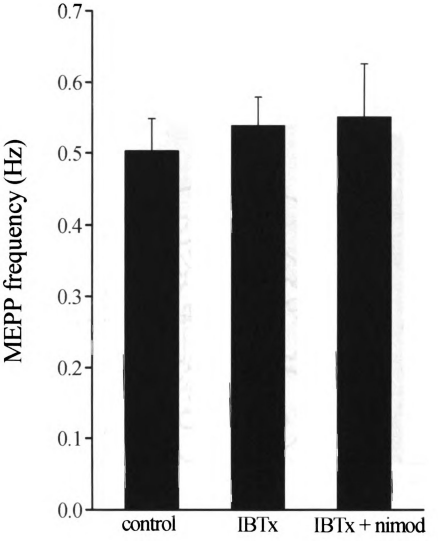

Figure 4.4. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on MEPP frequency from cut mouse hemidiaphragm preparations. Recordings were made in the absence of any drug treatments (control), following 1 hr incubation with 150 nM iberiotoxin alone, and following further incubation with 10μM nimodipine and 150 nM iberiotoxin for an additional 25 min. Each value represents the mean ± S.E.M. of at least 4 different preparations.

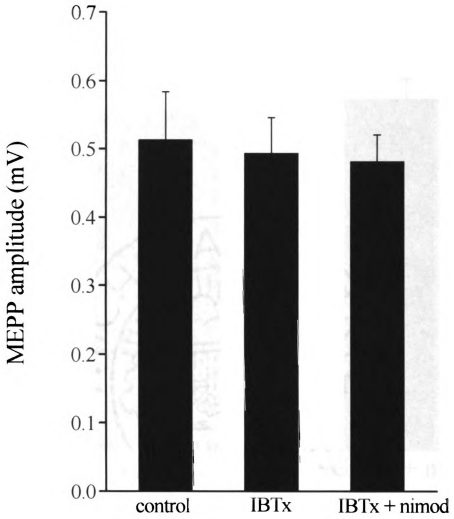

Figure 4.5. Effects of iberiotoxin (IBTx) and nimodipine (nimod) on MEPP amplitudes from cut mouse hemidiaphragm preparations. Recordings were made in the absence of any drug treatments (control), following 1 hr incubation with 150 nM iberiotoxin alone, and following further incubation with 10μM nimodipine and 150 nM iberiotoxin for an additional 25 min. Each value represents the mean ± S.E.M. of at least 4 different preparations.

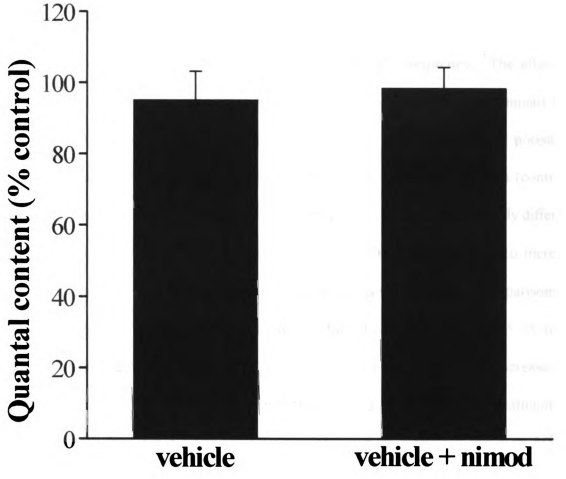

Figure 4.6. Effects of nimodipine (nimod) in the absence of iberiotoxin on quantal content of mouse hemidiaphragm end-plates. Recordings were first made in the absence of any drug treatments (control), then subsequently made after 1 hr incubation with iberiotoxin vehicle (0.01 % BSA) alone, and then following further incubation with 10 μM nimodipine for 25 min in the presence of iberiotoxin vehicle. MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from drug-treated preparations to that of control preparations. Each value represents the mean ± S.E.M. of at least 4 different preparations.

preparations enhanced quantal content to 134.7 ± 3.5 % of control (Fig. 4.7). Treatment of cut preparations with bay K 8644 in conjunction with iberiotoxin did not cause a significant difference from the effect of iberiotoxin alone (Fig. 4.8).

Effects of K_{Ca} and L-type Ca^{2+} channels on MEPP frequency. The effect of iberiotoxin and nimodipine on changes in MEPP frequency from motor nerve terminals was examined in the presence of varying concentrations of KCl. Resting membrane potentials recorded from uncut muscle preparations in the absence of any drug treatments (control), following incubation with iberiotoxin alone or with nimodipine were not significantly different from each other (Table 4.1). Although MEPP amplitudes (Fig. 4.9) appeared to increase slightly in the presence of nimodipine as KCl concentrations was increased, comparisons of results for all treatment groups within and between different KCl concentrations (5 -20 mM) revealed no significant differences. MEPP frequency, on the other hand, increased in conjunction with the increase in KCl concentration (Figs. 4.10, 4.11). Treatment of preparations with iberiotoxin alone or plus nimodipine had no significant effect on MEPP frequency in comparison to one another or to control treatment in physiological saline containing either 5,10, or 15 mM KCl (Fig. 4.10). However, when the physiological saline contained 20 mM KCl, iberiotoxin significantly increased MEPP frequency to approximately 125.6% of control treatment (Fig. 4.10). Further addition of nimodipine to preparations in the presence of iberiotoxin significantly reduced the enhancement of MEPP frequency observed with iberiotoxin alone approximately 102.2 % of control in the presence of 20 mM KCl (Fig. 4.10). However, MEPP frequency was not significantly different in the presence of nimodipine and iberiotoxin when compared with control treatment. Furthermore, MEPP frequency was

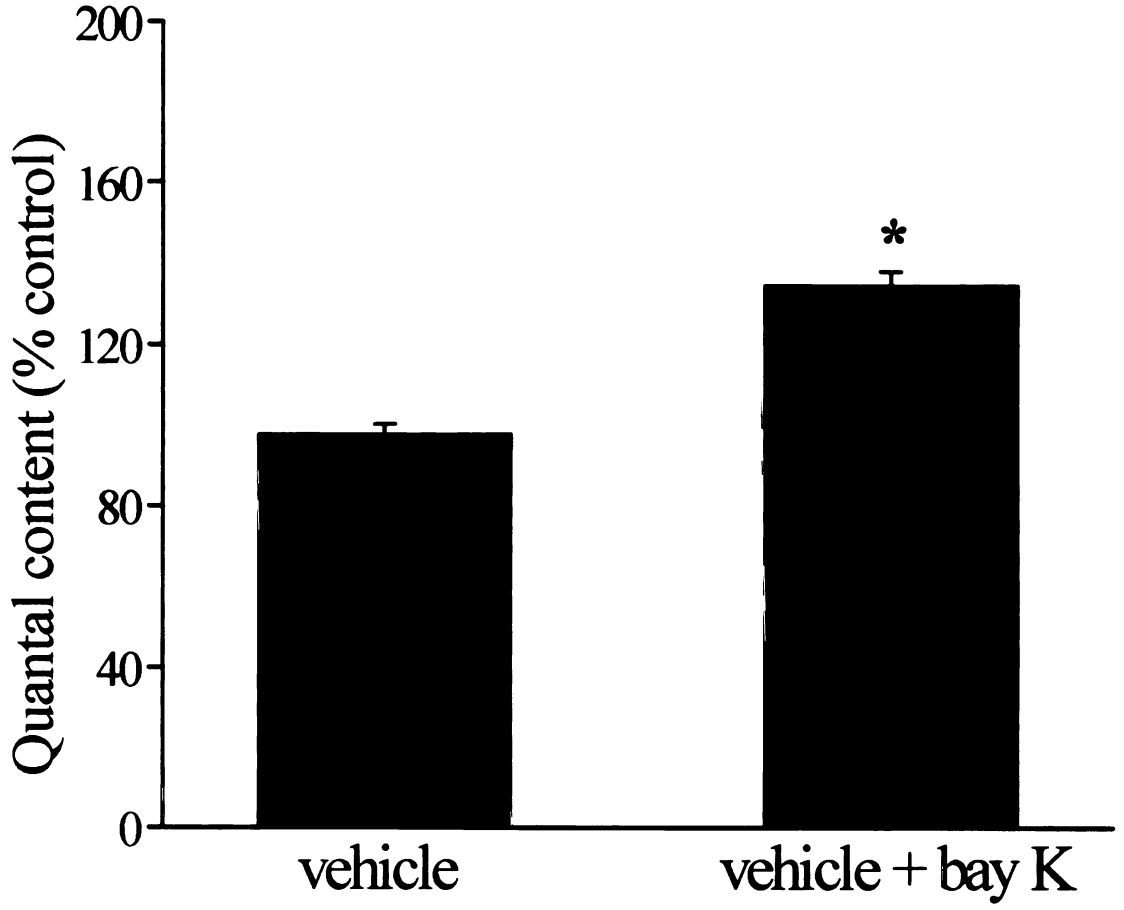

Figure 4.7. Effects of bay K 8644 (bay K) in the absence of iberiotoxin on quantal content of mouse hemidiaphragm end-plates. Recordings were first made in the absence of any drug treatments (control), then subsequently made after 1 hr incubation with iberiotoxin vehicle (0.01% BSA) and then following further incubation with 1 μM bay K 8644 for 25 min in the presence of iberiotoxin vehicle. MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from drug-treated preparations to that of control preparations. Each value represents the mean ± S.E.M. of at least 4 different preparations. The asterisk (*) indicates a value significantly different from either control or iberiotoxin vehicle ($P < 0.05$).

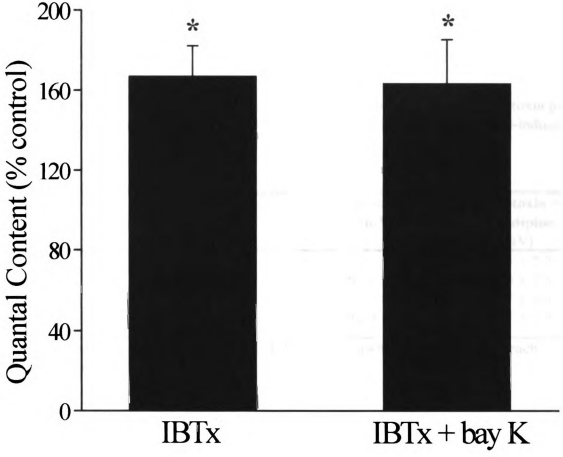

Figure 4.8. Effects of iberiotoxin (IBTx) and bay K 8644 (bay K) on quantal content of mouse hemidiaphragm end-plates. Recordings were first made in the absence of any drug treatments (control), then subsequently made after 1 hr incubation with 150 nM iberiotoxin and then following further incubation with 1 μM bay K 8644 for 25 min in the presence of iberiotoxin. MEPP and EPP amplitudes were recorded using standard intracellular techniques. EPPs were elicited at a frequency of 0.5 Hz. Quantal content was determined from each neuromuscular junction preparation using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from drug-treated preparations to that of control preparations. Each value represents the mean ± S.E.M. of at least 4 different preparations. The asterisk (*) indicates a value significantly different from control ($P < 0.05$).

Table 4.1

Muscle resting membrane potentials (RMP) from control, iberiotoxin, and iberiotoxin plus nimodipine treated-neuromuscular junction preparations at various levels of KCl-induced depolarization

[KCl] (mM)	Control (mV)	Iberiotoxin (mV)	Iberiotoxin + nimodipine (mV)
5	-70.1 ± 2.4[A]	-71.3 ± 2.0	-70.5 ± 2.0
10	-60.2 ± 2.0	-60.9 ± 3.1	-60.0 ± 2.6
15	-53.3 ± 2.1	-51.9 ± 1.8	-51.7 ± 1.0
20	-45.8 ± 1.3	-42.0 ± 1.7	-43.7 ± 1.9

[A] Each value represents the mean RMP ± S.E.M. from at least 4 preparations for each KCl concentration.

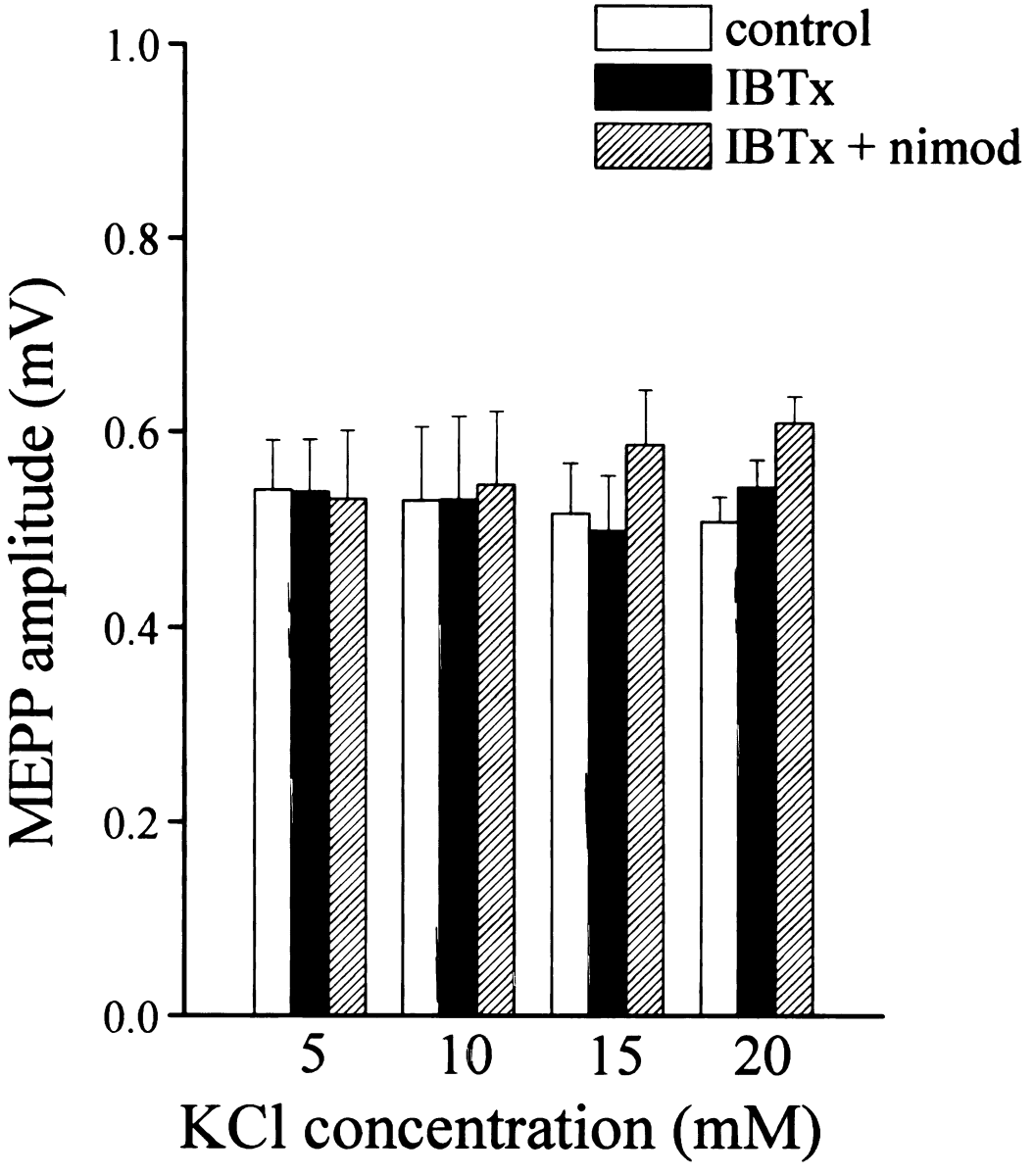

Figure 4.9. Comparative effects of iberiotoxin (IBTx) and nimodipine (nimod) on amplitudes of MEPPs recorded from muscle preparations in the presence of varying concentrations of KCl (5-20 mM). MEPP amplitudes were measured using standard intracellular techniques from untreated (control) preparations, following 1 hr incubation with 150 nM iberiotoxin, and then subsequently after 25 min incubation with 10 μM nimodipine in the presence of 150 nM iberiotoxin. Each value represents the mean ± S.E.M.. of at least 4 different preparations.

not significantly affected in the presence of nimodipine vehicle (0.05% ethanol) and iberiotoxin in comparison to iberiotoxin alone in physiological salines containing 20 mM KCl (data not shown). Incubation of uncut preparations with iberiotoxin vehicle alone or in the presence of nimodipine had no significant effect on MEPP frequency in comparison to control treatment in physiological saline containing 5,10, 15, or 20 mM KCl (Fig. 4.11).

E. *Discussion*

Normally, P/Q-, but not L-type channels are primarily involved in the nerve-evoked release of ACh from adult mammalian motor nerve terminals (Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997). L-type Ca^{2+} channels can, however, participate in ACh release from motor nerves during development (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001) and in the presence of certain pathological conditions (Katz *et al.*, 1996; Fratantoni *et al.*, 2000; Santafe *et al.*, 2000; Flink and Atchison, 2002, Giovannini *et al.*, 2002). In one such pathological condition, Lambert-Eaton myasthenic syndrome (LEMS), autoantibodies not only reduce the entry of Ca^{2+} into the motor nerve terminal (Lambert and Elmqvist, 1971; Fukunaga *et al.*, 1983; Hewett and Atchison, 1991), but also induce L-type Ca^{2+} channels to participate in ACh release (Flink and Atchison, 2002; Giovannini *et al.*, 2002).

Other conditions exist in which L-type channels can participate in ACh release as well. In the presence of specific pharmacological agents, normally silent L-type Ca^{2+} channels on or near the motor nerve terminal become involved in ACh release (Atchison, 1989; Hong and Chang, 1990; Urbano *et al.*, 2001). Taken together, it is therefore possible that in LEMS, involvement of L-type Ca^{2+} channels in release of ACh from motor nerve terminals, may in

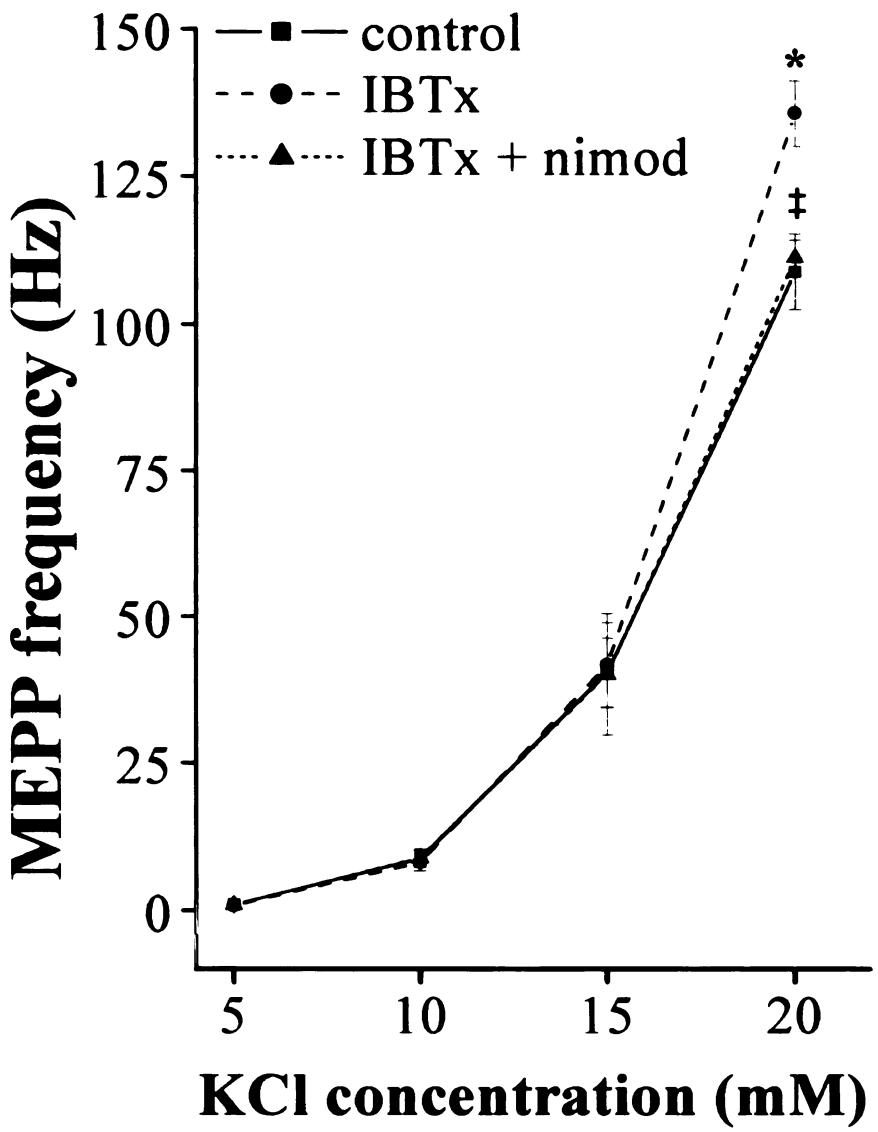

Figure 4.10. Comparative effects of iberiotoxin (IBTx) and nimodipine (nimod) on MEPP frequency at mouse hemidiaphragm preparations. Recordings were made from preparations before (control), after perfusion with iberiotoxin (150 nM) for 1 hr, and following perfusion with both nimodipine (10 μM) and iberiotoxin (150 nM) for an additional 25 min in buffers containing either 5-20 mM KCl. An equiosmolar substitution was made in the NaCl concentration to compensate for the varying KCl concentrations in the buffer. Each value represents the mean ± S.E.M. of at least 4 different preparations. The asterisk (*) and double dagger (‡) indicates a value significantly different from either control or iberiotoxin alone, respectively ($P < 0.05$).

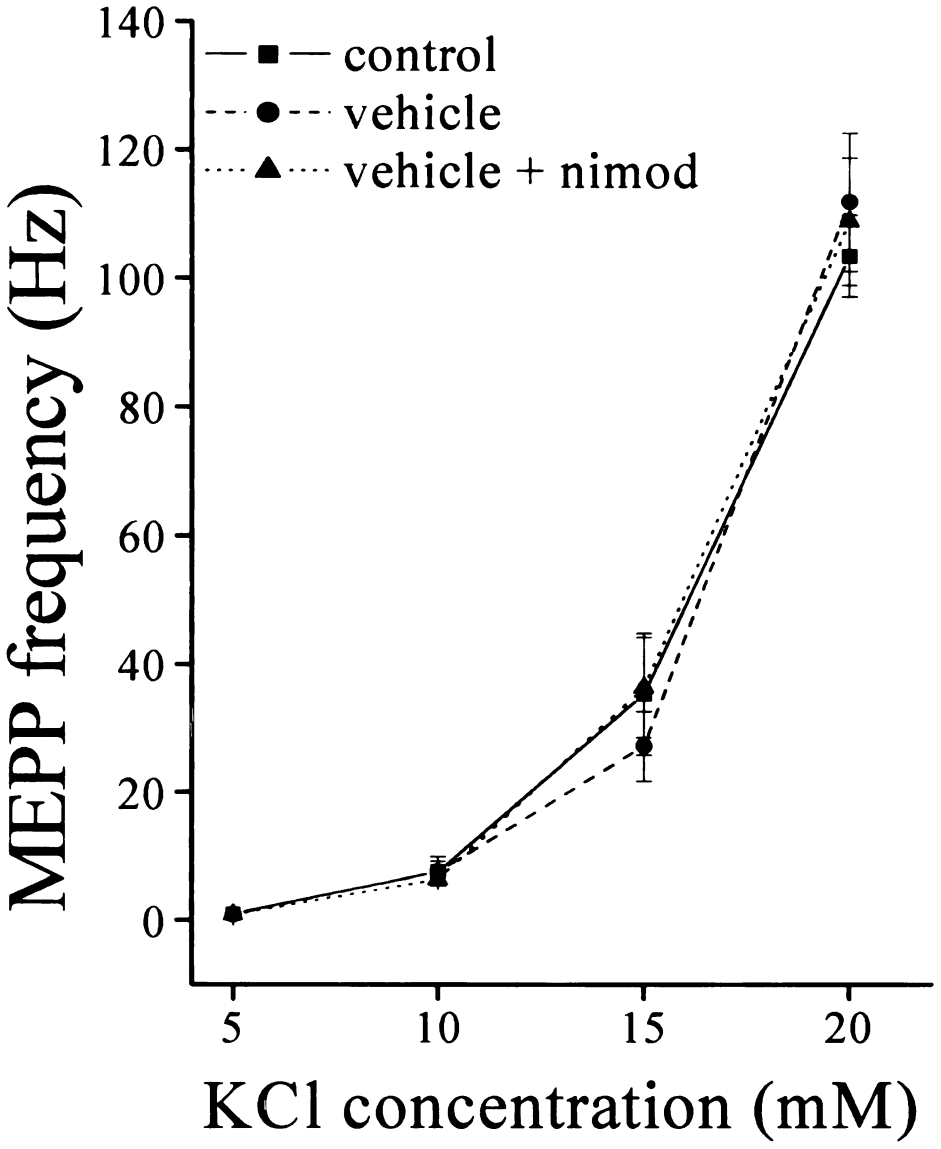

Figure 4.11. Effects of nimodipine (nimod) in the absence of iberiotoxin on MEPP frequency at mouse hemidiaphragm preparations. Recordings were made from preparations before (control), after perfusion with iberiotoxin vehicle (0.01 % BSA) for 1 hr, and following perfusion with both nimodipine (10 μM) and iberiotoxin vehicle (0.01% BSA) for an additional 25 min in buffers containing either 20, 15, 10, or 5 mM KCl. An equiosmolar substitution was made in the NaCl concentration to compensate for the varying KCl concentrations in the buffer. Each value represents the mean ± S.E.M. of at least 4 different preparations.

part be due to unmasking of silent channels.

One possible reason for involvement of the L-type Ca^{2+} channels in release of ACh from motor nerves during LEMS may involve a corresponding reduction in K_{Ca} currents at the nerve terminal. K_{Ca} channels are activated in the presence of Ca^{2+} and colocalized at active zone regions with Ca^{2+} channels normally involved in release of ACh (P/Q- type at mammalian and N-type at amphibian motor nerve terminals) (Robitaille and Charlton, 1992; Robitaille *et al.*, 1993; Xu and Atchison, 1996; Protti and Uchitel, 1997). Although the exact role of K_{Ca} channels is unknown, they are believed to aid in the repolarization of the nerve terminal following the incoming action potential and thus, limit the duration over which Ca^{2+} channels remain involved in transmitter release (Mallart, 1985; Robitaille and Charlton, 1992). In LEMS, reduced entry of Ca^{2+} into the nerve terminal may attenuate activation of colocalized K_{Ca} channels and in turn alter repolarization of the membrane. This prolonged depolarization of the nerve terminal may lead to changes in gating kinetics of L-type Ca^{2+} channels to the extent that they now become involved in ACh release. In fact, intense nerve terminal depolarization has been shown to alter the phenotype of Ca^{2+} channels involved in ACh release from motor (Hong and Chang, 1990; Correia-de-Sá *et al.*, 2000a,b) and sympathetic nerves (Somogyi *et al.*, 1997). The present study was designed, to examined if loss of functional K_{Ca} channels at motor nerve terminals could expose silent L-type Ca^{2+} channels involved in ACh release.

Incubation of muscle preparations with iberiotoxin, a K_{Ca} channel antagonist, significantly increased quantal content in comparison to untreated terminals. This finding is consistent with those of earlier reports (Robitaille and Charlton, 1992; Robitaille *et al.*, 1993;

Vatanpour and Harvey, 1995), and those in which K_{Ca} channel block affects perineurial Ca^{2+} currents recorded at motor nerve terminals (Xu and Atchison, 1996). Thus, functional antagonism of K_{Ca} channels influences Ca^{2+} current flow and in turn enhances the release of ACh.

Further, treatment of muscle preparations with nimodipine, a dihydropyridine-sensitive L-type Ca^{2+} channel antagonist in the presence of iberiotoxin reduced the enhanced ACh release caused initially by iberiotoxin alone. Therefore, it appears that normally silent L-type Ca^{2+} can participate in ACh release from motor nerve terminals lacking functional K_{Ca} channels. These findings are consistent with those reported by Hong and Chang (1990), in which L-type Ca^{2+} channels are involved in ACh release from mammalian motor nerve terminals pretreated with 3,4 diaminopyridine (DAP), which blocks voltage-dependent K^+ channels. Interestingly, block of L-type Ca^{2+} channels reduces the increased duration, but not the enhanced amplitude of EPPs caused by 3,4- DAP. Unlike 3,4-DAP, iberiotoxin had no effect up the duration of EPPs, but only affects the amplitude of EPPs. The reason for this difference is unclear, but may reflect functional and local differences between voltage-dependent-K^+ channels and K_{Ca} channels. Block of K_{Ca} channels, which are colocalized with voltage-gated Ca^{2+} channels, enhances transmitter release faster than that observed with block of voltage-gated K^+ channels (Vatanpour and Harvey, 1995). Also, voltage-dependent K^+, but not K_{Ca} channels, cause repetitive transmitter release following a single stimulation (Hong and Chang, 1990; Vatanpour and Harvey, 1995), which may influence the gating of L-type channels. The intimate colocalization of K_{Ca} with Ca^{2+} channels involved normally in release may allow K_{Ca} to have a faster and more direct effect upon these Ca^{2+} channels than that observed with loss of voltage-

gated K^+ channels alone. My findings and those reported by Hong and Chang (1990) are however, in contrast with those reported by Giovannini *et al.* (2002), where enhanced ACh from mouse motor nerve terminals in the presence of the voltage-dependent K^+ channel antagonist, 4-aminopyridine was unaffected by L-type Ca^{2+} channel antagonists. This discrepancy most likely reflects differences in experimental protocols.

The effect of iberiotoxin on MEPP frequency was also examined. Addition of 5,10,15, or 20 mM KCl to the buffer caused a corresponding depolarization of the endplate resting membrane potential and presumably of the nerve terminal as well. MEPP frequency increased significantly as the resting membrane potential became more depolarized. This was unaffected by iberiotoxin and/or nimodipine in buffer containing 5, 10, or 15 mM KCl. However, in the presence of 20 mM KCl, addition of iberiotoxin significantly further increased MEPP frequency, whereas further addition of nimodipine in the presence of iberiotoxin attenuated the enhanced release caused by iberiotoxin alone. The amplitude of MEPPs was unaltered in the presence of iberiotoxin at all concentrations of KCl tested. Thus, the effect of iberiotoxin on ACh release appears to occur only when the membrane is depolarized by KCl concentrations greater than 15 mM. This is consistent with the result on evoked release in which the membrane potential is markedly depolarized from rest. It is also consistent with the biophysical properties of L-type Ca^{2+} channels which require strong depolarization from rest to induce opening.

The exact mechanism involved in unmasking silent L-type Ca^{2+} channels during block of K_{Ca} channels is unclear, and may be multifaceted. K_{Ca} channels may exert a direct effect by altering localized membrane potentials and thus, prevent opening of L-type Ca^{2+} channels,

which require strong depolarization for activation (Miller, 1987; Tsien *et al.*, 1988; Zhang *et al.*, 1993). As such, recruitment of silent L-type Ca^{2+} channels involved in ACh release is evident during prolonged or high frequency stimulation of motor (Correia-de-Sá *et al.*, 2000b; Correia-de-Sá *et al.*, 2000a) and sympathetic nerves (Somogyi *et al.*, 1997) or during block of voltage-dependent K^+ channels (Hong and Chang, 1990). Also, L-type Ca^{2+} channels, which may be located at a site distinct from active zone regions, may require prolonged periods of openings in order to allow diffusion of Ca^{2+} through these channels to reach the release machinery (Miller, 1987; Elhamdani *et al.*, 1998).

Alternatively, activation of L-type Ca^{2+} channels in the presence of iberiotoxin may involve more indirect mechanisms. Enhanced release of ACh from mammalian motor nerves by α_1 adrenergic-receptor activation is abolished following functional antagonism of L-type channels (Wessler *et al.*, 1990). Intense and prolonged depolarization has been implicated to enhance Ca^{2+} currents by increasing phosphorylation of L-type Ca^{2+} channels (Sculptoreanu *et al.*, 1995). In the presence of muscarinic receptor-dependent activation of protein kinase C, L-type Ca^{2+} channel activity at adult rat major pelvic ganglia is increased. Furthermore, it has been postulated that at mammalian motor nerve terminals, L-type Ca^{2+} channels are in close proximity to A_{2A} adenosine receptors and activation of A_{2A} adenosine receptors during prolonged depolarization most likely unmasks L-type Ca^{2+} channels *via* activation of protien kinases (Correia-de-Sá *et al.*, 2000a). More direct evidence also supports the role of protein kinases in activating L-type Ca^{2+} channels involved in ACh release from mature mammalian motor nerves (Urbano *et al.*, 2001).

The ability of bay K 8644, a DHP-sensitive L-type channel agonist to enhance ACh

release in comparison to control treatments, further supports the notion that silent L-type channels exist at mammalian motor nerve terminals and corroborates previous reports (Atchison and O'Leary, 1997; Atchison, 1989). However, incubation of motor nerve terminals with bay K 8644 in the presence of iberiotoxin did not alter the release of ACh in comparison to iberiotoxin alone. Thus, if iberiotoxin simply unmasked silent L-type channels then addition of bay K 8644, which increases the duration of L-type Ca^{2+}channel openings (Nowycky *et al.*, 1985), should have further increased ACh release. It is possible that mechanisms involved in L-type channel activation or L-type channel involvement with ACh release become saturated in the presence of iberiotoxin and thus, bay K 8644 has no additional effect. Alternatively, iberiotoxin and bay K 8644 may act through similar mechanisms that reach a maximum state of activation in the presence of either drug alone. Although, iberiotoxin does not bind to L-type Ca^{2+} channels, loss of K_{Ca} channels in the presence of iberiotoxin may activate secondary pathways that act in a manner similar to that of bay K 8644 directly. The precise reason for the inability of bay K 8644 to enhance the iberiotoxin-induced release of ACh is still elusive, however.

In conclusion, loss of functional K_{Ca} channels, which presumably delays nerve terminal membrane repolarization, activates normally silent L-type Ca^{2+} channels involved in ACh release at adult mammalian motor nerves. The role which these silent L-type Ca^{2+} channels play at the adult mammalian motor nerve terminal remains unclear, but may offer a means to maintain a certain level of ACh release during periods of intense nerve stimulation or in certain pathological conditions, in which involvement of Ca^{2+} channels normally responsible for ACh release is impaired.

CHAPTER FIVE

INVOLVEMENT OF P/Q-TYPE Ca^{2+} CHANNELS IN RELEASE OF ACETYLCHOLINE FROM MOTOR NERVE TERMINALS FOLLOWING EXPOSURE TO LEMS PLASMA

A. *Introduction*

Voltage-dependent Ca^{2+} channels (VDCC) serve a vital role in neuronal activity and as such, couple the action potential to release of transmitters from the nerve terminal (Augustine *et al.*, 1987; Katz *et al.*, 1997). Multiple types VDCC have been identified based upon electrophysiological, molecular, and pharmacological techniques and thus, far include the following subtypes: T, L, N, R, P, and Q (Tsien *et al.*, 1988; Zhang *et al.*, 1993; Catterall, 1998; Newcomb *et al.*, 1998). Although the exact role of each VDCC subtype throughout the nervous system is still unclear, those channels involved in release of ACh from motor nerve terminals have been identified. However, the specific VDCC subtype coupled to release of ACh from motor nerve terminals vary based upon species (Sano *et al.*, 1987; De Luca *et al.*, 1991; Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997) and age (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001). During development, release of ACh from immature motor nerve terminals is controlled by Ca^{2+} entry through multiple subtypes of VDCC; immature motor nerve terminals from mice and rats utilize P/Q-, N-, and L-type Ca^{2+} channels to affect release of ACh (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001). On the other hand, mature motor nerves mainly possess one VDCC subtype involved in release of ACh. Based on pharmacological sensitivities to agatoxin IVA, a specific antagonist for P/Q-type Ca^{2+} channels and to ω-conotoxin GVIA, a specific antagonist for N-type Ca^{2+} channels, the VDCC subtypes involved in release of ACh at mature motor nerve terminals have been identified. As such, P/Q- and N- Ca^{2+} channel subtypes are involved in release of ACh from mature mammalian (Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997) and amphibian motor nerve terminals (Sano *et al.*, 1987), respectively.

In Lambert-Eaton myasthenic syndrome (LEMS), a neurological disorder affecting release of ACh, antibodies down-regulate multiple subtypes of VDCC. However, the P/Q-type Ca^{2+} channel appears to be affected preferentially (Lennon *et al.*, 1995; Garcia and Beam, 1996; Houzen *et al.*, 1998 Pinto *et al.*, 1998a; Engisch *et al.*, 1999; Pinto *et al.*, 2002). Based upon the VDCC subtype controlling release of ACh from mature mammalian motor nerve terminals and antibody specificities in LEMS, it is clear why neuromuscular weakness is the most common symptom reported by LEMS patients. Although direct access to the mammalian motor nerve terminal is precluded by its small size, indirect electrophysiological recordings have been used to determine the effect of passive transfer of LEMS to mice on the P/Q-type Ca^{2+} currents at the motor nerve terminal. Overall, Ca^{2+} current amplitudes are reduced from motor nerve terminals of mice exposed to LEMS plasma in comparison to those at motor nerve terminals exposed to plasma from disease-free individuals. However, the actions of agatoxin IVA, which normally antagonize the majority of P/Q-type Ca^{2+} currents are reduced at LEMS motor nerve terminals in comparison to controls (Xu *et al.*, 1988); agatoxin IVA reduces the amplitude of the P/Q-type Ca^{2+} currents at control motor nerve terminals by approximately 90%, whereas agatoxin IVA only attenuates the P/Q-type Ca^{2+} current amplitude by approximately 45% from LEMS motor nerve terminals. Thus, it appears that passive transfer of LEMS to mice reduces the number of functional P/Q-type Ca^{2+} channels. Furthermore, the percent Ca^{2+} current remaining following passive transfer of LEMS is greater than that observed at controls, indicating the possible expression of a Ca^{2+} current not normally observed.

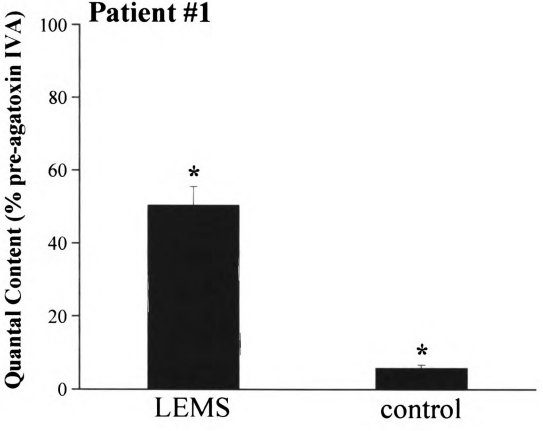

Figure 5.1. Effect of LEMS plasma on the functional P/Q-type Ca^{2+} channels involved in release of ACh from mature mouse motor nerve terminals. Diaphragm neuromuscular preparations were obtained from mice (20-22g) following injection of LEMS or control plasma for 30 days. Prior to injection with plasma, mice were first injected with 300 mg/kg cyclophosphamide in order to suppress the immune response to foreign proteins. Muscles were cut on either side of the main intramuscular nerve branch to prevent contractions that occur during nerve-stimulation. EPPs and MEPPs amplitudes were determined using conventional intracellular recording techniques. EPPs were elicited during 0.5 Hz stimulation of the phrenic nerve. Quantal content was determined using the ratio of the average EPP amplitude to the average MEPP amplitude. Values are expressed as the percentage of quantal content from plasma-exposed preparations after addition of 150 nM agatoxin IVA to those before addition of agatoxin IVA. The asterisks (*) indicates a value significantly different from pre-agatoxin IVA treated preparations ($P < 0.5$ Hz).

B. *Summary*

The effect of passive transfer of LEMS plasma to mice on the number of functional P/Q-type Ca^{2+} channels involved in release of ACh from the motor nerve terminal was examined (Fig. 5.1). Following injection of control plasma into mice for 30 days, agatoxin IVA reduced the nerve-stimulated release of ACh to approximately 90%. On the other hand, agatoxin IVA only inhibited the nerve-stimulated release of ACh by approximately 50% following passive transfer of LEMS to mice by daily injections for 30 days. These findings are consistent with those reported by Xu *et al.* (1998), in which passive transfer of LEMS to mice following 30 days of injections had a similar effect on the P/Q-type Ca^{2+} current at the motor nerve terminal. Furthermore, the ability of agatoxin IVA to reduce almost completely the nerve-stimulated release of ACh from control preparations also corroborates the findings reported elsewhere, which have shown that P/Q-type Ca^{2+} channels are the primary subtype of VDCC involved in release of ACh from mature mammalian motor nerve terminals. The effect of agatoxin IVA on release of ACh following injection of mice with LEMS plasma for 30 days in comparison to controls has two important implications: 1) the number of functional P/Q-type Ca^{2+} channels involved in nerve-stimulated release of ACh is reduced in LEMS and 2) the component of nerve-stimulated release of ACh unaffected by agatoxin IVA may reflect Ca^{2+} entry through a VDCC subtype not normally found at the mature mammalian motor nerve terminal.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

Summary and Conclusions

The overall aim of this dissertation was to examine the role of L-type Ca^{2+} channels in release of ACh from motor nerves following passive transfer of LEMS to mature mice. Although antibodies from patients with LEMS have been observed to down-regulate a variety of functional voltage-dependent Ca^{2+} channel subtypes, the P/Q-type channel appears to be the primary target (Lennon *et al.*, 1995; Garcia and Beam, 1996; Houzen *et al.*, 1998 Pinto *et al.*, 1998a; Engisch *et al.*, 1999; Pinto *et al.*, 2002). Furthermore, among the multiple VDCC subtypes known to exist, the P/Q-type channel appears to be the predominant channel subtype involved in release of ACh from mature mammalian motor nerve terminals (Uchitel *et al.*, 1992; Protti *et al.*, 1996; Katz *et al.*, 1997). Based on these findings, the paucity of nerve-stimulated release of ACh and resulting skeletal muscle weakness that ensues in patients with LEMS is therefore not surprising.

Passive transfer of LEMS to mature mice also reduces nerve-stimulated release of ACh stimulation (Lambert and Elmqvist, 1971; Lang et al., 1983; Kim, 1985; Prior and Newsom-Davis, 1985; Lambert and Lennon, 1988). Along these lines, LEMS antibodies injected into mature mice for 30 days attenuate P/Q-type Ca^{2+} current at motor nerve terminals (Xu *et al.*, 1998) However, L-type Ca^{2+} current, which is not normally found at mature mouse motor nerve terminals is present following passive transfer of LEMS for 30 days (Xu *et al.*, 1998). Not only is this finding in contrast with the known effects of LEMS antibodies, but represents expression of a Ca^{2+} current not normally observed.

It is possible that this novel L-type Ca^{2+} current may participate in ACh release and/or complex signaling pathways that have as of yet, no clearly identified function. However, based

on the effects of LEMS antibodies, it seems likely that this novel current is involved in release of ACh in order to compensate for the reduction in P/Q-type channels. Furthermore, during maturation of some mammalian motor nerves, not only P/Q-, but L-type Ca^{2+} channels participate in release of ACh as well (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002; Santafe *et al.*, 2002). At birth, developing muscle fibers are innervated by multiple motor nerves, which through a process of activity-dependent synaptic elimination, decrease until only one nerve is eventually associated with a single end-plate (Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2000; Santafe *et al.*, 2002). It appears that the function of multiple subtypes of VDCC found at motor nerve terminals differs depending on the eventual role of the motor nerve in synaptic transmission in mature mammals; motor nerves destined to become involved in release of ACh in mature mammals possess multiple VDCC subtypes, including P/Q- and L-types that are both involved in release of ACh throughout development. On the other hand, P/Q- and L-type Ca^{2+} channels located on motor nerve terminals destined for elimination are believed to be involved in activation of cytoplasmic proteases that aid in neuronal death and only weakly participate in release of ACh (Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001; Santafe *et al.*, 2002). Thus, motor nerves that release the largest amount of ACh during development survive, whereas those nerves that release minimal ACh eventually are eliminated. Although the role of multiple VDCC subtypes on surviving motor nerve terminals during development is unclear, it is believed they act to keep Ca^{2+} entry and thus, ACh release, at high levels until expression of P/Q-type Ca^{2+} channels becomes maximal (Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001; Santafe *et al.*, 2002). Based on these findings, L-type Ca^{2+} current observed after passive transfer of LEMS to

mature mice most likely reflects actions of multiple subtypes of VDCC on motor nerve terminals destined to survive development. Thus, it was my contention that this novel L-type Ca^{2+} current at motor nerve terminals of mice with LEMS may compensate for lost P/Q-type Ca^{2+} channels and become involved in release of ACh.

Following passive transfer of LEMS to mice by 30 days of daily injection, the spider toxin, ω-Aga IVA, at concentrations used to inhibit both P- and Q-type Ca^{2+} channels, reduced the nerve stimulated-release of ACh from motor nerves to a lesser extent than that observed when applied to control preparations; ω-Aga IVA inhibited the nerve-stimulated release of ACh by approximately 90% at neuromuscular junction (NMJ) preparations obtained from mature mice following injection of control plasma for 30 days, whereas ω-Aga IVA only reduced release of ACh by approximately 50% following injection of LEMS plasma into mature mice for 30 days. This finding is consistent with the effects of passive transfer of LEMS to mature mice on the P/Q-type Ca^{2+} currents (Xu and Atchison, 1998). The inability of ω-Aga IVA to reduce quantal content at LEMS NMJ preparations to the same extent as those observed from controls, suggests that LEMS plasma attenuates the number of functional P/Q-type channels at the motor nerve terminal of mature mice. Furthermore, the remaining quantal content observed after the addition of ω-Aga IVA to LEMS NMJ preparations indicates a possible role of another VDCC subtype involved in release of ACh. This finding lends support to my contention that L-type Ca^{2+} currents observed at motor nerve terminals following injection of LEMS plasma for 30 days into mature mice is involved in release of ACh.

However, in order to determine clearly the role of L-type Ca^{2+} current in release of ACh at LEMS motor nerves, the pharmacological sensitivity to the dihydropyridine L-type Ca^{2+}

channel antagonist, nimodipine was examined. As such, nimodipine reduced the nerve-stimulated release of ACh at NMJ preparations obtained from mature mice injected with LEMS plasma for 30 days, but did not affect release of ACh at NMJ preparations obtained from mice injected with control plasma for a similar duration. These findings support the idea that L-type Ca^{2+} current at motor nerve terminals in LEMS effect release of ACh.

This involvement of L-type Ca^{2+} channels in release of ACh from LEMS motor nerve terminals may represent an unmasking of silent L-type channels already present on the motor nerve terminal. Although L-type Ca^{2+} current is not normally observed at mature mouse motor nerve terminals, various mechanisms exist to unmask current through these channels, which can participate in release of ACh. L-type Ca^{2+} channels become involved in ACh release from mature mammalian motor nerve terminals following incubation with the dihydropyridine agonist, bay K 8644 by increasing the duration of channel openings (Atchison and O'Leary, 1987; Atchison, 1989) or during increased protein phosphorylation in conjunction with intracellular Ca^{2+} chelation (Urbano *et al.*, 2001).

Based on these observations, it seemed plausible that involvement of L-type Ca^{2+} channels in release of ACh following exposure of motor nerves to LEMS plasma results from unmasking of normally silent channels. While this possibility can not be rejected, experiments contained in this dissertation provide some evidence that L-type Ca^{2+} channel involvement in release of ACh from LEMS motor nerves is due to more than merely unmasking of silent channels. For instance, L-type Ca^{2+} channels did not become involved in release of ACh from motor nerves exposed to LEMS plasma for 2 or 24 hours. This finding could potentially be due to the differences in the extent of reduction of quantal content between 30 days injection and

2 or 24 hour exposures. Injection of mature mice for 30 days with plasma from patients with LEMS not only significantly reduced the nerve-stimulated release of ACh, but induced facilitation in the release of ACh during high frequency stimulation of the motor nerve as well. These findings were not observed using control preparations and are consistent with those reported in other model systems and those observed from EMG recordings of patients with LEMS (Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Jablecki, 1984; O'Neill and Newsom-Davis, 1988). Similarly, exposure of NMJ obtained from mature mice to LEMS plasma for 2 and 24 hours also induced the electrophysiological characteristics of LEMS. However, under the experimental conditions used to examine the effect of LEMS plasma on motor nerve terminals exposed for 30 days and 2 and 24 hours, differences in the extent of reduction of nerve-stimulated release of ACh were only significantly different between 2 hours and 30 day treatment regimens. The lack of significance between the other treatment groups may reflect the manner in which the effect of LEMS plasma on ACh release was measured. It is possible that injection of mice with LEMS plasma for 30 days reduces the number of functional P/Q-type Ca^{2+} channels involved in ACh release to a significantly greater extent compared to exposure for 24 hours to LEMS plasma. However, expression of L-type Ca^{2+} current following 30 days, but not 24 hours of LEMS exposure somewhat compensates for the reduced number of P/Q-type Ca^{2+} channels and thus, differences in nerve-stimulated release of ACh appear similar. It is possible therefore, that significant differences of LEMS antibodies on the motor nerve terminal are obscured among the different treatment groups. Nevertheless, 2 and 24 hour exposure of motor nerve terminals to LEMS plasma induces the overall reduction in nerve-stimulated ACh release to an appreciable extent without involving L-type Ca^{2+} channels

in ACh release. Thus, the involvement of L-type Ca^{2+} channels in release of ACh from motor nerve terminals of mature mice following injection of LEMS plasma for 30 days is most likely due to long-term processes, such as synthesis of new channel components and not simply unmasking silent channels.

The time course of development of L-type Ca^{2+} channel involvement in release of ACh from LEMS motor nerve terminals was examined further using NMJ preparations from mature mice injected with LEMS plasma for varying lengths of time. Nerve-stimulated release of ACh was reduced significantly following injection of mice with plasma from two patients (designated arbitrarily as "Patients 1 and 2") after 1- 20 days in comparison to animals injected with plasma from disease-free controls. However, L-type Ca^{2+} channels only became involved in nerve-stimulated release of ACh following injection of plasma for more than 20 days. Interestingly, plasma from a third patient with LEMS (designated "Patient 3") only significantly affected release of ACh after 10 days of treatment and did so without inducing the involvement of L-type Ca^{2+} channels. The reasons for the inability of this patient's plasma to induce L-type Ca^{2+} channel involvement in ACh release are unclear, but may relate to the antibody titer in the plasma, which was not measured. It is possible that if the antibody titer of this particular patient's plasma was lower than that of the other two patients a longer exposure time would be required. However, L-type Ca^{2+} channels did not become involved in ACh release even after 30 days of using this treatment with this patient's plasma.

Qualitative differences among the antibody specificity may also exist. This has been shown previously in studies in which antibodies to other proteins involved in ACh secretion such as synaptotagmin occur in plasma of some LEMS patients (Leveque *et al.*, 1992;

Takamori *et al.*, 1995; Takamori *et al.*, 2000a). Although antibody titer specificity for subtypes of VDCC was not directly measured in experiments comprising this dissertation, based on functional studies, it appears that all patients' plasma contained antibodies for P/Q-type Ca^{2+} channels because all three patients' plasma eventually impaired ACh release. However, it is possible that plasma from "Patient 3" possessed antibodies directed against not only P/Q-, but L-type Ca^{2+} channels as well. Therefore, L-type Ca^{2+} channel involvement in ACh release from motor nerves exposed to this patient's plasma might not become apparent. The involvement of L-type Ca^{2+} channels in release of ACh from motor nerves exposed to the other patients' plasma suggests that their antibody titers specific for L-type Ca^{2+} channels would have to be quite small or even non-existent. However, the possibility that these patients' plasma do not contain antibodies specific for L-type Ca^{2+} channels can not be fully excluded.

The time differences necessary to reduce significantly release of ACh by the various patients' plasma may further reflect differences in antibody titers. Based upon the findings of this dissertation, I would predict that "Patient 3" possessed the lowest antibody titers specific for P/Q-type channels. Alternatively, the VDCC-specific antibodies may have different avidities among the LEMS patients' plasma. In such a condition, antibody titers may be equal among the patients' plasma, but varying avidities for VDCC would differentially affect the time and extent of reduction in the release of ACh. If each LEMS patient's plasma contained similar antibody titers with differing avidities, then it appears that plasma from "Patient 3" contained antibodies with the lowest avidity. However, based upon the work presented in this dissertation a distinction between antibody titers and avidities among the different patients' plasma can not be made directly.

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In conjunction with the effect of each individual patients' plasma on release of ACh during low frequency nerve-stimulation, preparations obtained from mice injected for 1-20 days with plasma from "Patient 1" or "2" induced significant facilitation of ACh release during high frequency nerve-stimulation, whereas that of "Patient 3" did not induce facilitation until after day 10. Based upon these findings, it appears that a significant reduction in release of ACh during low frequency nerve-stimulation is necessary in order for facilitation of ACh release to occur. This finding lends support to the idea that Ca^{2+}-dependent mechanisms must exist at the motor nerve terminal to induce closure of VDCC during periods of intense nerve-stimulation, and facilitation only occurs when these mechanisms are not activated. This idea is not unique, and Ca^{2+}-dependent inactivation of multiple subtypes of VDCC is known to occur (Tareilus *et al.*, 1994). Although the function of this mechanism is not fully understood, it is believed to act not only as a means to control tightly transmitter release (Gutnick *et al.*, 1989), but also to prevent cytotoxicity and cellular death that results from the cytoplasm being overloaded with excess Ca^{2+} (Choi, 1988).

In addition to becoming involved in release of ACh during low frequency nerve-stimulation, L- type Ca^{2+} channels also appear to comprise a component of the facilitation observed following passive transfer of LEMS in the 20 day treatment regimen. However, the extent of involvement of L-type Ca^{2+} in release of ACh during low frequency stimulation appears greater than that which occurs during high frequency nerve stimulation. Although the reasons for this discrepancy are unclear, it is possible that mechanisms exist which reduce L-type VDCC function during high frequency nerve-stimulation to a greater extent than during low frequency nerve-stimulation.

In corroboration with the observations reported by other investigators, MEPP frequencies or amplitudes measured from NMJ preparations in buffers containing physiological concentrations of K^+ were not altered by exposure of the motor nerve to any of the LEMS patient or control plasma. This finding further supports the idea that the LEMS antibodies act on the presynaptic motor nerve terminal without altering vesicle size or contents. Although one could make an argument that LEMS antibodies affects vesicle size, contents and ACh receptors located at muscle end-plates such that MEPP amplitudes appear unchanged, these structures have been shown to be unaffected in LEMS (Molenaar *et al.*, 1982; Lang and Vincent, 1984). Furthermore, the findings that MEPP frequency is unaltered during passive transfer suggest that intraterminal Ca^{2+} buffering mechanisms are unaltered in LEMS.

In contrast to the observations during nerve-stimulation, L-type VDCC were not involved in spontaneous release of ACh at NMJ preparations obtained from mice injected with any of the LEMS patient or control plasma. This finding has multiple implications: 1) it is possible that the number of functional L-type channels is not as high as the number of functional P/Q-type channels, 2) L-type channels may be located at sites distinct from ACh release sites, 3) and/or mechanisms exist which have differential effects upon L- and P/Q-type channels depending on the membrane potential. Most likely, the lack of involvement of L-type VDCC in spontaneous release of ACh is due a combination of the all of the above mentioned reasons. However, experiments performed for this thesis did not examine the densities of L-type channels expressed during long-term exposure of motor nerves to LEMS plasma. Although involvement of L-type Ca^{2+} channels in release of ACh was examined, correlations between channel function and density can not be made. For example, L- and N-type channels are

expressed at adrenal chromaffin cells of rats in equal densities, however, secretion of catecholamines is reduced by L-, but not N-type channel antagonists (Lopez 1994). Thus, functional involvement of L-type Ca^{2+} channels in release of ACh may not be directly related to the density of channels found on the nerve terminal. However, experiments comprising this thesis were performed, which used pharmacological methods to determine the approximate location of L-type Ca^{2+} channels on the motor nerve terminal. DM-BAPTA, a rapid chelator of Ca^{2+}, was loaded into motor nerve terminals in order to test the hypothesis that P/Q-, but not L-type channels are in close proximity to ACh release sites. This was based on the premise that coupling between the normal complement of P/Q-type VDCC and release sites is so tight that entry of Ca^{2+} through these channels would activate release of ACh before being bound by DM-BAPTA (Urbano *et al.*, 2001). However, Ca^{2+} which entered through VDCC located at sites distinct from the release apparatus would be bound by DM-BAPTA prior to activating release of ACh. Thus, DM-BAPTA did not affect nerve-stimulated release of ACh from control preparations. However, the involvement of L-type VDCC in ACh release was abolished from motor nerve terminals loaded with DM-BAPTA. Based upon the observations that P/Q- type Ca^{2+} channels represent the normal VDCC subtype controlling nerve-stimulated release of ACh from mature mouse motor nerves before and after control-plasma exposure and DM-BAPTA has no effect on release of ACh from untreated (Urbano *et al.*, 2001) or control-plasma exposed motor nerves implies an extremely tight coupling between P/Q-type Ca^{2+} channels and ACh release sites. L-type VDCC, on the other hand, appear to be located at a site distinct from the release sites. However, based on the findings in this dissertation it is unclear if newly synthesized L-type Ca^{2+} channels are inserted into the motor nerve terminal membrane or

expressed on intraterminal structures (for example, the endoplasmic reticulum). Support for an intraterminal location of L-type Ca^{2+} channels is based on the following: bay K 8644 increases the frequency of spontaneous release of ACh at mouse NMJ preparations in Ca^{2+}-free medium (Pancrazio *et al.*, 1989), and immunostaining of motor nerve terminal membranes obtained from mature rats does not reveal the presence of L-type Ca^{2+} channels comprised of either $\alpha_{1D,C}$ subunits (Westenbroek, *et al.*, 1998). It is possible that L-type Ca^{2+} channels located in the motor nerve terminal membrane of mature rats are comprised of α_1 subunits of the S, R, or some other unidentified class or that the number of L-type Ca^{2+} channels is too low on the motor nerve terminal membrane to detect using immunostaining methods. Nevertheless, in the presence of membrane potentials close to the resting value of -80 mV, influx of Ca^{2+} through a small number of L-type channels may be buffered by intracellular proteins and sequestration mechanisms prior to diffusing to ACh release sites. On the other hand, during strong membrane depolarization to levels more positive than - 50 mV , which increases the probability of many L-type channel openings, a large amount of Ca^{2+} enters, which may overwhelm intracellular buffering mechanisms, and thus, affect release of ACh.

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 idea 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. 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 and Uchitel, 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 (Sugiura and Ko, 1997; Rosato Siri and Uchitel, 1999; Santafe *et al.*, 2001; Rosato-Siri *et al.*, 2002; Santafe *et al.*, 2002). 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.

The finding of differential localization of multiple VDCC subtypes is not unique to LEMS. BAC cells possess Q-type Ca^{2+} channels located in close proximity to catecholamine release sites and L-type Ca^{2+} channels located at a distinct site (Lara *et al.*, 1998). Similar findings have been observed at motor nerve terminals from neonatal (0-4 day old) rats (Rosato-Siri *et al.*, 2002); P/Q- and N-type channels both appear to be involved in ACh release, however, P/Q-, but not N-type channels remain involved in release of ACh from BAPTA-AM loaded nerve terminals, providing evidence for differential localizations of these channel subtypes. Differential localizations of multiple VDCC subtypes may allow fine tuning of transmitter release under varying stimuli or physiological conditions.

Although the normal lack of involvement of L-type Ca^{2+} channels in release of ACh from mature mammalian motor nerves is not fully understood, it is possible that P/Q-type Ca^{2+}

channels may be better suited for the precise nature of ACh release. Using a fluorescent Ca^{2+} ion indicator it has been observed that, entry of Ca^{2+} through VDCC involved in release of ACh occurs in microdomains instead of a general diffuse pattern (Llinas *et al.*, 1992). This control is related to the biophysical properties, location, and other secondary mechanisms that affect channel function. As such, the large single channel conductance of L-type channels and slow rate of voltage-dependent inactivation may not allow precise control of Ca^{2+} influx to occur in microdomains. This in turn may prevent tightly controlled release, thus requiring that L-type Ca^{2+} channels be located away from release sites. Differential localizations of multiple VDCC-subtypes may also reflect the amino acid sequences of comprising each channel subtype (Atlas, 2001); amino acid sequences and conformational 3-dimensional structure of P/Q-type Ca^{2+} channels may allow tight interactions with other proteins, such as synaptotagmin, that are directly associated with the release apparatus, whereas L-type Ca^{2+} may lack the necessary coupling sites. L-type Ca^{2+} channels found on non-motor nerves of the mammalian system are also generally believed not to be tightly coupled with fast transmitter release. Thus, it is not surprising to find L-type channels located at a site distinct from that of P/Q-type channels. However, in retinal bipolar cells, L-type Ca^{2+} channels appear to be associated with the release apparatus (Tachibana 1999). This finding may reflect differences in the α_1 subtype comprising L-type channels in retinal bipolar and those L-type channels or other proteins involved in ACh release from LEMS motor nerves. Currently, the subunit composition of native L-type Ca^{2+} channels involved in ACh release from motor nerves following passive transfer of LEMS is unknown. In addition, VDCC have been shown to be modulated directly by interactions with proteins comprising the release apparatus. Thus, normally silent L-type channels, which may

be located at sites distinct from the release apparatus, may not be involved in the release of ACh under normal conditions due to lack of modulation by proteins associated with the release apparatus.

The location of VDCC may be directly related to the function of the channel during development. Channels destined to be the primary subtype involved in ACh release from mature motor nerves are more closely associated with the release apparatus, while channels located at distinct sites have additional functions. L-type Ca^{2+} channels on immature motor nerve terminals may not only aid in release of ACh during periods of incomplete expression of P/Q-type channels, but also be involved in repressing development of motor nerves destined to be eliminated– functions, which could only arise due to the location of the channel. It also has been proposed that motor nerves from mice deficient in NCAM (neuronal cell adhesion molecule) exhibit two separate vesicle recycling pathways that resemble those found on immature and mature motor nerves (Polo-Parada *et al.*, 2001). Interestingly, L-type VDCC found in pre-terminal regions appear to be involved in the immature vesicle recycling pathway, but not involved in transmitter release from NCAM deficient motor nerves. It is possible that during exposure of motor nerve terminals to LEMS plasma, L-type Ca^{2+} channels assume the role they played during maturation based solely upon their location at the motor nerve terminal.

Based on the findings described thus far, a hypothetical model (Fig. 6.1) has been proposed to explain the involvement of L-type Ca^{2+} in ACh release from motor nerves exposed to LEMS plasma. Normally, tonic excitatory signals originating from the motor nerve terminal or post-synaptic muscle membrane activate the motor cell body to initiate the production and transport of L-type channel subunit proteins. Once these L-type Ca^{2+} channels subunits reach

the necessary site at the motor nerve terminal cytoplasm, they are assembled and inserted into the membrane. However, normally the excitatory signals that stimulate the cell body to begin channel production are inhibited tonically by multiple mechanisms, which may include signals originating from the pre- and post-synaptic structures. Consequently, Ca^{2+} influx through P/Q-type channels and ACh release may directly or indirectly act as negative regulators of the tonic excitatory signals to the cell body and/or as negative regulators of L-type channels already present at sites distinct from the release apparatus. During down-regulation of P/Q-type channels by antibodies from patients with LEMS, entry of Ca^{2+} through P/Q-type channels and release of ACh are reduced. This in turn leads to loss of signals that normally inhibit the tonic excitatory stimulation of L-type channel production in the cell body. Thus, in LEMS expression of L-type Ca^{2+} channels is increased. However, it is unclear if production of new P/Q-type channels also occurs in LEMS. It has been observed that during exposure of IMR cells to N-type VDCC antibodies, channel turnover is increased (Passafaro *et al.*, 1992). In conjunction with the vital role which P/Q-type Ca^{2+} channels play in release of ACh, it seems plausible that in LEMS, synthesis of new P/Q-type channels occurs in addition to production of new of L-type channels. However, P/Q-type Ca^{2+} channel subunit production and assembly may not be able to keep up with the continual loss of P/Q-type channels that occurs during exposure to LEMS antibodies. In such a situation, it would appear, therefore, that only new L-type channel subunits are synthesized.

Along these lines, Ca^{2+} influx has been observed to play a vital role in gene expression in many cellular model systems. For example, the amplitude and frequency of Ca^{2+} entry into B- and T-lymphocytes, respectively has been demonstrated to diferentially affect the expression

of multiple genes (Dolmetsch et al., 1997, 1998). The spatial and temporal effect of Ca^{2+} entry on nuclear responses also is VDCC subtype-specific—expression of syntaxin-1A in HEK293 cells is dependent on the presence of P/Q-, but not L-, N-, or T-type VDCC (Sutton *et al.*, 1999). On the other hand, activity of L-type Ca^{2+} channels, induces phosphorylation of CREB (Ca^{2+}/cAMP response element binding protein) in hippocampal neurons, whereas P/Q- and N-type channel activity does not alter CREB phosphorylation (Hardingham *et al.*, 1999; Nakazawa and Murphy, 1999). Thus, loss of functional VDCC as that which occurs in LEMS would likely alter expression of proteins comprising multiple VDCC subtypes.

The reason that synthesis of a VDCC subtype not normally involved in release occurs in LEMS is unclear, but could reflect in the mechanisms controlling all VDCC synthesis. It is possible that under conditions in which the levels of P/Q-type Ca^{2+} channels are reduced, signals activate a global increase in expression of multiple VDCC subtypes at the motor nerve terminal. Furthermore, it is also unclear why L-type channels are found at the motor nerve terminals, but not normally involved in release of ACh. It is possible that these channels are involved in some unidentified function at the motor nerve terminal and unrelated to release of ACh. However, under certain environmental conditions, these channels indirectly and accidentally become involved in ACh release strictly due to their location. Alternatively, these channels may merely represent residual channels, whose presence decreased, but was not eliminated totally during maturation.

Although relocation of newly synthesized and pre-existing L-type channels may occur in LEMS, this hypothesis was not tested in this dissertation. It appears unlikely, however,that the only mechanism involved is that of relocation of pre-existing channels. This is based upon

the long time necessary for L-type channels to become involved in release of ACh from LEMS-exposed motor nerve terminals. Experiments involving immunostaining with α1 subunits subtypes believed to comprise L-type channels of NMJ preparations obtained from mice injected with LEMS or control plasma for varying days, may clarify the role of relocation.

In addition to location, 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 terminal. 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 (Singer *et al.*, 1991; Neely *et al.*, 1993; Chien *et al.*, 1995; Brice *et al.*, 1997; Brice and Dolphin, 1999). Furthermore, differential modulation of multiple VDCC subtypes acts through 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 DRG 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 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

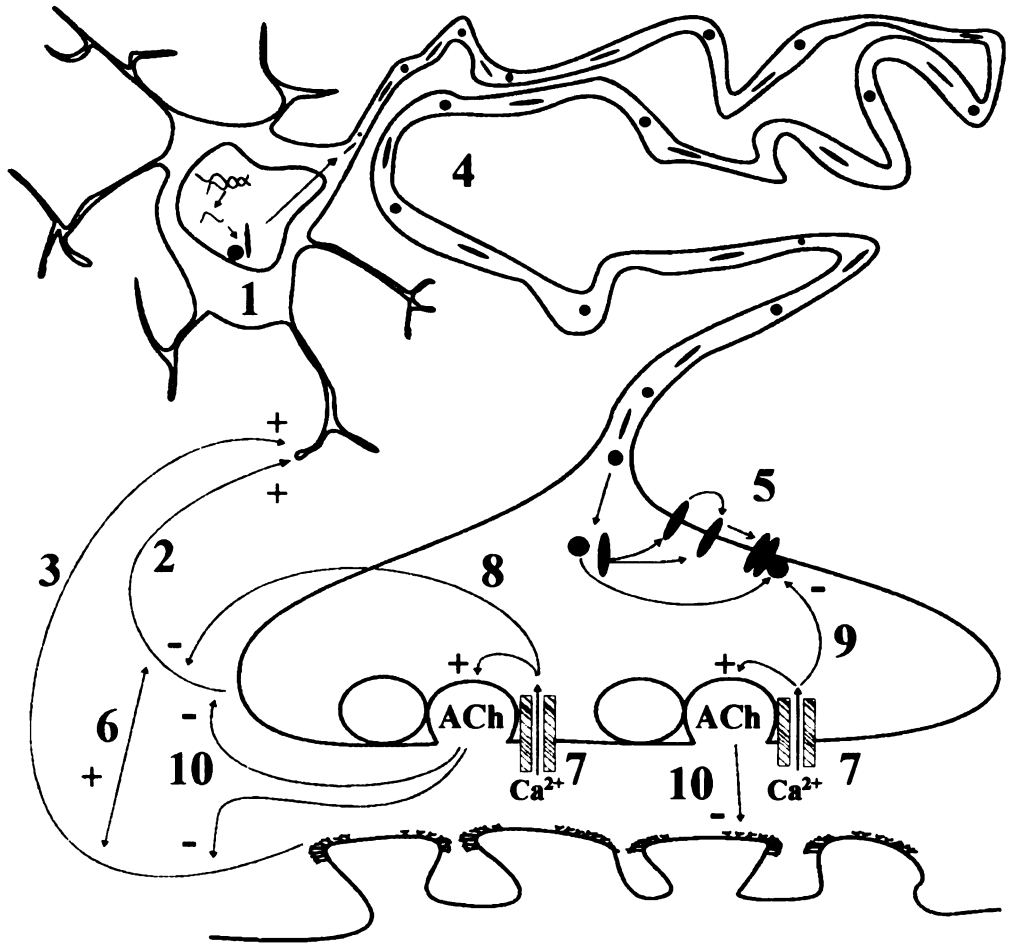

Figure 6.1. Hypothetical model depicting the development of functional L-type Ca^{2+} channels at adult mouse motor nerve terminals. L-type Ca^{2+} channel subunits are produced in the motor nerve cell body (1) in response to tonic excitatory signals originating from pre- (2) and/or post-synaptic (3) 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 post-synaptic 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 pre-synaptic 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 post-synaptic 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. LEMS antibodies reduce the number of functional P/Q-type Ca^{2+} channels, and subsequently reduce 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.

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 nerve terminal preparations (synaptosomes) 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 also 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). 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-Sá *et al.*, 2000). 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). Based on 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. The location of L-type channels at sites distinct from the release machinery

from LEMS-exposed motor nerve terminals most likely necessitates a large influx of Ca^{2+} through L-type channels in order to affect release of ACh. 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 K_{Ca} channels may enhance involvement of L-type Ca^{2+} channel-dependent release of ACh during LEMS. This idea is based on 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 (Mallart, 1985; Robitaille and Charlton, 1992; Robitaille *et al.*, 1993; Xu and Atchison, 1996; Protti and Uchitel, 1997). 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 decrease in the number of P/Q-type VDCC leads to a decrease in openings of colocalized K_{Ca} channels and 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.

Incubation of naïve neuromuscular preparations obtained from mature mice with the K_{Ca} channel antagonist, iberiotoxin, increased the nerve-stimulated release of ACh without altering MEPP amplitudes. However, iberiotoxin did not affect the spontaneous asynchronous release of ACh until the motor nerve terminal membrane was depolarized to approximately -42 mV

by exposure of NMJ preparations to buffers containing 20 mM KCl. Although all measurements pertaining to membrane potentials were made from the muscle, membrane potentials of the motor nerve terminal innervating the muscle were presumably similar. However, this may not be entirely true– mechanisms controlling membrane potentials may vary between muscle and nerve leading to differences in potentials between the two cell types. Nevertheless, the small size and inaccessibility of the motor nerve terminals precludes direct measurements, and thus, membrane potentials of the motor nerve terminal were assumed to be close to those measured from the muscle.

Enhanced release of ACh by iberiotoxin was much greater following electrical stimulation of the nerve than following K^+-induced depolarization of the nerve. This discrepancy most likely reflects than manner in which the motor nerve terminal was depolarized. Electrical stimulation of the nerve transiently depolarizes the motor nerve terminal membrane to ~ 0 mV. At such positive potentials, loss of K_{Ca} channel activation would affect the open probability of a greater number of VDCC than that observed when the membrane potential is ~ -42 mV. Thus in the presence of iberiotoxin, release of ACh is higher during electrical stimulation of the nerve. In addition, electrical stimulation of the nerve induces a large depolarization of the membrane that occurs rapidly and transiently, whereas, under the conditions used in this dissertation, K^+-induced depolarization of the motor nerve terminal produces modest and continuous membrane depolarization. During continual membrane depolarization, involvement of a large proportion of VDCC in release of ACh may be limited by voltage-dependent and/or other secondary channel inactivating mechanisms.

During functional antagonism of K_{Ca} channels, a significant number of normally silent

L-type channels become involved in ACh during electrical or K^+-induced nerve (at KCl 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. This decrease in activation of K_{Ca} channels, 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 support of 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+} current, but that of K_{Ca} current 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 generaly affected (Garcia and Beam, 1996; Garcia *et al.*, 1996).

L-type Ca^{2+} current involved in release of ACh during LEMS may be influenced directly or indirectly by K_{Ca} channels. 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 with one another. In support of the latter argument, differential colocalization of VDCC subtypes and K_{Ca} channels 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} channels on parasympathetic nerves.

Based on pharmacological sensitivities, K_{Ca} channels found on mature mammalian motor nerve terminals are most likely of the BK type and are sensitive to intracellular Ca^{2+} and to 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 likely that in LEMS, lack of colocalization of K_{Ca} channels 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 Figure 6.2 and most likely acts in concert with the hypothetical model depicted in Figure 6.1 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 VDCC subtypes, including the N-type. However, based on 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 also may occur, but due to a stronger preference of LEMS antibodies for not only P/Q-, but N- in comparison to L-type Ca^{2+} channels, the turnover of N-type channels may not occur fast enough to allow them to become

involved in release of ACh.

The involvement of L-type channels in release of ACh from motor nerves following passive transfer of LEMS to mice likely occurs in humans as well. Although direct evidence for a similar findings in humans has not been reported, indirect evidence supporting the role of L-type Ca^{2+} channel development at motor nerve terminals in LEMS has been observed. 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 a patient with LEMS (Krendel and Hopkins, 1986). It is likely, 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, 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.

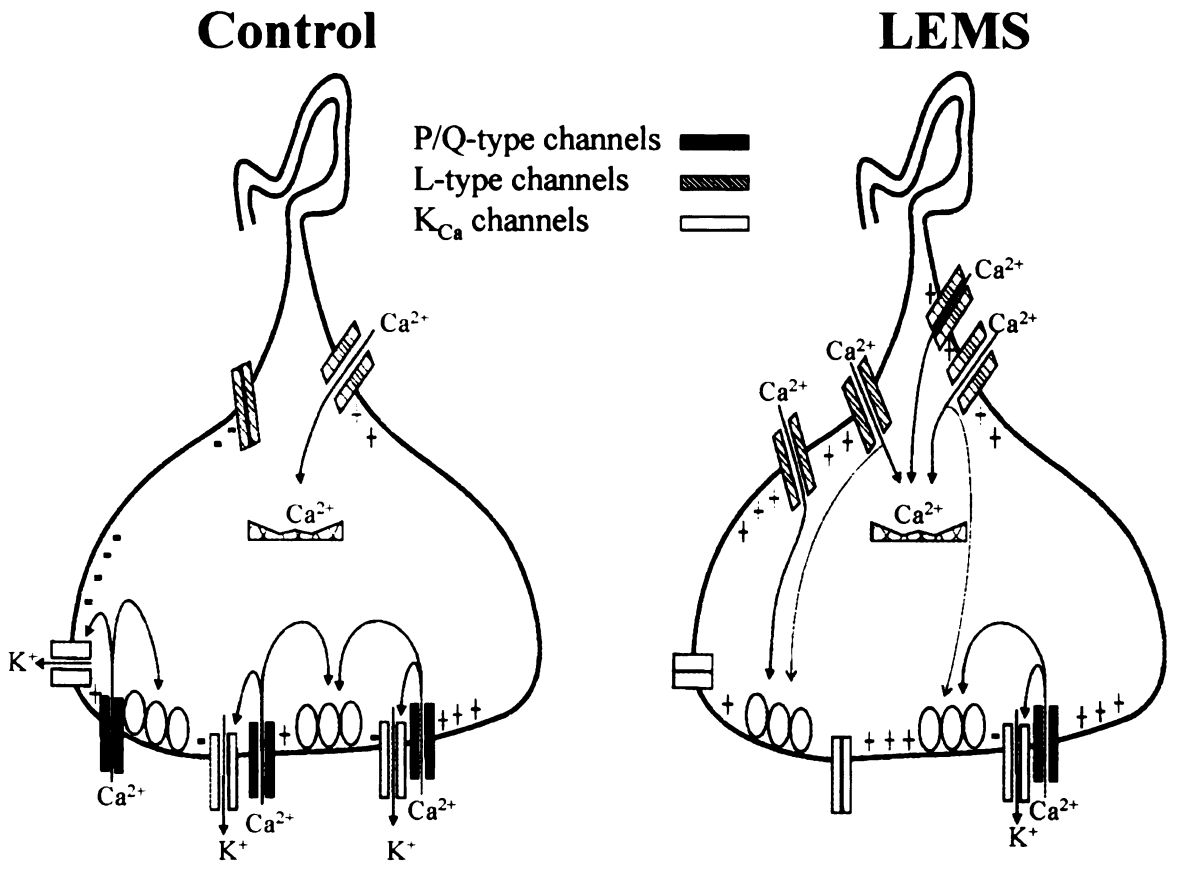

Figure 6.2. Hypothetical model representing the role of K_{Ca} channels in LEMS at the mature motor nerve terminal of mice. In **Control** environments, P/Q-type Ca^{2+} channels are the primary channel subtype controlling ACh release from motor nerve terminals of mature mice. Although Ca^{2+} entry 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 is 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.

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