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EFFECTS OF LAMBERT-EATON MYASTHENIC IMMUNOGLOBULIN ON SYNAPTOSOMAL CALCIUM CHANNELS: SPECIFICITY, SERUM DEPENDENCE AND SUBTYPE

By

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ABSTRACT

EFFECTS OF LAMBERT-EATON MYASTHENIC IMMUNOGLOBULIN ON SYNAPTOSOMAL CALCIUM CHANNELS: SPECIFICITY, SERUM DEPENDENCE AND SUBTYPE

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Sandra Jeanne Hewett

Lambert-Eaton Myasthenic Syndrome (LEMS) is a presynaptic disorder of neuromuscular transmission. The reduction in nerve evoked release of transmitter at the neuromuscular junction of patients with LEMS is thought to be caused by an autoantibody to motor nerve terminal Ca^{2+} channels. The overall goal of this dissertation was to test the hypothesis that a circulating autoantibody in the serum of patients with LEMS interferes with the function of nerve terminal Ca^{2+} channels associated with transmitter release. To test this a model system of isolated nerve terminals from mammalian CNS (synaptosomes) was used.

Serum and plasma from several patients and IgG from a single patient with LEMS reduce $^{45}Ca^{2+}$ influx into synaptosomes. The antibody appeared specific for Ca^{2+} channels as it was unable to disrupt $^{22}Na^{+}$ uptake into or $^{86}Rb^{+}$ efflux from Na^{+} or K^{+} channels, respectively. Synaptosomal membrane integrity as well as synaptosomal membrane potential were unaltered following incubation with the LEMS autoantibody.

In synaptosomes, the presence of human serum is important in mediating a functional change in Ca^{2+} channel activity by LEMS IgG. In the absence of

human serum, LEMS IgG did not reduce uptake of $^{45}Ca^{2+}$ into synaptosomes. Therefore, the role of the serum component complement in the pathogenesis of LEMS was assessed. Neither the membrane attack complex nor the alternative pathway of complement contributed to the disruption of Ca^{2+} channel function by LEMS IgG and serum in synaptosomes. In contrast, the C3 component of the complement cascade appeared to be important in mediating Ca^{2+} channel dysfunction.

The pharmacological determination of the Ca^{2+} channel subtype affected by IgG from a patient with LEMS was next examined. The density of binding sites for [^{3}H]-verapamil but not [^{3}H]-nitrendipine nor [^{125}I]-ω-conotoxin was reduced by approximately 45% following incubation with LEMS IgG alone or LEMS IgG and serum. This suggests that the IgG molecule itself interacts at this site. Verapamil is presumed to act as an L-type Ca^{2+} channel antagonist but recent evidence suggests that it may not be as selective as was previously thought.

The studies in this dissertation were the first to examine specifically the ability of antibody from LEMS patients to interact with nerve terminal Ca^{2+} channels. This work has strengthened the hypothesis that Ca^{2+} channels are the antigenic target, provided new insight into the mechanism of Ca^{2+} channel dysfunction, and explored pharmacologically the subtype of Ca^{2+} channels in a nerve terminal preparation affected by the LEMS autoantibody.

To my family, with all my love
Especially my parents, Ann and Dennis Simas
and
my husband, Jim

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PREFACE

Some of the data contained in this dissertation have been published previously. Chapter Three appeared as Hewett and Atchison (1991).

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LIST OF ABBREVIATIONS

ACh - acetylcholine

AChE - acetylcholinesterase

ANOVA - analysis of variance

APC - alternative pathway of complement

AZPs - active zone particles

B_{max} - maximum number of binding sites

BSA - bovine serum albumin

BoTX - botulinum toxin

BTX - batrachatoxin

Ca^{2+} - calcium

$[Ca^{2+}]_e$ - extracellular concentration of calcium

ChAT - choline acetyltransferase

CMAP - compound muscle action potential

CNS - central nervous system

CgTx - ω-conotoxin GVIA

CVF - cobra venom factor

DHP - dihydropyridine

diS-C_2(5)- 3,3'-diethyldicarbocyanine iodide

DMSO - dimethyl sulfoxide

DRG - dorsal root ganglion

EGTA - ethyleneglycol bis-(6-aminoethyl ether)-N,N,N,N'-tetra acetic acid

EPP - endplate potential

EtOH - ethanol

Fab - monovalent antigen binding fragment of IgG (papain-digested)

$F(ab)'_2$ - divalent antigen binding fragment of IgG (pepsin-digested)

FTX - funnel web spider toxin

HEPES - N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid

IgG - immunoglobulin G

Igs - total immunoglobulin

K^+ - potassium

$[K^+]_e$ - extracellular concentration of potassium

kDa- kilodalton

K_d - equilibrium dissociation constant for ligand binding

LDH - lactate dehydrogenase

LEMS - Lambert-Eaton Myasthenic Syndrome

MAC - membrane attack complex

MEPP - miniature endplate potential

MG - myasthenia gravis

Mg^{2+} - magnesium

MHC - major histocompatability complex

mRNA - messenger ribonucleic acid

msec - millisecond

Na^+ - sodium

NaCN - sodium cyanide

$NiCl_2$ - nickel chloride

NMJ - neuromuscular junction

PAGE - polyacrylamide gel electrophoresis

PKC - protein kinase C

SCC - small cell carcimona of the lung

SDS - sodiumdodecylsulfate

sec - seconds

SEM - standard error of the mean

synaptosomes - isolated nerve terminals from rat forebrain

TBA - tetrabutylammonium chloride

TEA - tetraethylammonium chloride

T_h cells - helper T lymphocytes

T_s cells - suppressor T lymphocytes

TTX - tetrodotoxin

VER - veratridine

4-AP - 4-aminopyridine

CHAPTER ONE: INTRODUCTION

A. *General Introduction*:

Lambert-Eaton myasthenic syndrome (LEMS) is an autoimmune disorder characterized symptomatically by a generalized muscle weakness. The neuromuscular junction (NMJ) was implicated as the site of the abnormality in LEMS when no aberration could be found in the responses of the motor nerve or muscle to direct electrical stimulation. The presynaptic terminal, specifically, was implicated as the site of action of the LEMS autoantibody when analysis of the NMJ in biopsy specimens from patients with the syndrome demonstrated that the postsynaptic membrane responds normally to acetylcholine (ACh). Since a presynaptic defect could involve an abnormality in transmitter synthesis, storage, or release, a great deal of effort has been expended in order to determine which of these processes is affected. Experimental evidence indicates that both synthesis and storage of ACh are normal. Thus, the abnormality appears to be caused by a defect in neurotransmitter release. It has been suggested that membrane calcium (Ca^{2+}) channels are the antigenic target for the LEMS autoantibody.

The initial sections of Chapter One will introduce normal neuromuscular transmission. These sections provide a description of the components of the neuromuscular junction as well as the dependence of transmission on $[Ca^{2+}]_e$ ions. Ca^{2+} channel subtypes believed to be involved in neurotransmitter release, in general, are discussed in detail. This is followed by a detailed discussion of the autoimmune, neuromuscular disorder, LEMS with attempts to delineate the stage at which normal functioning is altered by a pathological process. The final sections

of this chapter discuss the normal immune response focussing on the mechanisms of self- non-self recognition and how the breakdown of self-tolerance can lead to autoimmunity.

B. *Normal Neuromuscular Transmission*:

1. Synaptic transmission

The synapse formed between motor nerves and muscle cells is known as the neuromuscular junction (NMJ). The NMJ can be considered a transducing mechanism, in which electrical signals (nerve impulses) are transduced to chemicals signals (neurotransmitter release) and then back to electrical signals (muscle action potentials). The transmission of an impulse from the motor nerve to the muscle fiber involves a complex sequence of events. The structures and steps involved in neuromuscular transmission are shown in Figure 1.1.

The presynaptic part of the transducer consists of the nerve terminal. The terminal rests in a depression in the surface of the muscle fiber, the postsynaptic part of the transducer, and is separated from the muscle by the synaptic cleft (Bowden and Duchen, 1976). The nerve terminal contains large numbers of synaptic vesicles, each containing approximately 10,000 to 20,000 molecules of ACh (Potter, 1970; Kuffler and Yoshikami, 1975). Each vesicle represents one quantum of ACh (del Castillo and Katz, 1956; Martin, 1966; Hubbard, 1970). Transmitter release from the nerve terminal occurs both spontaneously and as a result of invasion of the axon terminal by an action potential. The spontaneous

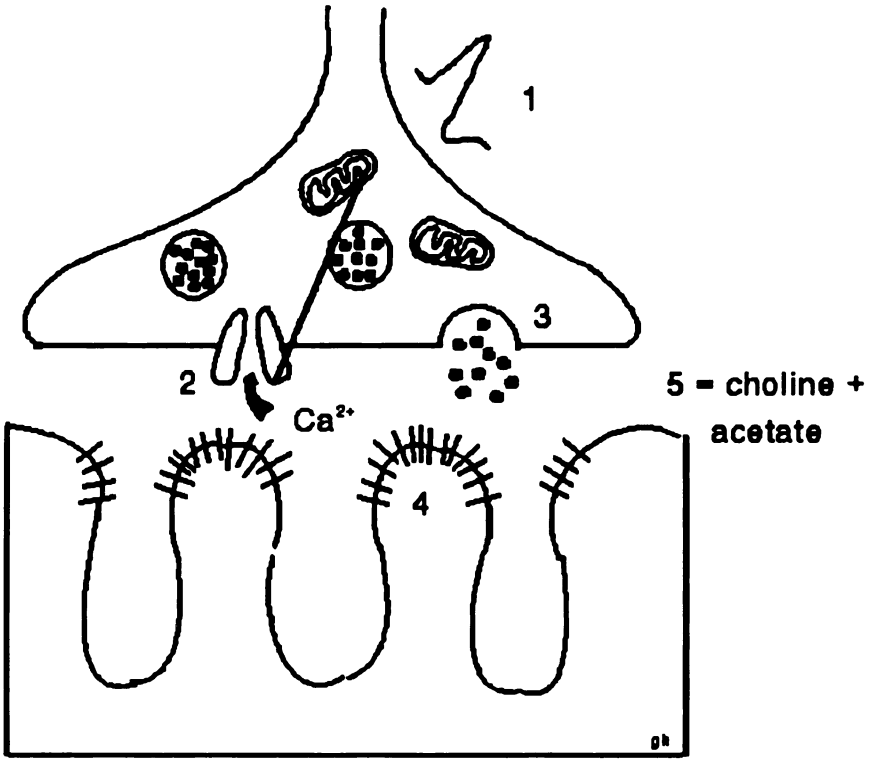


FIGURE 1.1. Neuromuscular Transmission

Following stimulation of the nerve, an action potential will be propagated down the axon, leading to depolarization of the nerve terminal (1). Voltage-sensitive Ca^{2+} channels open and allow Ca^{2+} ions to enter into the terminal (2). An increase in intraterminal Ca^{2+} triggers the fusion of synaptic vesicles with the plama membrane and the subsequent release of ACh into the synaptic cleft (3). ACh binds to nicotinic ACh receptors in the muscle membrane resulting in an excitatory postsynaptic potential (4). Diffusion of ACh away from the receptors and hydrolysis of ACh by AChE rapidly terminates the ACh-receptor combination (5).

release of one quantum of ACh leads to tiny depolarizations of the postsynaptic membrane known as miniature end-plate potentials (MEPPs) (Fatt and Katz, 1951; Boyd and Martin, 1956a; Liley, 1956). Stimulated release of many quanta of ACh leads to an excitatory postsynaptic potential which at the NMJ is termed an endplate potential (EPP) (Fatt and Katz, 1959; Takeuchi, 1963). The number of vesicles released per nerve impulse is known as the quantal content of the EPP.

Action potentials are propagated along the axon of the motor neuron by movement of Na^+ and K^+ into and out of the axon through their respective channels (Hodgkin and Huxley, 1952). Once the action potential reaches the axon terminal, it causes a depolarization of the terminal membrane (Brigant and Mallart, 1982; Mallart, 1985). As a result, voltage-dependent Ca^{2+} channels in the nerve terminal open allowing extracellular Ca^{2+} ions to enter down their electrochemical gradient (Katz and Miledi, 1967a,b; Llinas *et al.*, 1981). It is thought that these channels are clustered in specialized areas or active zones, where vesicles containing neurotransmitter are arranged in close apposition to the cell membrane for subsequent release. (Heuser *et al.*, 1974, 1979; Ceccarelli and Hurlbut, 1980; Pumplin *et al.*, 1981; Robitaille *et al.*, 1990). Ca^{2+} entry produces the near simultaneous exocytotic release of large numbers of quanta of ACh from the active zone area.

The presence of $[Ca^{2+}]_e$ is required for any form of depolarization to release ACh from nerve terminals. This requirement is not connected to the propagation

of action potentials into nerve terminals, for in conditions of Ca^{2+} deprivation, action potentials can still be recorded even though no ACh release occurs (Katz and Miledi, 1965; Dodge *et al.*, 1969). The sequence of molecular events linking depolarization-dependent Ca^{2+} influx to the release of neurotransmitters from nerve terminals is unknown. However, interactions between Ca^{2+} and intracellular Ca^{2+}-binding proteins are thought to trigger directly or indirectly the fusion of synaptic vesicles with the cell membrane, resulting in the release of neurotransmitter into the synaptic cleft (Augustine *et al.*, 1987).

One model suggests that activation of a Ca^{2+}-binding protein in turn activates a protein kinase which phosphorylates a protein or proteins essential for the release of neurotransmitter (Krueger *et al.*, 1977; DeLorenzo *et al.*, 1979; Dekker *et al.*, 1989*a*). Evidence for this centers on studies which demonstrate that the phosphorylation of synapsin I and B-50 (GAP-43) by Ca^{2+}/calmodulin-dependent protein kinase II and Protein kinase C, respectively, correlate with neurotransmitter release. (Llinas *et al.*, 1985; Robinson and Dunkley, 1985; Dekker *et al.*, 1989*a*; Sihra *et al.*, 1989; Dekker *et al.*,1990; Nichols *et al.*, 1992). Furthermore, antibodies to B-50 or injection of deposphorylated synapsin I inhibit release from the nerve terminal (Llinas *et al.*, 1985; Dekker *et al.*, 1989*b*).

Synaptophysin, synaptotagmin, synexin, calpactin and other members of the calelectrin family possess a cytoplasmic Ca^{2+}-binding site and have also been implicated in the control of neurotransmitter release (Augustine *et al.*, 1987; Smith and Augustine, 1988; Brose *et al.*, 1992). Depolarization of nerve terminals is

accompanied by the activation of a calcium-stimulated protein phosphatase, as well. The physiological significance of the rapid dephosphorylation of the phosphoproteins, dephosphin, P96 and P139 (Robinson *et al.*, 1987; Sihra *et al.*, 1992) has yet to be determined.

Once released, ACh crosses the synaptic cleft and binds to nicotinic ACh receptors located on the crests of the synaptic folds across from release sites (Landau, 1978; Ceccarelli and Hurlbut, 1980). The interaction between ACh and its receptor produces a conductance change in the postsynaptic membrane, markedly increasing the membranes permeability to Na^+ and K^+ (Takeuchi and Takeuchi, 1959; Takeuchi, 1963). This produces a local depolarization in the end-plate (Fatt and Katz, 1951; Boyd and Martin, 1956*b*). EPPs of sufficient magnitude spread from the endplate region to the surrounding muscle fiber membrane initiating an action potential. This leads to contraction of the muscle (Hubbard, 1973).

Diffusion of ACh away from the receptors and hydrolysis of ACh by acetylcholinesterase (AChE) rapidly terminates the ACh-receptor combination. The choline molecules liberated by AChE action are actively taken up by nerve terminals for resynthesis into ACh (choline + acetyl CoA) by choline acetyltransferase (ChAT)(Collier and Macintosh, 1969; Potter, 1970; Kuher and Murrin, 1978).

2. Calcium channel subtypes

Voltage-dependent Ca^{2+} channels occur in most neurons and in several other cell types. As stated above, influx of $[Ca^{2+}]_e$ through membrane ionic channels is the critical step for coupling the action potential to transmitter secretion (Katz and Miledi, 1965; 1967*a,b*; Dodge and Rahamimoff, 1969). Evidence indicates that more than one type of calcium channel exists (Nowycky, *et al.*, 1985*a,b*; Perney, *et al.*, 1986). This discovery has prompted questions as to which subtype(s) is (are) involved with the release of neurotransmitter. With the exception of some invertebrate neurons, characterization of the type of channel that exists at the active zone is impossible as nerve terminals are too small for direct recording techniques. Therefore, electrophysiological and pharmacological characterizations of the various types of calcium channels were made in the cell soma with the assumption that somal events model events occurring in the nerve terminal. Thus, the exact identity of the subtypes of calcium channels in the nerve terminal remains unknown. It is possible that the characteristics of calcium channels in the terminal may differ from those in the cell body (Leonard *et al.*, 1987; Suszkiw *et al.*, 1989; Charlton and Augustine, 1990).

Three distinct channel subtypes have been described in a dorsal root ganglion (DRG) preparation (Nowycky *et al.*, 1985*a,b*; Fox *et al.*, 1987). The channel subtypes, T, L, and N are distinguishable by their differential kinetics of activation and inactivation, as well as their pharmacological sensitivity to agonists and antagonists. A fourth type has been defined in mammalian Purkinje cells

(guinea pig cerebellum) and in presynaptic terminals of the squid giant synapse (Llinas *et al.*, 1989; Lin *et al.*, 1990). This channel differs, both electrophysiologically and pharmacologically, from the previously defined T-, L- and N-type and is termed the P-type channel (for Purkinje)(Llinas *et al.*, 1989; Lin *et al.*, 1990). The biophysical and pharmacological profiles of the different Ca^{2+} channel subtypes are summarized in Table 1.1.

T-type ("transient") channels activate at membrane potentials more positive than -70 mV, have very small unitary conductances, and inactivate quickly and completely during a maintained depolarization. L-type ("long-lasting") channels activate at postive test potentials (> -10 mV), have a very large unitary conductance, and show little inactivation during a 200 msec depolarization . N-type ("neither T- nor L-") channels activate at positive test potentials (> -20 mV), have a smaller unitary conductance than the L channel and decay with time constants ranging from 50-100 msec (Fox *et al.*, 1987). Finally, P-type channels activate at relatively positive test potentials (-30 to -20 mV) and decay with a time constant of 650 msec (Llinas *et al.*, 1989).

Pharmacological differences exist between the Ca^{2+} channel subtypes also. The dihydropyridine (DHP) class of organic Ca^{2+} channel agonists and antagonists such as Bay K 8644 and nitrendipine are presumed to interact with a site directly on or associated with L-type Ca^{2+} channels (Nowycky *et al.*, 1985a) whereas N- and T- channels are unaffected by submicromolar concentrations of these drugs. The phenylalkylamine, verapamil and the benzothiazepine, diltiazem also appear

Table 1.1

Pharmacological and Biophysical

Characteristics of Mammalian Ca^{2+} Channel Types

	T	N	L	P
Activation range (mV)	> -70	> -20	> -10	-30--20
Inactivation	fast	moderate	slow	slow
Kinetics	τ = 20-50 ms	τ = 95 ms	τ = > 250 ms	τ = 650 ms
Divalent Cation sensitivity	$Ni^{+} > Cd^{2+}$	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$	Cd^{2+}, < 100 μM
DHPs	-	-	+	-
ωCgTx	-	+	-*	-
FTX	-	-	-	+
Single Channel conductance (pS)	8	13	25	13

* Avian and amphibian L-type Ca^{2+} are sensitive to CgTx (Olivera *et al.*, 1985; Marqueze *et al.*, 1988).

Data for N-, L-, T-channels modified from Tsien *et al.*, 1988. P-channel data compiled from Llinas *et al.*, 1989 and Lin *et al.*, 1990.

to act as an L-type antagonist but have separate binding sites from that of the DHPs (Miller, 1987; Glossmann and Striessnig, 1988). It is believed that three drug receptor sites on this one channel are linked to one another via heterotrophic allosteric coupling mechanisms (Hofmann *et al.*,1987; Kamp and Miller, 1987; Glossman and Striessnig, 1988)

In mammalian neurons, N-type channels are blocked by ω-conotoxin GVIA (CgTx), a peptide purified from the venom of the marine snail *Conus geographus* (McCleskey *et al.*, 1987; Kasai *et al.*, 1987). Both N- and L-type channels in avian and amphibian preparations are blocked by CgTx at sites which appear to be distinct from DHP binding sites (Olivera *et al.*, 1985; Abe *et al.*, 1986; Dooley *et al.*, 1987; Marqueze *et al.*, 1988). CgTx lacks potency on the invertebrate nervous system, however (McCleskey *et al.*, 1987). T channels are DHP- and CgTx-insensitive but can be blocked by amiloride and octanol (Tsien *et al.*, 1988; 1991).

The T-., L-, and N-type of Ca^{2+} channels can be characterized by their sensitivity to inorganic ions as well. Inorganic heavy metal cations such as zinc, nickel, mercury, lead, copper, and cadmium block all three subtypes of channels (Hagiwara and Byerly, 1981; Augustine *et al.*, 1987). There is considerable variation in the sensitivity of Ca^{2+} channels to these ions, however. For instance, in chick DRG neurons, cadmium blocks N- and L-type channels with a K_d of 10 μM but about ten times more cadmium is required to block T-type channels (Nowycky *et al.*, 1985a; Fox *et al.*, 1987). Conversely, T-type channels are blocked more readily by nickel than either N- or L-channels (Tsien *et al.*, 1988).

The peptide, ω-Agatoxin and the polyamine, FTX are potent Ca^{2+} channel blockers derived from the venom of the funnel-web spider *Agelenopsis aperta* (Adams *et al.*, 1990; Llinas *et al.*, 1989). Subtypes of ω-Agatoxins are distinguished as type I, type II, and type III. All except type III toxins block Ca^{2+} channels in most insect motor nerve terminals and neuronal cell bodies at nanomolar concentrations (Adams *et al.*, 1990; Bindokas *et al.*, 1991). On the other hand, types II and III but not I toxins, block CgTx-sensitive channels in chick brain synaptosomes (Adams *et al.*, 1990; Venema *et al.*, 1992). Evidence exists that Agatoxins blocks DHP-sensitive Ca^{2+} currents in rat sensory neurons (Scott *et al.*, 1990; Mintz *et al.*, 1991) and in cardiac muscle as well (Liebowitz *et al.*, 1990; Mintz *et al.*, 1991).

P-type Ca^{2+} channels found in Purkinje cells and in the squid giant synapse (Llinas *et al.*, 1989; Mintz *et al.*, 1992) are blocked by ω-Aga-IVa and FTX respectively (Llinas *et al.*, 1989; Mintz *et al.*, 1992) but not by DHPs or CgTx (Llinas *et al.*, 1989; Charlton and Augustine, 1990). FTX also reduces a Ca^{2+} current observed following injection of total rat brain mRNA into *Xenopus* oocytes (Lin *et al.*, 1990). This current is insensitive to DHP- agonists, antagonists, and CgTx as well (Leonard *et al.*, 1987). These results suggest that the channel type expressed from rat brain mRNA in the oocyte may be similar to the P-type channel.

Despite considerable research, the channel type(s) involved in neurotransmitter release are still not clearly defined. Again, this is due to technical limitations of current techniques. However, results of some neurochemical (Miller,1987) and electrophysiological (Atchison and O'Leary, 1987; Hirning *et al.*,

1988) studies suggest that neurotransmitter release is modulated by both the L- and N- type channel. At rat sympathetic neurons and neuromuscular junctions, L-type Ca^{2+} channels can have a modulatory role in neurotransmitter release (Perney *et al.*, 1986; Atchison and O'Leary, 1987) but do not contribute to nerve-evoked release under normal conditions (Perney *et al.*, 1986; Atchison, 1989). In contrast, L-type channels have been implicated in release of substance P from rat sensory neurons (Perney *et al.*, 1986) and chick sensory neurons (Rane *et al.*, 1987; Holz *et al.*, 1988), growth hormone and prolactin release from rat anterior pituitary cells (Stojilkovic *et al.*, 1988) and ^{3}H-norepinephrine release from undifferentiated PC12 cells (Kongsamut and Miller, 1986). N-type channels have been reported to play a dominant role in mediating evoked-release of norepinephrine from rat sympathetic neurons (Perney *et al.*, 1986; Hirning *et al.*, 1988), differentiated PC12 cells (Kongsamut and Miller, 1986) and various areas of the central nervous system (Dooley *et al.*, 1987).

Data exist that suggest the P-type Ca^{2+} channel may also mediate Ca^{2+} entry associated with transmitter release. As discussed previously, Ca^{2+} channels at the squid giant synapse have pharmacological and functional properties unlike those of T-, L-, or N-type Ca^{2+} channels (Charlton and Augustine, 1990). They are DHP- and CgTx-insensitive but partially sensitive to FTX. Therefore, Ca^{2+} channels in the squid giant synapse appear similar to the P-type isolated from mammalian Purkinje cells (Llinas *et al.*, 1989; Charlton and Augustine, 1990) and likely mediate release in these preparations. Likewise, CgTx and DHPs were unable to block K^{+}-

evoked release of ^{3}H-aspartate from rat hippocampal slices or release of ACh from murine motor nerve terminals either (Anderson and Harvey, 1987). Mammalian neuromuscular transmission was effectively abolished by FTX, however. FTX blocked presynaptic Ca^{2+} currents, nerve-evoked neurotransmitter release and muscle contraction from a murine nerve-muscle preparation (Uchitel *et al.*, 1992). This suggests that channels which are neither N- or L-type but possibly P- are involved in neurotransmitter secretion from some mammalian nerve terminals. To date, there is no evidence indicating a role for T-type channels in neurosecretion.

It should be noted that the characteristics attributed to Ca^{2+} channel subtypes do not always fit neatly into one of the above catagories. Thus, the original classification of Ca^{2+} channels into T-, L-, N-, and now P- subtypes is likely an oversimplification. It is possible that functional heterogeneity among channel subtypes as well as additional types of channels exist. This is supported by the isolation from rat brain of four different types of cDNA that code for putative Ca^{2+} channels (Snutch *et al.*, 1990). Characterization of the primary structure suggest that several distinct voltage-gated Ca^{2+} channel genes exist (Snutch *et al.*, 1990; Mori *et al.*, 1991; Snutch *et al.*, 1991). Additionally, species and tissue differences in the type of Ca^{2+} channel involved in neurotransmitter release are evident (Brush *et al.*, 1987; Nayler, 1988; Maggi *et al.*, 1990).

C. *Disorders of Neuromuscular Transmission*

As the above review was meant to indicate, there are many steps in the process of synaptic transmission at the neuromuscular junction. Thus, there are

many ways in which the process can be disrupted. Disorders of neuromuscular transmission vary in etiology, frequency and clinical presentation. All produce muscle weakness through involvement of the neuromuscular junction, with a preference for certain muscle groups. These disorders may affect the neuromuscular junction presynaptically (the nerve terminal), postsynaptically (the muscle endplate) or both pre-and post-synaptically. This dissertation will focus on the presynaptic, neuromuscular disorder Lambert-Eaton Myasthenic Syndrome and will attempt to delineate the stage at which normal functioning is altered by a pathological process.

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

1. Clinical Features

The principal symptoms of Lambert-Eaton Myasthenic Syndrome (LEMS) are weakness and easy fatigability of limb muscles, which temporarily improves in response to voluntary exercise. Tendon reflexes are reduced or absent at rest (Eaton and Lambert, 1957; Rooke *et al.*, 1960; Lambert *et al.*, 1961; Wise and McDermot, 1962; Elmqvist and Lambert, 1968). Patients frequently complain of dry mouth, urinary hesitancy, constipation, sexual impotence, and impaired accomodation of the eye (Rubenstein *et al.*, 1979; Mamdani *et al.*, 1985; Heath *et al.*, 1988; Khurana *et al.*, 1988; Manji *et al.*, 1990). These symptoms are indicative of a cholinergic dysautonomia and therefore demonstrate that the defect is not limited to striated muscle but can affect smooth muscle and glands as well. Drugs

that increase transmitter release, such as guanidine, 4-aminopyridine and 3,4-diaminopyridine give patients with LEMS symptomatic relief (Oh and Kim, 1973; Lundh, 1983; Newsom-Davis and Murray, 1984; McEnvoy, *et al.*, 1989). Serious toxic effects, however, limit their use. AChE inhibitors have minimal benefit (Eaton and Lambert, 1957; O'Neill *et al.*, 1988).

LEMS occurs in two groups of patients. In both groups, male Caucasians (median age of 54 yrs) predominate (Elmqvist and Lambert, 1968; O'Neill *et al.*, 1988). In one group, all have some type of malignancy, the most common (60-70%) being small cell carcinoma of the lung (SCC)(Eaton and Lambert, 1957; Lambert *et al.*, 1961; Wise and MacDermot, 1962; Elmqvist and Lambert, 1968; O'Neill *et al.*, 1988). Symptoms of LEMS can precede the diagnosis of SCC by 5 months to 4 years (median 10 months) (O'Neill *et al.*, 1988; Anderson *et al.*,1990) and sometimes improve when the tumor is either treated or removed (Ingram *et al.*, 1985; Oh, 1989; Chalk *et al.*, 1990). The second group of patients have no detectable malignancy but present with identical clinical signs.

LEMS is generally accepted as being a paraneoplastic disease, a nervous system dysfunction in patients with cancer that is not caused by metastases (Vincent *et al.*, 1989; Posner, 1991). Because the disease occurs in the absence of tumor as well as in its presence, it follows that the tumor favors the development of the condition but cannot in itself be the only cause (Lambert and Rooke, 1965; Chad and Recht, 1991).

2. Electrophysiology

a. Clinical

Electromyography is diagnostic and conventionally consists of the recording of muscle action potentials generated following surface stimulation of nerves from living patients (Harvey and Masland, 1941; Eaton and Lambert, 1957). An electromyographic feature that characterizes LEMS is a significant reduction in the amplitude of the compound muscle action potential (CMAP). Low frequencies of nerve stimulation (1 per sec, 1Hz) result in a progressive decrease in the amplitude of the CMAP while a marked increase in the response is noted when the nerve is stimulated at higher frequencies (10 to 50 Hz) (Figure 1.2). An increase in response amplitude can also be demonstrated after brief periods of voluntary contraction of the muscle (Eaton and Lambert, 1957; Elmqvist and Lambert, 1968; Swift, 1981; Jablecki, 1984).

Nerve conduction velocities in LEMS patients are within the range of normal values and remain normal at repetitive rates of stimulation (Eaton and Lambert, 1957; McQuillen and Johns, 1967; Elmqvist and Lambert, 1968). This suggests the defect in transmission is not due to failure of action potential generation or propagation.

Direct stimulation of muscle fibers also reveals normal conduction velocities. Repetitive stimulation at increasing frequencies do not produce the typical changes

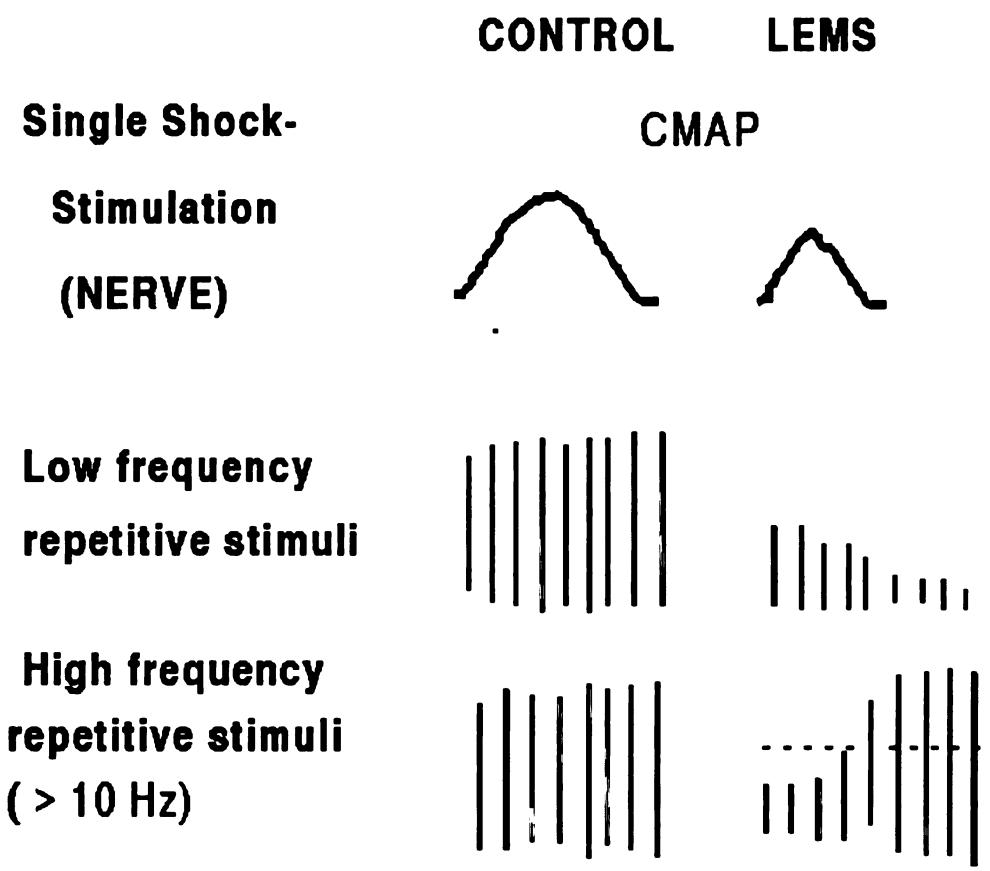


Figure 1.2. Electromyographic Characteristics of LEMS

This is a schematic representation of the EMG characteristics of LEMS. As you can see, there is a significant reduction in the CMAP. At low frequencies of stimulation there is a progressive decrease in the CMAP while a marked increase in the response is noted at higher frequencies of stimulation.

seen with indirect stimulation of the muscle via the nerve (McQuillen and Johns, 1967; Elmqvist and Lambert, 1968). Thus, the reduction in CMAP amplitude at low frequencies of stimulation and the increase in amplitude at high frequencies of stimulation do not arise from the muscle itself.

b. Experimental

To investigate the electrophysiological basis for the electromyographical observations, intracellular microelectrode recordings were made from biopsied intercostal muscle taken from patients with LEMS. The number of ACh-containing quanta released per nerve impulse (quantal content) was markedly reduced (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971; Cull Candy *et al.*, 1980). This suggests that *in vivo* the EPP would fail to achieve the threshold depolarization required for generating an action potential in the muscle membrane. As was seen electromyographically for the CMAP, repetitive stimulation of the nerve at 30 Hz increased quantal content.

Spontaneous release of neurotransmitter was also recorded. MEPP amplitudes and frequencies were similar to the MEPPs recorded in normal tissue indicating resting quantal release of ACh was normal. (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971). Resting, non-quantal release of ACh was decreased, however (Lang *et al.*, 1984; Molenaar *et al.*, 1987).

3. Biochemistry

Additionally, the sensitivity of LEMS muscle to ACh (Lambert and Elmqvist, 1971; Lindstrom and Lambert, 1978; Cull Candy *et al.*, 1980) as well as the number of ACh receptors present are normal (Lindstrom and Lambert, 1978). Also, the reduced quantal content is not due to a decreased synthesis or storage of neurotransmitter because the ACh and ChAT content of the nerve terminal are normal (Molenaar *et al.*, 1982; Molenaar *et al.*, 1987). Thus, these potential factors which could have contributed to the neuromuscular weakness found in LEMS patients have been ruled out. The evidence so far strongly suggests a selective effect on the mechanism of ACh release.

4. Morphology

a. Morphometric analysis

Electron microscopic, morphometric analysis of biopsied endplate regions typically consists of determining the nerve terminal area, the number of synaptic vesicles per unit nerve terminal area, the mean synaptic vesicle diameter, the presynaptic membrane length and the postsynaptic membrane length *per* nerve terminal (Loud, 1962; Engel, 1970; Engel and Santa, 1971; Hesselmans *et al.*, 1992). Engel and Santa (1971) reported that both methylene blue-stained intramuscular nerves and cholinesterase-stained endplates showed no definite abnormalities. Electron microscopy demonstrated that the mean nerve terminal area and the mean synaptic vesicle count *per* unit area were not significantly

different from control values. When the mean synaptic vesicle diameter was calculated for each biopsy, control and LEMS means did not differ significantly either (Engel and Santa, 1971).

One abnormality noted, however, was an overdevelopment of the postsynaptic region. Analysis showed a significant increase in the mean area of clefts and folds per nerve terminal and in the mean postsynaptic to presynaptic membrane length ratio (Engel and Santa, 1971). Multiplication of endplate areas is a well known phenomenon in disorders of neuromuscular transmission such as botulinum toxin (BoTx) intoxication (Duchen, 1971), myasthenia gravis (MG; in some cases) (Coërs and Tellerman-Toppet, 1976) and congenital myasthenia with decreases in ACh receptors (Vincent *et al.*, 1981). The increase in contact between muscle and nerve is likely to be compensatory to the deficiency in ACh release (LEMS and BoTx intoxication) or ACh receptors (myasthenia gravis).

In contrast, Hesselmans and colleagues (1992) report that both postsynaptic areas and postsynaptic membrane lengths of NMJs are significantly less in LEMS than in controls (Hesselmans *et al.*, 1992). This has also been observed in patients with MG and in the chronic phase of MG's experimental model as well (Engel and Santa, 1971; Engel and Tsujihata, 1976). A factor which may have influenced the differences between Hesselmans' and Engel's studies is the duration of the disease before muscle biopsy was performed. In Hesselmans' study, the onset of symptoms was at most 1.5 years before biopsy. In the study by Engel and Santa (1971) duration of symptoms ranged from 3 to 11 years. Since newly-created

NMJs have small postsynaptic regions which in time increase, perhaps only short-term effects of ACh release blockade were studied by Hesselmans *et al.*, (1992).

b. Ultrastructural analysis

Ultrastructural changes in the motor nerve terminal associated with LEMS were first reported by Fukunaga and coworkers (Fukunaga *et al.*, 1982; 1983). Measurements of the density of the active zones and large intramembraneous active zone particles (AZPs) were made in freeze-fracture preparations of biopsied human intercostal muscle. Their results indicated that both the active zones and the AZPs were strikingly scarce and disorganized (Fukunaga *et al.*, 1982). Prejunctional membranes taken from patients with LEMS had fewer active zones/μm^2; fewer number of active zone particles/μm^2; less particles/active zone; and a 3.6 fold increase in the number of large particles aggregated into clusters/μm^2. All other membrane particles studied appeared normal. Since the average decrease in the AZPs *per* unit of membrane area was comparable to the average decrease in quantal content of the EPP in LEMS, Fukunaga and colleagues concluded that a reduction in AZPs in LEMS accounted for the diminished probability of ACh release. (Fukanaga *et al.*, 1983). Active zones are topographically related to the sites of synaptic vesicle exocytosis, and thus are believed to be the sites at which neurotransmitter release occurs (Heuser *et al.*, 1974; 1979). Thus, electron micrographic findings in LEMS are consistent with a presynaptic disorder of neuromuscular transmission as postulated from the results of microelectrode studies.

E. *Lambert-Eaton Myasthenic Syndrome: Autoimmunity*

1. Clinical and experimental evidence

Compelling evidence has indicated that the etiology and pathogenesis of LEMS is autoimmune. Clinically, beneficial effects of plasma exchange (Newsom-Davis and Murray, 1984) and immunosuppressive drugs (Streib and Rothner, 1981; Newsom-Davis and Murray, 1984) have been demonstrated. Furthermore, LEMS has frequently been shown to accompany one or more autoimmune disorders (Gutmann *et al.*, 1972; Lennon *et al.*, 1982; Peris *et al.*, 1990).

The autoimmune basis and involvement of a humoral factor was demonstrated with certainty when the electrophysiological features of the disorder were transferred passively to mice by injection of humoral substances from affected patients (Denys *et al.*, 1972; Lang *et al.*, 1981; Newsom-Davis *et al.*, 1982; Lang *et al.*, 1983*a*; Kim, 1985; Kim, 1986; Lambert and Lennon, 1988). Plasma (Newsom-Davis *et al.*, 1982; Kim, 1985), crude immunoglobulin fractions (Lang *et al.*, 1983*a*; Kim, 1986; Lambert and Lennon, 1988) and purified IgG (Denys *et al.*, 1972; Kim, 1986) are all effective in transferring the disease with the production of characteristic *in vitro* electrophysiologic abnormalities. Mice treated with IgG from patients exhibiting LEMS also had a significantly lower number of active zones and AZPs (Fukanaga *et al.*, 1983; Nagel *et al.*, 1988). Furthermore, immunolocalization of LEMS IgG was demonstrated in the region of presynaptic active zones of mice (Fukuoka *et al.*, 1987).

It has been proposed that the AZPs correspond to voltage-sensitive calcium

channels of the presynaptic membrane (Pumplin *et al.*, 1981; Robitaille *et al.*, 1990; Tarelli *et al.*, 1991; Cohen *et al.*, 1991). Indirect evidence to support this assumption is as follows: (1) presynaptic voltage clamp studies in the squid giant synapse demonstrated a very short latency between Ca^{2+} entry and postsynaptic response, suggesting that Ca^{2+} channels are located at the site of vesicle accumulation and neurotransmitter release (Llinas *et al.*, 1976); (2) synaptic vesicle exocytosis captured by quick freezing occurs near the AZPs (Heuser *et al.*, 1979); (3) AZPs continue to specify sites of exocytosis even when dispersed by a low Ca^{2+}-medium (Ceccarelli *et al.*, 1979); and (4) in the squid synapse, the maximum presynaptic Ca^{2+} current appears to be related to the number of AZPs (Pumplin *et al.*, 1981).

Direct evidence for this contention is the presynaptic localization of CgTx-sensitive Ca^{2+} channels at the frog neuromuscular junction (Robitaille *et al.*, 1990; Tarelli *et al.*, 1991; Cohen *et al.*, 1991). CgTx- immunoreactivity revealed a pattern composed of regularly spaced bars running parallel to each other and perpendicularly to the major axis of the nerve terminal. This array corresponds to the ordered arrangement of active zones in this species. A correspondence was also found between the mean number of active zones and fluorescent bars present within the same length of axolemma (Tarelli *et al.*, 1991). If AZPs correspond to voltage-dependent Ca^{2+} channels, the reduced neurotransmitter release by nerve impulses in LEMS patients could be related to reduced entry of calcium into the nerve terminal.

2. The Antigenic Target

a. Calcium channel hypothesis

Autoantibodies that interfere with Ca^{2+}-dependent neurotransmitter release may do so by three distinct mechanisms. The LEMS autoantibody could act on (1) Ca^{2+} entry through voltage-gated Ca^{2+} channels, (2) intraterminal Ca^{2+} metabolism or (3) the exocytotic release process. Results of several studies indicate that LEMS autoantibodies interact with voltage-dependent Ca^{2+} channels (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988; Kim and Neher, 1988; Peers *et al.*, 1990). Other studies have rendered the other possibilities unlikely (Lang *et al.*, 1983; Kim, 1985; 1986; 1987; Wray *et al.*, 1987; Lambert and Lennon, 1988).

Except for the well-understood requirement of Ca^{2+} ions for prejunctional ACh release (Katz and Miledi, 1965; Dodge and Rahamimoff, 1969), specific molecular events leading to exocytosis are largely unknown. Because of this, it is difficult, if not impossible, to evaluate a possible dysfunction in the release mechanism itself. However, indirect effects on release mechanisms can be studied with pharmacological manipulation. Nerve evoked release of neurotransmitter from passively transferred mice was studied over a range of $[Ca^{2+}]_e$ concentrations (Kim, 1987; Wray *et al.*, 1987). NMJs from LEMS IgG-treated mice showed the characteristic decrease in quantal content at both low and physiological Ca^{2+} concentrations when compared to control IgG-treated mice. However, at unphysiologically high Ca^{2+} concentrations the number of quanta released by nerve stimulation in LEMS-treated NMJs reached the same plateau value as did

control-treated NMJs. This suggests that the number of available quanta was not different in passively transferred LEMS and the exocytotic release process itself was not impaired (Kim, 1987; Wray *et al.*, 1987).

The second possibility that intracellular mechanisms governing Ca^{2+} homeostasis may operate abnormally in LEMS is also not supported. An abnormality of this type would result in a decline in both nerve-evoked and spontaneous release of neurotransmitter. In human LEMS as well as passively transferred murine LEMS, no appreciable reduction in either MEPP frequency or amplitude is seen (Lang *et al.*, 1983; Kim, 1985; 1986; Lennon and Lambert, 1988).

With the exclusion of the latter two possibilities, and with the evidence presented thus far, the "calcium channel hypothesis" appears to be the most plausible explanation for the pathophysiology of LEMS. Numerous studies have helped to clarify the neuromuscular deficit associated with LEMS.

Incubation of rat cortical synaptosomes with whole serum or high molecular weight serum fractions (>10,000 daltons) from patients with LEMS, for as little as three minutes, decreased the amount of [^{3}H]ACh released during depolarization (Meyer *et al.*, 1986). Although this study did not measure Ca^{2+} channel activity, it was important in that it indicated that acute exposure of synaptosomes to serum or serum fractions taken from LEMS patients could elicit responses qualitatively similar to those obtained in chronic passive transfer experiments. Also, antigens presumed to reside on human and rodent nerve terminals appear to share a common epitope recognized by the LEMS autoantibody. Likewise, Login *et al.*,

(1987) exposed rat anterior pituitary cells to immunoglobulin isolated from a LEMS patient, and found that release of growth hormone and prolactin was significantly reduced by LEMS IgG compared with control IgG (Login *et al.*, 1987). Again, no assessment of ion channel activity was made but secretion from these cells is Ca^{2+}-dependent (Stojilkovic *et al.*, 1988).

Roberts *et al.*, (1985) cultured human small cell carcinoma cells (SCC) in the presence and absence of LEMS IgG for seven days. Following the incubation period, $^{45}Ca^{2+}$ uptake induced by 30 sec of K^+ depolarization was measured. The cells treated with LEMS IgG exhibited a significant decrease in stimulated uptake compared with untreated cells or with cells grown in control IgG (Roberts *et al.*, 1985). These studies were confirmed by De Azipurua *et al.*, (1988*a*) and Lang *et al.*, (1989*b*) who in addition demonstrated that PC12 cells were similarly affected (Lang *et al.*, 1989*a*; 1989*b*).

Kim and Neher (1988) applied IgG from LEMS patients to isolated bovine chromaffin cells from the adrenal medulla. Electrophysiological recordings indicated a 40% reduction in the current carried through calcium channels. Sustained inward Ca^{2+} currents in undifferentiated mouse neuroblastoma x rat glioma hybrid cells (NG108-15) were reduced by LEMS IgG also (Peers *et al.*, 1987). Taken altogether, these various results provide strong evidence for membrane calcium channels as a target site for the autoantibody in LEMS.

However, recent evidence suggests that synaptotagmin may also be a LEMS antigen (Leveque *et al.*, 1992). A 58 kDa antigen, identified as

synaptotagmin by a partial amino acid sequence, was detected by probing Western blots of partially purified calcium channels from rat brain with LEMS plasma and IgG. Synaptotagmin is a synaptic vesicle protein believed to be involved in docking synaptic vesicles to the plasma membrane at the active zone (Augustine, 1987; Leveque *et al.*, 1992; Brose *et al.*, 1992). As this protein was recognized in what was thought to be partially purified Ca^{2+} channels, Leveque and colleaques (1992) believe that synaptotagmin not only binds to the plasma membrane but is tightly associated with the Ca^{2+} channel as well. As such, alterations in synaptotagmin function could indirectly alter Ca^{2+} channel activity (Leveque *et al.*, 1992).

However, several deficiencies in this study are evident. First, synaptotagmin is a putative transmembrane protein with the N-terminus oriented toward the interior of synaptic vesicles and the C-terminus at least partially inserted into the lipid bilayer (Perin *et al.*, 1990). In order for the circulating antibody to bind, one must presume that this protein is exposed at the surface of the nerve terminal following exocytosis. Secondly, synaptotagmin and calcium channels may have a common epitope which can be recognized via SDS-PAGE followed by Western blot analysis but which is normally concealed. Finally, the possibility that LEMS sera contains antibodies that only recognize calcium channel associated polypeptides in their undenatured form can not be excluded.

b. Calcium channel subtype affected by LEMS IgG

The finding that LEMS IgG inhibits Ca^{2+} entry in motor nerve terminals (Lang *et al.*, 1987), SCC (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988*a*; Lang *et al.*, 1989*a*; 1989*b*), chromaffin (Kim and Neher, 1988), anterior pituitary (Login *et al.*, 1987), NG108-15 (Peers *et al.*, 1987) and PC12 cell lines (Lang *et al.*, 1989*a*; 1989*b*) indicates that the antibodies recognize an epitope that is present on voltage-dependent Ca^{2+} channels from several sources. The use of these cell lines have led to assumptions concerning the subtype of Ca^{2+} channel putatively affected by this antibody.

LEMS IgG was shown to reduce Ca^{2+} currents with characteristics of L-type channels in NG108-15 and chromaffin cells (Kim and Neher, 1988; Peers *et al.*, 1987). Secretion from anterior pituitary and undifferentiated PC12 cells is believed to be mediated by L-type channels also (Kongsamut and Miller, 1986; Login *et al.*, 1987; Stoljilkovic *et al.*, 1989). Data from studies utilizing SCC cell lines (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988*a*; 1988*b*) suggest an effect on both L- and N-type channels, respectively. Finally, LEMS autoantibodies have been shown to immunoprecipitate ^{125}I-CgTx labeled voltage-gated Ca^{2+} channels (N-type) from a human neuronal cell line (Sher *et al.*, 1989).

The results from these studies would suggest that the L- and N- type Ca^{2+} channel are putative antigenic targets for the LEMS autoantibody. Although these studies have proven useful in determining the antigenic target in the cell soma, the identity of Ca^{2+} channels in the nerve terminal remains unclear. What is clear, is

the types of Ca^{2+} channels at mammalian nerve terminals do not appear to be either N- or L-type and are possibly of the P-type (Anderson and Harvey, 1987; Sano *et al.*, 1987; Charlton and Augustine, 1990; Uchitel *et al.*, 1992).

3. Pathophysiological Mechanisms

The exact mechanism by which LEMS IgG exerts its pathological effects has not been fully established. In bovine adrenal chromaffin cells treated with LEMS IgG, kinetic and single channel studies indicate that the pathogenic autoantibodies neither modify the kinetics of Ca^{2+} channel activation and inactivation nor single channel open time nor conductance (Kim and Neher, 1988). Also, compared with control serum, pooled LEMS sera from 5 patients reduced the binding of the Ca^{2+} channel antagonist ^{125}I-ω-conotoxin (CgTx) by 22% following overnight incubation with the human neuronal cell line, IMR-32 (Sher *et al.*, 1989). Thus, it appears as if the antibody-induced dysfunction of Ca^{2+} channels occurs in an "all or none" fashion. Each channel is either subject to a complete functional block or no blockade at all. Therefore, the pathogenic action of the LEMS autoantibody is characterized by a reduction in the number of **functional** Ca^{2+} channels. Functional alterations could be achieved by several mechanisms and may act in concert.

a. Direct inhibition

Bound, pathogenic autoantibodies could reduce the number of functional Ca^{2+} channels by direct inhibition. This does not appear to be the case in some

systems, however. The application of LEMS plasma to an *in vitro* organ culture for up to three hr had no effect on nerve-evoked quantal release (Kim *et al.*, 1988). When mice were studied after various periods of IgG treatment, the $t_{½}$ for the reduction in quantal content was at least 4-6 hr (Lambert and Lennon, 1988), or longer (Prior *et al.*, 1985). However, it just may be that more than three hr of incubation is needed in order to gain access to, and inhibit the function of prejunctional Ca^{2+} channels in some systems. For example, only a two hr incubation of chromaffin cells with LEMS antibodies resulted in significant reductions in whole cell Ca^{2+} currents (Kim and Neher, 1988) while incubation of synaptosomes with LEMS sera for as little as three min decreased the amount of [^{3}H]-ACh released during depolarization (Meyer *et al.*, 1986).

b. Antigenic modulation

In view of the morphologic alterations depicting the scarcity of AZPs, it would appear that the LEMS autoantibody eventually destroys or eliminates Ca^{2+} channels. Antibody-induced antigenic modulation could result in loss of channels by initiating accelerated channel degradation. Ca^{2+} channel crosslinkage by LEMS IgG could be an initial process required for final channel elimination. This process has been observed by Nagel *et al.*, (1988) and Lang *et al.*, (1987). These investigators observed that divalent $F(ab)'_2$ (antigen-binding) fragments of LEMS autoantibodies which retain the antigen binding capacity of normal IgG effectively transferred the disease to mice, whereas monovalent Fab fragments failed to do so. This phenomenon was verified by measurements of AZP density (Nagel *et al.*,

1988) and quantal content of the EPP (Lang *et al.*, 1987). In the terminals treated with LEMS IgG or LEMS F(ab)'$_2$, quantal content was reduced; monovalent LEMS Fab had no significant effect (Lang *et al.*, 1987). There was also a significant decrease in the density of active zones and AZPs. In the AZPs remaining, interparticle distance was reduced (Nagel *et al.*, 1988). This was considered to be an early event associated with disorganization and eventual destruction of active zones and AZPs (Fukuoka *et al.*, 1987).

c. Complement-mediated destruction

Alternatively, loss of channels could be due to complement-mediated channel destruction. Activation of complement plays a central role in mediating postsynaptic membrane damage in animal models of the autoimmune neuromuscular disorder, myasthenia gravis (MG) as well as in the human disease state (Engel *et al.*, 1977; Sahashi *et al.*, 1978). It seems possible that complement activation could be important in LEMS as well.

Complement is a complex series of proteins which form a triggered enzyme system in the plasma. Figure 1.3 provides a summary of the complement components and their interactions. Some of the complement components are designated by the letter "C" followed by a number. The complement system may be activated by two major pathways (classical and alternative) that proceed in a sequential fashion. Activation of individual components of complement depends upon activation of a prior component or components in the sequence. Fragments of native complement components are either released into the fluid phase or bind

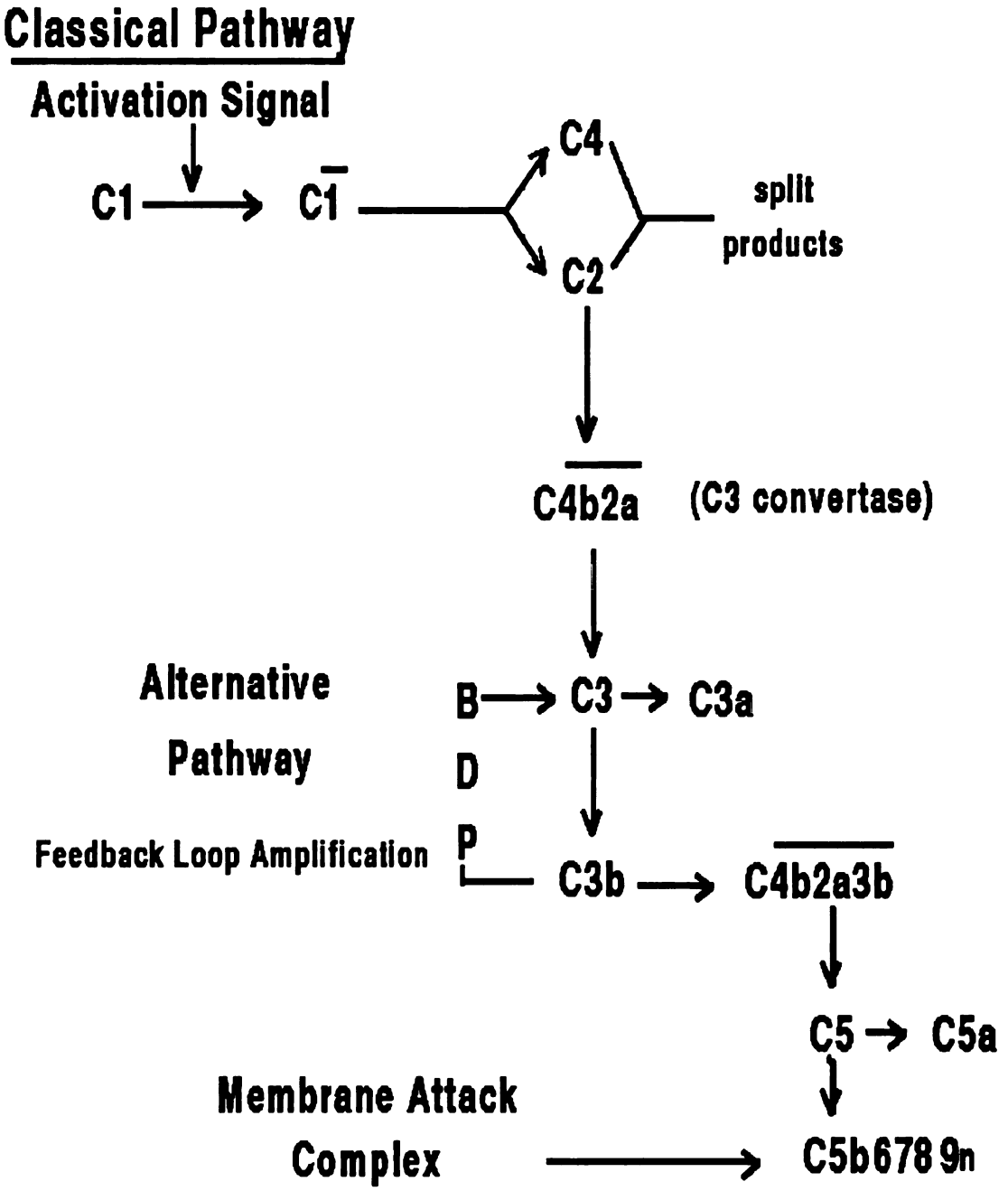


Figure 1.3. Complement.

Scheme of the reaction mechanisms following activation of the complement cascade. For detailed explanation please see text (pp. 32-35).

to other complement components or suitable surfaces such as immune complexes and cell membranes (Law and Levine, 1977; Capel *et al.*, 1978; Takata *et al.*, 1984).

The classical pathway of complement activation is typically initiated by the union of antibody and antigen. The antigen can be either soluble or a constituent of a cellular or extracellular surface. Whether an antigen-antibody reaction activates complement depends upon the antibody molecule. Most immunoglobulins of the IgM or IgG (with the exception of IgG4) subclass activate the classical pathway (Loos, 1987).

Antigen binds to Fab (antigen-binding) regions of immunoglobulins. Together, these molecules undergo a conformational change that permits binding and activation of the first component of complement, C1. Activated C1 cleaves C4 and C2, by limited proteolytic reactions. Cleavage of C4 results in the formation of a small peptide, C4a and a large fragment, C4b, which can bind either to the immune (antigen-antibody) complex or to the surface of the antigen. C2 is cleaved into a small peptide, C2b and a large fragment, C2a, which binds to C4b. This complex, C4b2a, (C3 convertase) possesses new enzymatic activity that is capable of cleaving the third component of complement, C3.

Proteolytic cleavage of C3 yields a small fragment, C3a and a larger fragment, C3b, which possesses the ability to bind to immune complexes, as well as to a wide variety of surfaces (Law and Levine, 1977; Capel *et al.*, 1978; Takata *et al.*, 1984). Once bound, C3b imparts a new specificity to C4b2a, enabling this

enzyme to function as a C5 convertase. Bound C3b is also recognized by phagocytic cells (Hostetter *et al.*, 1984), and functions as an important intermediate in the activation of the alternative pathway (Gõetze, 1987).

Cleavage of C5 by C5 convertases, constitutes the final proteolytic reaction in the classical pathway. A small fragment, C5a, is released into the fluid phase, but the larger fragment, C5b, binds to immune complexes and to a variety of surfaces. Whether bound or in the fluid phase, C5b forms a stable macromolecular complex with the complement components C6, C7, and C8. This complex induces orientation between two or more molecules of the terminal component C9. This leads to unfolding of C9 and insertion into the lipid bilayer and polymerization to a "membrane attack complex" (MAC). MACs form transmembrane channels that permit bidirectional flow of ions and ultimately macromolecules producing cellular injury and/or lysis. (Porter and Reid, 1978; Bhakdi and Tranum-Jensen, 1978).

The alternative pathway (APC) is a non-specific, natural defense system against pathogenic microorganisms (Joiner *et al.*, 1984; Gõetz *et al.*, 1987). In addition, it is an amplification system for increasing C3b depositon and C3/C5 convertase activity after complement activation through the classical pathway (Joiner *et al.*, 1984). Once activated, the biological sequence of events of the APC does not differ from that of the classical pathway.

Attempts to clarify the participation of complement in the pathogenesis of LEMS have yielded negative results. Passive transfer was attempted in mice which had received cobra venom factor (CVF) (Lennon and Lambert, 1988) and in mice

congenitally deficient in C5 (Prior *et al.*, 1985; Lennon and Lambert, 1988). CVF is a potent complement activator, which interacts with Factor B of the alternative pathway to form a stable C3 convertase (Göetz and Müller-Eberhard, 1971). This leads to the consumption of plasma C3 and the later components of the complement cascade. The defect in neuromuscular transmission in mice receiving LEMS IgG was evident in mice of normal and deficient complement status (Prior *et al.*, 1985; Lambert and Lennon, 1988). These results suggest that the effects of LEMS IgG may not be due to complement activation. This unaltered response in the absence of complement activity does not, however, mean that it is not involved when present. Most of the effects of IgG or the alternative pathway activators on cells are greatly enhanced in the presence of complement (Rother, 1987).

F. *The Immune Response*

As stated previously, the pathogenic alterations in neuromuscular transmission in LEMS are caused by an autoantibody circulating in the plasma of patients with the disorder. Alterations in the regulation of the normal immune response could lead to recognition of self-components as foreign inducing autoimmunity. The next section will discuss the normal immune response with emphasis on mechanisms which disrupt regulatory pathways leading to faulty self-non-self recognition. Determinations of the mechanisms of autoimmunity in concert with determination of the antigenic target could have important therapeutic implications for LEMS patients or any patient with autoimmune disease.

1. Cellular interactions in the immune system

The immune system can be considered simplistically the body's defense against foreign invaders. An intricate series of cellular events must occur in order for the body to mount an effective immune response. Typically, an immune response is generated by the coordinated interaction of lymphocytes and antigen-presenting cells (Fig 1.4)(see Miller *et al.*, 1971). All of these cells derive from the bone marrow, from a pluripotent stem cell (Roit, 1988*a*).

One class of lymphocytes consists of B lymphocytes, so called because maturation of these cells occurs in the bone marrow. B lymphocytes are the only cells capable of producing antibodies (Nossal *et al.*, 1968; Jacobson *et al.*, 1970).

The second major class of lymphocytes are T lymphocytes, which arise in the bone marrow and then migrate to and mature in the thymus. It is in the thymus that the T lymphocyte undergoes a selection event in which "learning" takes place for recognition of self-encoding molecules known as the major histocompatibility complex (MHC). T lymphocytes are further subdivided into functionally distinct populations, the helper (T_h) cell and the suppressor/cytotoxic T (T_s) cell. T_h cells promote immune responses to antigen whereas T_s cells function to suppress such a response (Schwartz, 1987; Roit, 1988*a*).

Cells responsible for antigen presentation are usually of the phagocytic monocyte-macrophage lineage (Adler *et al.*, 1966; Unanue and Askonas, 1968; Unanue and Cerottini, 1989). Other antigen-presenting cells include the Langerhans cells in the skin, dendritic cells of the lymph nodes and spleen, and

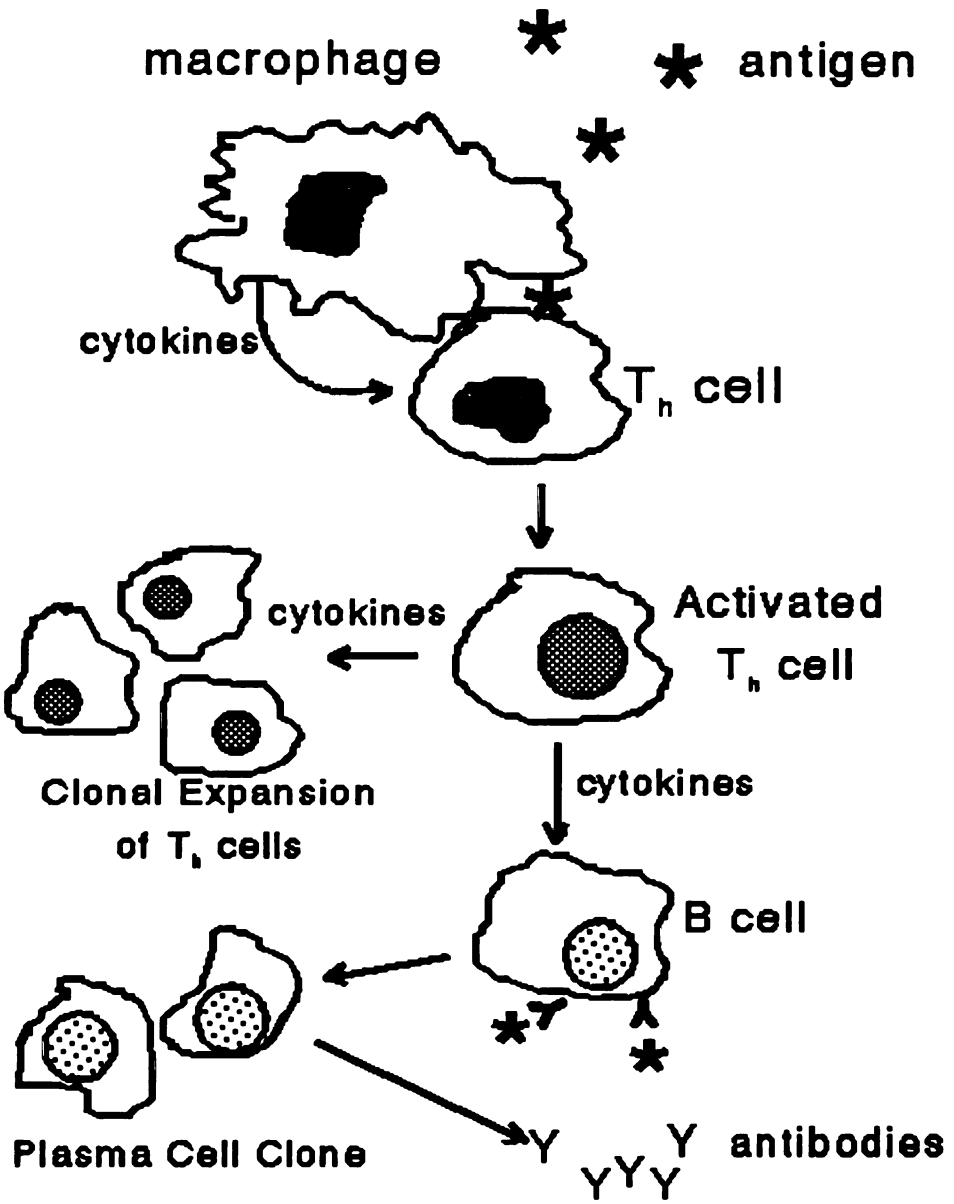


Figure 1.4. Immune Response

The immune response is initiated when antigen is processed and presented to a T_h lymphocyte by a macrophage. The T_h cell becomes activated and releases a variety of soluble factors (cytokines) which act on both B and T lymphocytes to augment the immune response. Once triggered by antigen and T_h cell cytokines, B cells proliferate forming a large clone of plasma cells which make antibody.

the interdigitating follicular cells of the thymus (Dalton and Bennett, 1992).

Normally, the interaction between the T_h lymphocyte and the antigen-presenting cell is the first event in an immune response. Helper T cells have an unusual specificity for antigens. They recognize only peptide antigens that are attached to MHC molecules expressed on the surfaces of antigen-presenting cells (Lamb and Feldman, 1984). The T cell does not recognize native antigen, however. Instead, the antigen-presenting cell goes through a series of events known as antigen processing, whereby native antigens are internalized, partially degraded and immunogenic fragments are returned to the cell surface (Unanue and Askonas, 1968; Unanue and Cerottini, 1989). In addition, the antigen-presenting cell releases protein hormones called cytokines to co-stimulate the T_h cell. In response to stimulation, T_h cells also secrete cytokines, whose function is to promote the proliferation and differentiation of the T cells themselves as well as other cells including B cells (Schwartz, 1987).

Once triggered by antigen and T_h cell cytokines, B cells undergo successive waves of proliferation to build up a large clone of plasma cells which make antibody that is antigen-specific (Jacobson *et al.*, 1970). In addition, B cells are able to recognize some antigens without any T cell help. Certain antigens, such as bacterial lipopolysaccharides, have the ability to activate a proportion of the B cell population by their inherent mitogenic potential (Roit, 1988a). B cells have the ability to produce $\approx 10^8$ different antibodies (Max, 1989). However, each B cell can produce only a single heavy and a single light chain, which interact to make an

antibody with a unique specificity (Tonegawa, 1983).

2. Self- non-self recognition

The key to effective immunity is the elimination of pathogens without concomitant host injury. The finely tuned interaction among lymphoid cells, cytokine molecules and foreign influences determines immune reactivity. This finely tuned modulation of the immune system is crucial since excessive activation can lead to allergies and autoimmune diseases and oversuppression may promote infectious diseases and cancer (Homo-Delarche *et al.*, 1991). Curiously enough, autoreactive B and T cells are present normally (Dighiero *et al.*, 1987; Rosenkrantz *et al.*, 1987). The body has homeostatic mechanisms to prevent them from being triggered under normal circumstances. Immunologic tolerance to self in T and B lymphocytes can be induced by two basic mechanisms: (1) deletion, through cell death; and (2) functional inactivation (anergy) without physical deletion.

a. Clonal Deletion

The clonal deletion theory of self-tolerance was first proposed by Burnet (1959) to occur during fetal or early postnatal life. Autoreactive clones of lymphocytes (both B and T) were believed to be eliminated during the stage in development immediately after the lymphocyte expresses its antigen receptor and before it becomes competent to respond positively to antigenic stimulation (Lederberg, 1959). In this way autoreactive clones are filtered out.

The original theory was modified by Allison *et al.*, (1971) and Weigle (1971)

who independently suggested that self-tolerance depends upon intrathymic elimination of self-reactive T lymphocytes while B cell responsiveness to self antigens remained intact. In this way, autoreactive T cells are unable to collaborate with B cells to generate autoantibodies. The models put forth to explain this were based on receptor affinity (for review see Sprent *et al.*, 1988). T cells in the thymus which bind with high affinity to MHC molecules on the surface of intrathymic macrophage cells are eliminated. Those capable of reacting with MHC plus antigen but which only bind with poor affinity to self molecules alone, survive.

The idea of clonal deletion has been now been verified for both T and B cells using transgenic mice (Kisielow *et al.*, 1988; Sha *et al.*, 1988; Nemazee and Bürki, 1989). However, clonal deletion may play a relatively minor role in B cell tolerance (Nemazee and Bürki, 1989; Goodnow *et al.*, 1990). Because, autoreactive T and B cells can be recovered from normal humans and animals (Dighiero *et al.*, 1987; Rosenkrantz *et al.*, 1987), the process of clonal deletion must be incomplete. Other mechanisms must act to limit the potentially catastrophic immune response against self.

b. Clonal Anergy

***i.* Induced by Suppression**

Removal of anti-self effector lymphocytes may be attributable to elimination in some cases but may be due to the presence of peripheral blood regulatory lymphocytes in others. Self-tolerance based on the induction of T_s cells has been postulated by Rose (1988). In this model, cells are functionally inactivated without

being physically deleted. Self-antigens are considered normally to induce mostly suppressor (rather than helper) T cells with the ability of suppressing that particular immune response (for review see Cruse and Lewis, 1992). This model allows for the existence of a reservoir of cells potentially capable of autoimmune behavior.

Clonal anergy has been demonstrated in the B cell repertoire as well (Weigle, 1973; Metcalf and Klinman, 1976; Nossal, 1989; Goodnow *et al.*, 1990). Studies in tissue culture demonstrate that B lymphocytes exposed to antigens in the absence of T_h lymphocytes can be induced into a state of nonresponsiveness. Subsequent challenge of the B cells with antigen in the presence of T_h cells produces no response (Metcalf and Klinman, 1976).

***ii.* Induced by Signal Failure**

Bretscher and Cohn (1970) suggested the need for two signals to activate a lymphocyte: one, antigen receptor occupation; the other, a co-stimulatory signal delivered by a second cell. In the case of B lymphocytes, the second signal comes from activated T_h cells. In the case of T_h lymphocytes, the second signal comes from antigen-presenting cells. Tolerance induction in this model was postulated to be delivery of signal one in the absence of signal two (Bretscher and Cohn, 1970).

Inactivation of antigen-specific lymphocytes as a consequence of abortive antigen presentation may indeed play a role in self-tolerance. In healthy organisms, self-antigens may be presented without accessory signals (cytokines, MHC, adhesion molecules) necessary to induce clonal proliferation and differentiation. This would leave the T cell unchanged and possibly unable to respond later to an

adequate stimulus (Bretscher and Cohn, 1970; Lamb and Feldman, 1984; Parker and Eynon, 1991). In this way, immunocompetent, autoreactive T cells could be inactivated. Similiarly, B lymphocytes whose receptors are cross-linked by certain multivalent antigens alone are rendered non-responsive, whereas simultaneous costimulatory signals by T cell cytokines lead to activation and differentiation (Nossel, 1983).

3. Breakdown of self-tolerance

The state of self-tolerance may be an amalgam of many processes including (but not limited to) clonal deletion and specific suppressor cell networks. If lymphocyte/monocyte action is not limited by protective regulatory pathways then these cells will either directly or indirectly attack innocent bystander cells, disrupting tissue structure and leading to autoimmune disease. Direct attacks include the release of biologically active agents (enzymes, peroxides, cytotoxic cytokines etc.) from monocytes or cytotoxic lymphokines from T lymphocytes. Indirect attacks may be mediated through antibodies released from B cells and/or activation of the complement system (Grossman *et al.*, 1991).

Autoimmune diseases are chronic and in some cases severe. They are clearly multifactorial in origin with genetic, immune, endocrine and environmental elements contributing to their development (Rose, 1988; 1989; McGregor, 1990; Dalton and Bennett, 1992). A variety of factors have been identified that are both indirectly and directly responsible for the inappropriate activation of immune

effector cells (Bottazzo *et al.*, 1983; Cruse and Lewis, 1987; Sarvetnick *et al.*, 1988; Sinha *et al.*, 1990; Grossman *et al.*, 1991; Kronenberg, 1991). Figure 1.5 summarizes schematically some of the initiators of the pathogenesis of autoimmune disease. These factors are often interrelated and can act in concert to give rise to pathogenic autoimmunity.

a. Decreased Suppression

Reversal of anergy might form part of the spectrum of autoimmunity. Antigen specific T_s cells responsible for blocking the effects of autoreactive T_h cells could be rendered ineffecient and anergy would be abrogated by allowing signals to reach the B lymphocyte. Distortion in the helper/suppressor cell ratio could produce an advantage to helper components (Krosgrud and Perkins, 1977; Tyan, 1977). Defects of T_s cells have been described in systemic lupus erythematosus, rheumatoid arthritis, MG, and autoimmune hemolytic anemia (Shoenfield and Isenberg, 1989*a*). In addition, aging alone has profound effects on thymic function and may alter the balance of helper/suppressor cells (Serge and Serge, 1987).

b. Modification of an autoantigen

Self antigens may be altered by a variety of chemicals. Patients receiving chronic administration of the antihypertensive drug, α-methlydopa, have a higher incidence of developing autoimmune hemolytic anemia. This may be attributable to a modification of the erythrocyte surface in such a way as to provide stimulation of B cells (Rose, 1988; Roit, 1988*b*; Shoenfeld and Isenberg, 1989*b*). The addition of a foreign determinant to native proteins may provide a mechanism for T cell

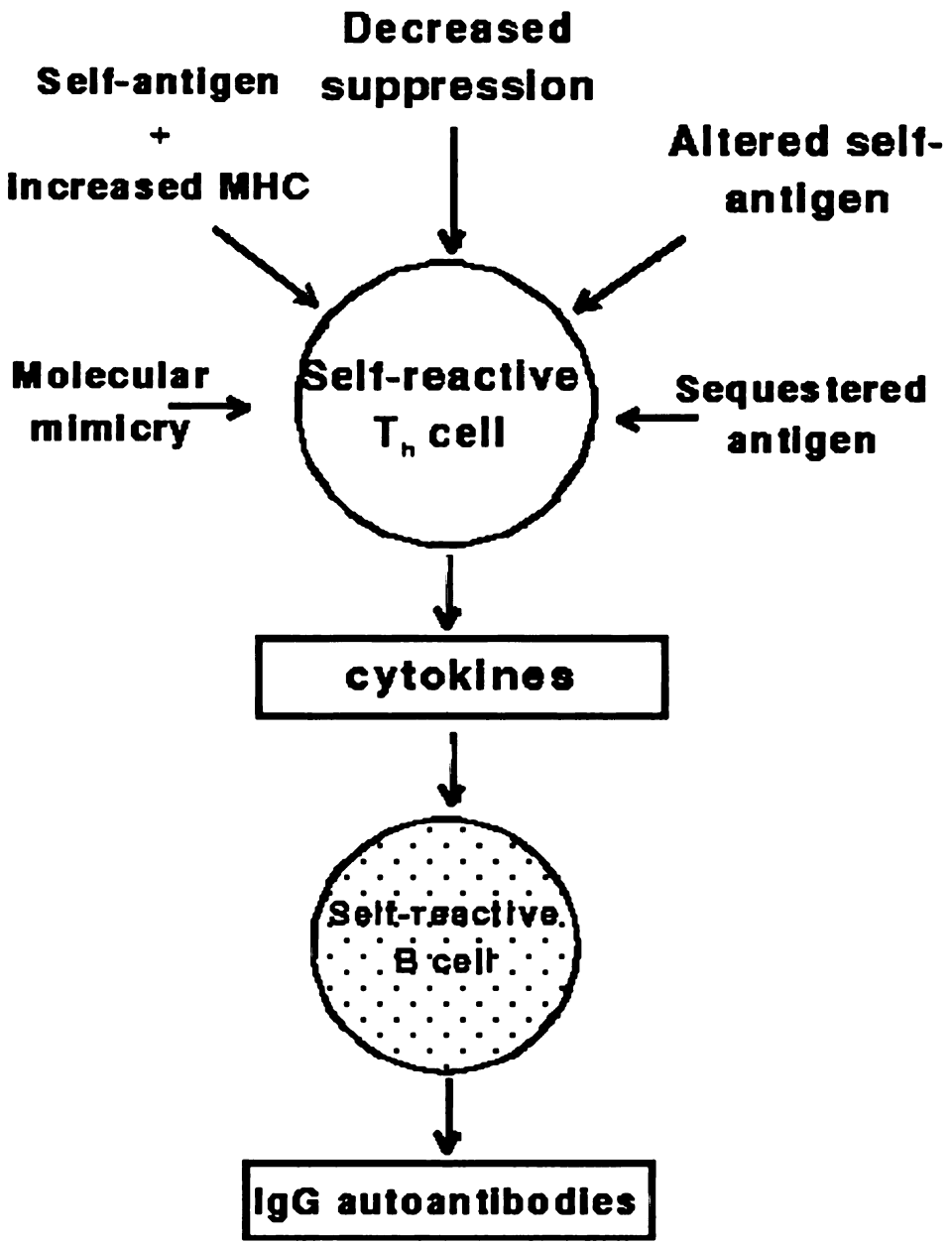


Figure 1.5. Pathogenesis of autoimmune disease.

Autoimmunity may be initiated by a variety of mechanisms involving alterations in immune regulation, changes in auto-antigens or both. These factors are often interrelated and may act in concert to give rise to pathogenic autoimmunity.

recognition.

MG has been described in some patients on penicillamine (Bucknell *et al.*, 1975; Czlonkowska, 1977) and a model of penicillamine-induced MG has been produced in animals (Bever *et al.*, 1982; Bever and Asofsky, 1991). It is thought that penicillamine either causes a generalized alteration in immunoregulation (Bluestone and Goldberg, 1973; Epstein *et al.*, 1979) and/or penicillamine becomes covalently linked to host proteins causing antigenic changes which lead to an immune response against the host protein, in this case, acetylcholine receptors (Dawkins *et al.*, 1981; Bever *et al.*, 1982).

c. Self antigen plus aberrant self-encoding molecules

In many autoimmune diseases there is an overexpression of MHC molecules on the target tissue (Shoenfield and Isenberg, 1989*b*). Because MHC molecules are known to be important in antigen-presentation, these observations led to the proposal that abnormal, excessive or ectopic self-encoding molecule expression leads to the presentation of autoantigen to autoreactive T_h cells (Bottazzo *et al.*, 1983). The basic principles of this hypothesis have been verified experimentally using the thyroid as a model. It was shown that interferon-γ induced MHC expression in thyrocytes; that these cells could act as antigen-presenting cells; and that, in autoimmune disease, many of the infiltrating T cells specifically recognize these thyrocytes (Londei *et al.*, 1984; 1985).

d. Release of sequestered antigens

Anatomic alterations may lead to the exposure of antigens that are normally sequestered and concealed from the immune system. Because of this sequestration, individuals may not be immunologically tolerant to such antigens. Therefore, if such self antigens are released and interact with immunocompetent lymphocytes, specific immune responses may develop (Kronenberg, 1991). Examples of anatomically- sequestered antigens are intraocular proteins and sperm.

e. Cross-reactivity

***i.* antigenic mimicry**

Many examples are known in which potential autoantigenic determinants are present on an exogenous cross-reacting antigen which provokes autoantibody formation (Shoenfield and Isenberg, 1989*b*). An antiserum raised against an antigen can cross-react with a partially related antigen which bears one or more similar determinants. The more determinants that are similar, the greater the cross-reactivity (Roit, 1988*b*).

Infection by certain microorganisms may result in antigenic mimicry. Immune reactivity against microbial antigens may encompass the stimulation of T_h lymphocytes capable of reacting with self. Alternatively, structural mimicry between selected antigens of microorganisms and mammalian tissue may result in cross-reactivity of the antibody response (Rose, 1989). Well known examples of the association of autoimmune disease with infection are the role of *streptococcal*

infection with the development of rheumatic fever (Cruse and Lewis, 1987; Roit, 1988*b*) and the onset of diabetes mellitus type 1 following mumps (Coxsackie) virus infection (King *et al.*, 1983).

***ii.* aging**

Studies have also demonstrated a progressive decline in the immune response associated with aging. For example, there are several changes in effector functions of the T cell compartment (Krosgrud and Perkins, 1977; Tyan, 1977) and there is a consistent decrease in the amount and in the average affinity of antibody produced by B cells in aged animals (Price and Makinodan, 1972; Nordin and Makinodan, 1974; Callard *et al.*, 1977). Paradoxically, these changes are accompanied by an increased incidence of autoantibodies (Goidl, 1987). Thus, the aging immune system is poorly regulated.

The decline in antigen responsiveness associated with aging has been characterized by the production of antibody with decreased average affinity and decreased heterogeneity of the antibody affinity (Callard *et al.*, 1977; Klinman, 1981). This decrease in heterogeneity in the aged is seen preferentially as the loss of medium- to high-affinity antibody. Thus, the expressed antibody repertoire is principally composed of low-affinity antibody (Doria *et al.*, 1978; Goidl, 1987). The etiology of the increase in antibodies that recognize self-components could be explained then by inappropriate cross-reactive low-affinity antibodies.

G. *Autoimmune LEMS and the role of cancer*

It is now well established that self-reactivity is a common phenomenon, but the conversion from autoimmunity to autoimmune disease requires a variety of additional factors. The mechanisms of autoimmunity are topics for which theories and hypotheses outnumber facts. A major difficulty in defining the mechanisms of human autoimmune disease has been the inability to identify the antigens that initiate the autoimmune response. As a result the specific etiology of most autoimmune diseases, in general, and of LEMS, in particular, is unknown.

Muscle weakness is often a feature of systemic malignancy, but is frequently dismissed as a non-specific complaint in a chronically debilitated patient (Gomm *et al.*, 1990). A variety of non-metastatic neuromuscular disorders, including cerebellar degeneration, peripheral neuropathy and myopathy may be associated with malignant neoplasm, in particular with lung cancer (Brain *et al.*, 1951; Henson *et al.*, 1954; Gomm *et al.*, 1990) .

As mentioned previously, an association of LEMS with SCC occurs in 60% of LEMS patients (Eaton and Lambert, 1957; Lambert *et al.*, 1961; Wise and MacDermot, 1962; Elmqvist and Lambert, 1968; O'Neill *et al.*, 1988). Early suggestions were made that SCC tumors, produced biologically active peptides which interferred with neuromuscular transmission (Ishikawa *et al.*, 1977). These studies, however, where unable to be duplicated. Further studies of the association of LEMS with neoplasms have proposed that an IgG autoantibody is produced in response to antigens on the surface of SCC tumor cells which cross-react with

Ca^{2+} channel determinants on the presynaptic motor nerve terminal (Newsom-Davis *et al.*, 1982; Lennon *et al.*, 1982; Vincent *et al.*, 1989).

In support of this hypothesis, oat (small) cells are thought to be of neuroectodermal origin and have many of the biochemical markers of neurons (Gadzar *et al.*, 1981; Moody *et al.*, 1981; Tapia *et al.*, 1981). Electrophysiological recordings from, and radioactive $^{45}Ca^{2+}$ flux and Fura-2 measurements in small cells in tissue culture indicate that SCC cells have voltage-dependent Ca^{2+} channels (McCann *et al.*, 1981; Roberts *et al.*, 1985; De Aizpurua *et al.*, 1988; Sher *et al.*, 1990). Furthermore, SCC cells grown in LEMS IgG containing medium exhibited significantly less K^+-stimulated Ca^{2+} influx than control IgG-treated cells (Roberts *et al.*, 1985; De Aizpurua *et al.*, 1988).

More recently, the immunocytochemical characteristics of small cell carcinoma with and without associated LEMS was assessed by Morris *et al.*, (1992). In LEMS-associated SCC, there were numerous macrophages at the periphery of the tumor and many in the tumor mass. This contrasted with the non-LEMS cases in which there were fewer macrophages both in the periphery and in the tumor. Lymphocyte aggregates were also seen adjacent to the tumor in LEMS patients with fewer seen in non-LEMS tumors. Analysis of the lymphocyte populations using subset-specific monoclonal antibodies revealed that the majority of lymphocytes were B cells. Fewer T cells were seen and there was little difference in proportions of T_h/T_s cells in either LEMS or non-LEMS tumors. The marked macrophage and lymphocyte infiltration in LEMS-associated SCC raises

the possibility that it is indeed the tumor in LEMS that is provoking the immune response (Morris *et al.*, 1992).

Clinical evidence for neurological improvement after treatment or excision of the tumor would be consistent with this fact. Specific tumor therapy (resection and/or irradiation) **may** be followed by improvement or remission of the neurological deficit but this has not been generally noted (Ingram *et al.*, 1984; Ferroir *et al.*, 1989; Chalk *et al.*, 1990; Oh, 1990). Furthermore, interpretation of the data from these studies can be difficult as many of the patients receiving specific tumor therapy are also receiving pharmacological treatment for LEMS. Therefore, it seems likely that the tumor may be the initial antigenic target, but this can not be stated with certainty.

The clinical and electrophysiological features of LEMS do not show any consistent differences between those with underlying SCC and those who do not. Thus, the disease which is homogeneous in its clinical expression has more than one triggering factor. In about 60% of the cases, this factor appears to be SCC while in the remainder the factor(s) are unknown. One possibility is that LEMS patients without detectable cancer had a small occult tumor which was successfully eliminated by the immune response (Vincent *et al.*, 1989).

CHAPTER TWO

SYNAPTOSOMES AND SYNAPTOSOMAL ION CHANNELS

A. ***General Introduction***:

The exact identity of the antigenic target of the autoantibody which results in LEMS is unknown. Studies of the association of LEMS with SCC suggest that an antibody is produced in response to antigens on the surface of SCC tumor cells and that these antibodies cross-react with Ca^{2+} channel determinants on the presynaptic motor nerve terminal (Newsom-Davis *et al.*, 1982; Lennon *et al.*, 1982; Vincent *et al.*, 1989). However, there is reason to believe that Ca^{2+} channels from other nerve terminals may also be susceptible to the the putative blocking actions of the LEMS autoantibody (Meyer *et al.*, 1986). LEMS immunoglobulin (Ig) appears to interact with voltage-dependent Ca^{2+} channels in a number of model cell systems (Roberts *et al.*, 1985; Login *et al.*, 1987; De Azipurua *et al.*, 1988*a*, 1988*b*; Kim and Neher, 1988; Lang *et al.*, 1989*a*; Peers et al., 1990). Assumptions concerning the subtype of Ca^{2+} channel affected by the LEMS autoantibody have been made based on the subtypes found in these various cell lines. However, the exact identity of the subtype(s) of Ca^{2+} channel affected in nerve terminals remains unknown. Again, it is not only possible but probable that characteristics of Ca^{2+} channels in the terminal differ from those in the cell body (Leonard *et al.*, 1987; Suszkiw *et al.*, 1989; Charlton and Augustine, 1990; Uchitel *et al.*, 1992).

Passive transfer of the electrophysiological characteristics of LEMS to rodents by chronic injection of IgG isolated from LEMS patients permits only indirect experimental investigation of the putative site of action of the LEMS autoantibody. This is due to the fact that motor nerve terminal Ca^{2+} channels are

not sufficiently accessible nor amenable to direct recording techniques. Since the low density of nerve terminals in skeletal muscle makes them impratical to isolate, an alternative model of centrally isolated nerve terminals (synaptosomes) may be useful to examine this problem at a more molecular level.

B. *Synaptosomes*

1. General Description

Synaptosomal suspensions are heterogeneous preparations of intact, isolated nerve terminals. They are prepared from brain homogenates using density gradient centrifugation techniques (Gray and Whittaker, 1962). Synaptosomes may be formed by a process of pinching-off so that the cytoplasm is never at any time exposed to the isolation medium, or conversely by resealing of the fractured membrane after rupture of the axon with brief exposure of cytoplasm to isolation medium (Gray and Whittaker, 1962; Bradford, 1975). Whatever the mechanism, the results are completely sealed cytoplasmic sacs of 1-2 μM in diameter retaining all of the cell organelles visible in the intact terminal *in situ*, including mitochondria, endoplasmic reticulum and synaptic vesicles (Gray and Whittaker, 1962; Dodd *et al.*, 1981). Also, many metabolic and synthetic pathways remain intact in synaptosomes. These include glycolysis, the Kreb's cycle and neurotransmitter synthesis (de Belleroche and Bradford, 1973; Bradford, 1975). Synaptosomes maintain transmembrane ion gradients and therefore a membrane potential. By using a potentiometric, fluorescent dye, investigators estimate the presence of a

membrane potential in the -55 to -65 mV range (Blaustein and Goldring, 1975; Hare and Atchison, 1992). High K^+ concentrations and veratrum alkaloids, such as veratridine, depolarize the synaptosomal membrane and induce Na^+ and Ca^{2+} influx, K^+ efflux, and Ca^{2+}-dependent transmitter release (Bradford *et al.*, 1973; Nachshen and Blaustein, 1980; Tamkun and Catterall, 1981; Draupeau and Blaustein, 1983; Bartschart and Blaustein, 1985; Nachshen, 1985). When synaptosomes are incubated in a glucose-containing medium at 37°C, they remain viable for up to four hours after isolation (Bradford, 1975). Incubation at lower temperatures or maintenence of synaptosomes on ice significantly increases their viability.

Synaptosomes have been used widely in receptor binding assays (Turner and Goldin, 1985; Dunn, 1988; Marqueze *et al.*, 1988; Suszkiw *et al.*, 1989), and for studies of nerve terminal channel function (Nachshen and Blaustein, 1979, 1980; Bartschat and Blaustein, 1980; Tamkun and Catterall, 1981) and neurotransmitter synthesis and release (Bradford *et al.*, 1973; Drapeau and Blaustein, 1983; Floor, 1983; Woodward *et al.*, 1988). Results from such experiments have contributed significantly to knowledge of the neurochemical events that occur at the presynaptic nerve terminal.

2. Synaptosomal ion channels

a. Sodium channels

Influx of Na^+ through voltage-dependent channels is involved in action potential generation and propagation. A variety of toxins from both plants and

animals specifically interact with voltage-dependent Na^+ channels in excitable membranes. Saxitoxin (STX) and tetrodotoxin (TTX) specifically block these channels while batrachotoxin (BTX) and veratridine (VER) persistently activate voltage-sensitive Na^+ channels. The latter two toxins will also depolarize excitable cells by increasing Na^+ permeability (for review see Catterall, 1980). The use of these neurotoxins as specific pharmacological probes has made it possible to establish biochemical methods to study Na^+ channel properties in excitable tissues and in synaptosomes.

Synaptosomal Na^+ channels were indirectly demonstrated by VER-stimulated $^{45}Ca^{2+}$ uptake (Blaustein, 1975; Nachshen and Blaustein, 1979), VER-stimulated neurotransmitter release (Blaustein, 1975; Cotman *et al.*, 1976) and VER- and BTX- induced synaptosomal depolarization (Krueger and Blaustein, 1980). Direct evidence for the presence of Na^+ channels in synaptosomes was demonstrated by Krueger and Blaustein (1980) and Tamkun and Catterall (1981), who reported VER-stimulated uptake of $^{22}Na^+$ into synaptosomes. This uptake was TTX-sensitive. The use of these toxins has enabled these investigators to conclude that Na^+ channels in synaptosomes retain functional properties that are identical with those in intact neurons (Krueger *et al.*, 1980; Tamkun and Catterall, 1981).

b. Potassium channels

Potassium channels located in the presynaptic nerve terminal control excitability and neurotransmitter release and thus have the potential to be key regulators of synaptic transmission. Previous work has shown that synaptosomes

contain a variety of K^+ channels whose function can be studied with $^{86}Rb^+$ efflux methods (Bartschat and Blaustein, 1985*a*,*b*). The various channels can be distinguished on the basis of their kinetic and pharmacological characteristics (Bartschat and Blaustein, 1985*a*,*b*; Benishen *et al.*, 1988) and correspond to known conductances determined in cell systems by electrophysiological techniques.

Rat brain synaptosomes contain at least four distinct types of K^+ efflux mechanisms: (1) a resting K^+ permeability, seen with 5 mM K^+ solutions; (2) a fast, inactivating, voltage-dependent K^+ channel sensitive to block by tetraethylammonium (TEA), tetrabutylammonium (TBA) and 4-aminopyridine (4-AP); (3) a slowly inactivating, voltage-dependent K^+ channel which is less sensitive to TEA, TBA and 4-AP; and (4) a Ca^{2+}-activated K^+ channel (Bartschat and Blaustein, 1985*a*).

Rb^+ passes through these various types of channels as well as K^+ itself. It is accumulated in the cell by the Na^+/K^+ ATPase and is released under depolarizing conditions (Bartschart and Blaustein, 1985*a*,*b*). Since $^{86}Rb^+$ has a longer half-life (days) than $^{42}K^+$ (hours), it is a more practical choice for assessing K^+ channel activity in synaptosomes or intact cells.

c. Calcium channels

Ca^{2+} plays an essential role in the control of a wide range of cellular functions. Normally, cells maintain an $[Ca^{2+}]_i$ in the range of 100 nM while the $[Ca^{2+}]_e$ is typically in the low millimolar range. A transient rise in the $[Ca^{2+}]_i$ can

trigger many biological responses, including excitation-contraction coupling and neurotransmitter release. One way in which $[Ca^{2+}]_i$ can be rapidly increased is by the opening of voltage-sensitive Ca^{2+} channels in the cell membrane, allowing Ca^{2+} to flow down its electrochemical gradient. Ca^{2+} channels can be classified into various subtypes differing dramatically in the mechanism of inward current decay during a maintained depolarization and in their sensitivity to pharmacological agonists and antagonists (see Chapter One).

Radiotracer flux studies, using $^{45}Ca^{2+}$, have been useful in demonstrating Ca^{2+} influx into synaptosomes. Voltage-dependent uptake of Ca^{2+} into synaptosomes consists of two distinct phases (Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1983; Nachshen, 1985). The "fast" phase of influx occurs during the first second of depolarization. It is sensitive to block by lanthanum (< 1 μM) and nickel (40 μM) and inactivates during the the first 2-5 sec of K^+-induced depolarization (Nachshen and Blaustein, 1980; Nachshen, 1985). The second or "slow" phase of Ca^{2+} entry remains active for 20-90 sec after depolarization and is insensitive to block by lanthanum (Nachshen and Blaustein, 1980).

The "slow" phase of influx is believed to be mediated primarily by a reversed Na^+/Ca^{2+} exchanger with a residual slowly inactivating Ca^{2+} channel component (Blaustein and Oborn, 1975; Nachshen *et al.*, 1986). Removal of Na^+_o largely reduces "slow" influx (Turner and Goldin, 1985; Suszkiw *et al.*, 1986; Shafer and Atchison, 1989). The "fast" phase of influx is presumably mediated by voltage-dependent Ca^{2+} channels (Nachshen and Blaustein, 1980; Suszkiw and O'Leary,

1983). Uptake in this phase is independent of external Na^+ (Shafer and Atchison, 1989) and is associated with neurotransmitter release (Drapeau and Blaustein, 1983; Floor, 1983; Suszkiw and O'Leary, 1983; Nachshen, 1985; Leslie *et al.*, 1985).

Much like Ca^{2+} channels in other cell systems, voltage-dependent Ca^{2+} channels in synaptosomes undergo both voltage- and Ca^{2+}-dependent inactivation (Suszkiw and O'Leary, 1983; Suszkiw *et al.*, 1986; 1989) and are sensitive to block by inorganic, multivalent metal cations (Nachshen, 1984). The most potent blockers are lanthanum, cadmium and lead. The block is rapid in onset and is not attributed to changes in either the membrane or surface potential. Inhibition of Ca^{2+} flux by these cations is relieved by increasing $[Ca^{2+}]_o$ suggesting that the inhibitory ions compete with Ca^{2+} and directly block passage of Ca^{2+} ions through the Ca^{2+} channel (Nachshen, 1984). The antibiotics neomycin and polymixin B block synaptosomal Ca^{2+} channels in a manner that is reversed by increasing $[Ca^{2+}]_o$ also (Atchison *et al.*, 1988).

In addition to inorganic metals, the organic metals methylmercury (Atchison *et al.*, 1986; Shafer and Atchison, 1989; Hewett and Atchison, 1992) and ethylmercury (Hewett and Atchison, 1992) block $^{45}Ca^{2+}$ influx into synaptosomes in a manner that is irreversible by increasing $[Ca^{2+}]_o$ (Atchison *et al.*, 1986; Hewett and Atchison, 1992). It appears that both charge and lipophilicity are important in determining the blocking characteristics of mercurials, as only positively charged mercurials blocked synaptosomal Ca^{2+} channels and only organic mercurials do

so in a voltage-dependent manner (Hewett and Atchison, 1992).

Finally, the organic Ca^{2+} channel blocker, verapamil also decreases $^{45}Ca^{2+}$ influx into synaptosomes (Nachshen and Blaustein, 1979; Åkerman and Nicholls, 1981; Norris *et al.*, 1983; Nachshen, 1985; Carvalho *et al.*, 1986). Single-channel recordings of synaptosomal Ca^{2+} channels in lipid bilayers confirm that lanthanum, cadmium and verapamil are potent blockers of synaptosomal Ca^{2+} channels (Nelson, 1985).

***i.* Ca^{2+} channel subtypes**

Synaptosomes are likely to contain more than one subtype of Ca^{2+} channel but the types present have yet to be identified clearly. Block of Ca^{2+} channels by verapamil suggests that L-type channels may be involved in Ca^{2+} influx. This is a subject of much controversy as DHPs, also L-type antagonists, do not have consistent effects on $^{45}Ca^{2+}$ influx into synaptosomal preparations. DHP antagonists bind to synaptosomal preparations with high affinity (Boles *et al.*, 1984; Greenberg *et al.*, 1984; Turner and Goldin, 1985; Wei and Chang, 1985; Carvalho *et al.*, 1986; Suszkiw *et al.*, 1986; Dunn, 1988). However, a number of investigators have found evidence for both DHP-sensitive and -insensitive pathways mediating synaptosomal Ca^{2+} influx and neurosecretion.

Middlemiss and Spedding (1985) reported that submicromolar concentrations of the DHP agonist, Bay K 8644, could augment K^+-evoked neurotransmitter release from cortical brain slices. The augmented release was blocked by the DHP antagonist, nifedipine (Middlemiss and Spedding, 1985).

Woodward and Leslie (1986) and White and Bradford (1986) independently obtained similar results in synaptosomes (Woodward and Leslie, 1986; White and Bradford, 1986; Woodward *et al.*, 1988*a*). From the above studies, it appears that L-type Ca^{2+} channels in the CNS may modulate neurotransmitter release but do not contribute under normal circumstances. As you may recall, this was the conclusion drawn from studies using rat sympathetic neurons as well as rat NMJs (Perney *et al.*, 1986; Atchison, 1989).

In contrast, various investigators report direct block of synaptosomal K^+-stimulated Ca^{2+} influx and neurotransmitter release by nifedipine, nimodipine and nitrendipine (Turner and Goldin, 1985; White and Bradford, 1986; Dunn, 1988). Still, others report no effect of DHPs on K^+-stimulated Ca^{2+} uptake into synaptosomes (Nachshen and Blaustein, 1979; Daniell *et al.*, 1983; Wei and Chang, 1985; Suszkiw *et al.*, 1986; Reynolds *et al.*, 1986; Vickroy *et al.*, 1992). Thus, the relationship of DHP receptors to synaptosomal Ca^{2+} channel function is still uncertain.

Synaptosomes also contain specific high-affinity receptors for the N-type channel antagonist, CgTx. (Abe *et al.*, 1986; Marqueze *et al.*, 1988; Wagner *et al.*, 1988; Scheer, 1990). Polymixin B and several aminogycoside antibiotics potently inhibit binding of radiolabeled CgTx to guinea-pig cerebral cortex membranes (Knaus *et al.*, 1987) and to rat forebrain synaptosomes (Wagner *et al.*, 1988). Rapid $^{45}Ca^{2+}$ flux measurements into rat brain synaptosomes demonstrates only partial antagonism ($\approx$ 20-40%) of voltage-dependent Ca^{2+} channels by CgTx,

however (Reynolds *et al.*, 1986; Suszkiw *et al.*, 1987; Woodward *et al.*, 1988*b*; Suszkiw *et al.*, 1989). Thus, N-type channels may mediate only a proportion of Ca^{2+} uptake and neurotransmitter release from synaptosomes.

Because depolarization-induced Ca^{2+} entry into rat brain synaptosomes is largely resistant to both CgTx and DHPs, it appears that synaptosomes contain other high-voltage-activated channels which may be neither N- nor L-type (Lundy *et al.*,1992). In addition, Suszkiw and coworkers (1989) demonstrated that it was not possible to clearly classify the Ca^{2+} channels into the T-, L- or N-type based on their activation and inactivation kinetics (Suszkiw *et al.*, 1989). In some respects, Ca^{2+} influx into synaptosomes has characteristics similar to currents mediated by P-type channels (Leonard *et al.*, 1987; Llinas *et al.*, 1989; Suszkiw *et al.*, 1989). Likewise, the P-type Ca^{2+} channel blockers, ω-agatoxin IVA and FTX, are potent blockers of K^+-induced $^{45}Ca^{2+}$ uptake into rat brain synaptosomes (Mintz *et al.*, 1992; Uchitel *et al.*, 1992). These findings suggest that P-type Ca^{2+} channels may be involved in transmitter release from mammalian synaptosomes, but this requires verification.

C. Overall Aim

It is reasonably clear that LEMS is due to autoimmune attack directed against the nerve terminal in affected patients. However the exact identity of the target remains speculative. In large part this is due to the inaccesibility of the putative target site, the voltage-dependent Ca^{2+} channel in the presynaptic motor

nerve terminal. *The overall objective of this dissertation was to test the hypothesis that a circulating autoantibody in the plasma of patients with LEMS interferes with nerve terminal Ca^{2+} channels associated with neurotranmitter release.* To test this, the model system of isolated nerve terminals from mammalian CNS was used. Several lines of pharmacological evidence suggest close similarity between Ca^{2+} channels in synaptosomes and those at motor nerve terminals. CgTx and DHPs are unable to block release of ACh from murine motor nerve terminals (Anderson and Harvey, 1987; Atchison, 1989; Protti, 1991). Rapid $^{45}Ca^{2+}$ flux measurements in rat brain synaptosomes have shown that the voltage-dependent Ca^{2+} channels that mediate transmitter release are, in most but not all cases, DHP insensitive (Nachshen and Blaustein, 1979; Turner and Goldin, 1985; Suszkiw *et al.*, 1986; Woodward and Leslie, 1986) and at best are only partially antagonized by CgTx (Reynolds, 1986; Suszkiw *et al.*, 1989) as well. However, mammalian neuromuscular transmission and voltage-dependent Ca^{2+} channels in synaptosomes are effectively blocked by FTX (Uchitel *et al.*, 1992). FTX blocked presynaptic Ca^{2+} currents, nerve-evoked transmitter release and muscle contraction from a murine nerve-muscle preparation as well as K^+-stimulated $^{45}Ca^{2+}$ influx into rat brain synaptosomes (Uchitel, 1992). Thus, synaptosomes may be an useful model system in which to study the potential interaction of the autoantibody in LEMS with Ca^{2+} channels involved in neurotransmitter release.

1. Specific Aim 1

Incubation of rat cortical synaptosomes with serum or high molecular weight serum fractions from patients with LEMS decreased the amount of [^{3}H]-ACh released during depolarization (Meyer *et al.*, 1986). This study was important in that it indicated that acute exposure of synaptosomes to the serum or serum fractions from LEMS patients could elicit responses qualitatively similar to those obtained in chronic passive experiments in mice. It did not measure Ca^{2+} channel activity, however. Therefore, studies were performed to determine whether acute application of plasma and serum from patients with LEMS could reduce depolarization-dependent uptake of Ca^{2+} into rat forebrain synaptosomes. The results from these studies are presented in Chapter Three.

2. Specific Aim 2

Chronic injection of mice with plasma, crude Igs or purified IgG from patients with LEMS was effective in transferring both the electrophysiological and morphological characteristics of the disease (Denys *et al.*, 1972; Lang *et al.*, 1981; Newsom-Davis *et al.*, 1982; Fukunaga *et al.*, 1983; Kim, 1985; 1986; Lennon and Lambert, 1988; Nagel *et al.*, 1988). This led to the conclusion that the abnormality in LEMS was due to the production of IgG autoantibodies. The objective of the studies in Chapter Four was to test whether acute application of IgG isolated from a patient with LEMS could disrupt synaptosomal Ca^{2+} channels, and if so, whether other ion channels in nerve terminals were similarly affected. In addition, studies

were performed to determine if non-specific membrane damage contributed to the pathogenesis of LEMS.

3. Specific Aim 3

Activation of the serum component complement plays a central role in mediating postsynaptic membrane damage in animal models of the autoimmune neuromuscular disorder MG, as well as in the human disease state (Engel *et al.*, 1977; Sahashi *et al.*, 1978). Attempts to clarify the participation of complement in the pathogenesis of LEMS have so far yielded negative results (Prior *et al.*, 1985; Lennon and Lambert, 1988). As it is possible that complement activation may be important in LEMS, studies were undertaken to assess the role of serum in the pathogenesis of Ca^{2+} channel dysfunction induced by LEMS IgG in isolated nerve terminals from mammalian CNS. The results from these studies are presented in Chapter Five.

4. Specific Aim 4

LEMS immunoglobulin (Ig) appears to interact with voltage-dependent Ca^{2+} channels in a number of model cell systems (Roberts *et al.*, 1985; Login *et al.*, 1987; Peers *et al.*, 1987; De Azipurua *et al.*, 1988*a*, 1988*b*; Kim and Neher, 1988; Lang *et al.*, 1989*a*). Although, these studies have proven useful in the characterization of the subtype of Ca^{2+} channel affected by the LEMS autoantibody in the cell soma, the exact identity of the subtype(s) of Ca^{2+} channel affected in

nerve terminals remains unknown. Therefore, attempts to characterize pharmacologically the subtype of Ca^{2+} channel in synaptosomes that is affected by the LEMS autoantibody are presented in Chapter Six.

CHAPTER THREE

EFFECT OF SERUM AND PLASMA FROM PATIENTS WITH LAMBERT-EATON MYASTHENIC SYNDROME ON DEPOLARIZATION-INDUCED UPTAKE OF $^{45}Ca^{2+}$ INTO RAT CORTICAL SYNAPTOSOMES

A. *Summary*

The reduction in nerve-evoked release of transmitter at the neuromuscular junction of patients with LEMS is thought to be caused by a circulating autoantibody to calcium channels of presynaptic motor nerve terminals. Studies were undertaken to determine whether acute application of plasma and serum from patients with LEMS or SCC would reduce depolarization-dependent uptake of $^{45}Ca^{2+}$ into isolated nerve terminals of the CNS. Net potassium-stimulated influx was reduced by sera and plasma from five patients with LEMS but not by sera from four patients with SCC. Lactate dehydrogenase (LDH) release from synaptosomes incubated with plasma or serum from patients with LEMS was not increased over control indicating the reduction in $^{45}Ca^{2+}$ uptake was not a result of disruption of synaptosomal membrane integrity. These results are significant because: (1) they demonstrate that acute exposure to a circulating factor in sera/plasma from a patient with LEMS is sufficient to inhibit Ca^{2+} channel activity in isolated nerve terminals, as opposed to chronic regimens used in other models for the disease; (2) they indicate that the existence of SCC alone in some patients is insufficient to trigger a LEMS-like autoimmune response; (3) they suggest that Ca^{2+} channels of nerve terminals secreting different transmitters may share common epitopes recognized by the LEMS autoantibody; and (4) they suggest that synaptosomes will be useful in determining the neurochemical site and specificity of the LEMS autoantibody.

B. *Introduction*

LEMS is a presynaptic disorder of neuromuscular transmission frequently associated with SCC (Eaton and Lambert, 1957; Wise and MacDermot, 1962; Elmqvist and Lambert, 1968). The disease is characterized by a reduction in Ca^{2+}-dependent quantal release of ACh in response to nerve stimuli (Lambert and Elmqvist, 1971; Cull-Candy *et al.*, 1980). There is no evidence of a defect in ACh synthesis nor of decreased sensitivity of the postsynaptic membrane to ACh (Lambert and Elmqvist, 1971; Lindstrom and Lambert, 1978; Cull-Candy *et al.*, 1980). Muscle fiber excitability and nerve conduction appear normal (Pascuzzi and Kim, 1978). Thus, LEMS is presumed to result from defective transmitter release.

Evidence for an autoimmune origin of LEMS exists (Lang *et al.*, 1981; Lennon *et al.*, 1982; Newsom-Davis and Murray, 1984). The ability to transfer the human neuromuscular defect to mice by injections of either plasma or immunoglobulin derived from LEMS patients (Denys *et al.*, 1982; Newsom-Davis *et al.*, 1982; Lang *et al.*, 1983a; Lang *et al.*, 1984; Kim, 1985; Kim, 1986; Lambert and Lennon, 1988) supports the hypothesis that circulating autoantibodies to some presynaptic component are present in patient serum and mediate the pathogenesis of this disease. Evidence suggests that these autoantibodies interact with voltage-dependent Ca^{2+} channels since they have the ability to alter Ca^{2+} flux in several cell lines of diverse origin (Roberts *et al.*, 1985; Login *et al.*,1987; Peers *et al.*, 1987; Kim and Neher, 1988; De Azipurua *et al.*, 1988). These studies have proven useful in the characterization of the LEMS autoantibody's antigenic target in the cell

soma. However, studies which specifically examine the ability of the LEMS autoantibody to interact with Ca^{2+} channels in nerve terminals are lacking. This is important as it is possible that the type of Ca^{2+} channel in the terminal may differ from that in the cell body (Suszkiw *et al.*, 1989; Charlton and Augustine, 1990; Uchitel *et al.*, 1992).

In the present investigation, we sought to determine whether acute application of plasma and serum from LEMS patients could disrupt Ca^{2+} channel activity in presynaptic nerve terminals from the CNS. Synaptosomes maintain the morphological and functional properties of intact nerve terminals and several studies suggest that Ca^{2+} channels in the CNS resemble those in the periphery (Anderson and Harvey, 1987; Nachshen and Blaustein, 1979; Reynolds *et al.*, 1986; Suszkiw *et al.*, 1986;1989; Atchison, 1989; Protti *et al.*, 1991; Uchitel *et al.*, 1992). LEMS patients do not, however, normally exhibit CNS symptomology because IgG is a high molecular weight protein ($\approx$150 kDa) that does not readily pass the blood brain barrier. If the autoantibody in the sera of patients with LEMS could gain access to the calcium channels of the CNS by incubation *in vitro*, it might be possible to induce a passive transfer of the nerve terminal dysfunction to central neurons.

C. *Materials and Methods*

1. Animals

Male Sprague-Dawley rats (Harlan; 175-200g) were maintained on a 12 hr light-dark cycle in a temperature- and humidity-controlled room for at least 3 days

prior to use. Standard laboratory rodent chow and water were provided *ad libitum*.

2. Serum/Plasma Samples

Sera and plasma samples from patients diagnosed as having LEMS were kindly provided by the following persons: Andrew Massey, M.D., Dept of Neurology, University of Kentucky Medical Center, Lexington,KY; Mark Glasberg, M.D., Henry Ford Hospital, Dept of Neurology, Detroit, MI; David Johnson, M.D., Vanderbilt University, Nashville, TN; Donald Sanders, M.D., Division of Neurology, Duke University Medical Center, Durham, NC; Daniel Watson, M.D., Dept of Neurology, Johns Hopkins School of Med., Baltimore, M.D.; Eva Feldman, M.D., Dept. of Neurology, University of Michigan, Ann Arbor, MI and Daniel Drachman M.D., Dept of Neurology, John Hopkins School of Med., Baltimore, M.D.

Plasma and serum samples from patients with SCC were provided by David Johnson, M.D., Vanderbilt University, Nashville, TN and purchased from the Cooperative Tissue Network, Columbus, OH. Control plasma from patients with no history of neurological or immunological disease was purchased from the American Red Cross, Lansing MI.

3. Preparation of Synaptosomes

Synaptosomes were prepared from whole brain (minus the brainstem and cerebellum) of male Spraque-Dawley rats according to a modified method of Gray and Whittaker (1962). Forebrains were homogenized in 10 ml of 0.32 M unbuffered sucrose and centrifuged at 1000xg, 4°C, for 10 min. The supernatant was

decanted and the pellet discarded. The supernatant was next centrifuged at 10,000xg for 20 min and the resulting pellet resuspended in fresh 0.32 M unbuffered sucrose. The synaptosomal fraction was further purified by layering onto a sucrose density gradient consisting of 0.8 and 1.2 M unbuffered sucrose, and centrifuged at 69,000xg for 2 hr. The synaptosome-enriched fraction was subsequently removed from the interface between the 0.8 and the 1.2 M layers of sucrose, and diluted 1:1 (v/v) with a physiological saline buffer. The synaptosomal suspension was then washed free of sucrose by centrifugation at 10,000xg for 10 min and resuspended in the physiological saline to an approximate concentration of 15-25mg protein/ml as determined by the method of Lowry *et al.*, (1951).

Following isolation, equal aliquots of the synaptosomal suspension were incubated at 37°C for one hr, while shaking, in a pure O_2 atmosphere with varying concentrations of either plasma or sera (expressed as " % of total incubation volume") from patients with no known neurological or immunological illness (control), SCC in which no neurological involvement was observed, or LEMS. The proper volume of physiologic saline buffer was added to maintain a constant reaction volume.

4. Solutions

The solutions used in the $^{45}Ca^{2+}$ uptake experiments contained the following: *physiological saline buffer* (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; *d*-glucose, 10; and HEPES, 10; *depolarizing K^+ solutions* (mM): NaCl, 72.5; KCl, 77.5; $MgCl_2$, 1; $CaCl_2$, 0.04; *d*-glucose, 10; and HEPES, 10; and *quench solution* (mM): KCl, 5;

$MgCl_2$, 2; EGTA, 1; *d*-glucose, 10; HEPES, 10; and N-methyl-*d*-glucamine, 145. *Non-depolarizing K^+ solutions* were identical to depolarizing K^+ solutions except that $[K^+]_e$ was 5 mM, and 72.5 mM choline chloride was added to maintain iso-osmolarity.

The LDH reaction mixture contained (mM): K_2HPO_4, 41.2; KH_2PO_4, 6.8; Na^+ pyruvate, 0.6; NADH, 0.2 ; and $NaHCO_3$, 1.1.

5. Measurement of $^{45}Ca^{2+}$ Uptake

Uptake of $^{45}Ca^{2+}$ was measured as described previously (Atchison *et al.*, 1986). Fifty μl of the synaptosomal suspension was added to 50 μl of either depolarizing or non-depolarizing K^+-buffer containing 1 μCi of $^{45}Ca^{2+}$ (New England Nuclear, Boston, MA) and uptake was measured for 10 sec. The uptake of $^{45}Ca^{2+}$ associated with transmitter release occurs within the first 2 sec and accounts for 80% of the uptake measured over a 10 sec period (Nachshen and Blaustein, 1980; Drapeau and Blaustein, 1983). Therefore, these data accurately reflect the uptake of $^{45}Ca^{2+}$ associated with voltage-dependent Ca^{2+} channels in the synaptosomes as reported previously (Nachshen and Blaustein, 1980; Drapeau and Blaustein, 1983; Suszkiw *et al.*, 1986; Suszkiw *et al.*, 1989; Shafer and Atchison, 1989).

The reaction was stopped by the addition of 2 ml of cold quenching solution followed by suction-filtration of the sample through 0.45 μM HVLP Millipore filters (Millipore, Bedford, MA). Radioactivity remaining on the filters in low $[K^+]_e$ solution was subtracted from that obtained in high $[K^+]_e$ solutions as depolarization-

independent background. Experiments were replicated five to seven times. Values obtained for any particular experiment were the average of three replicates. Readings of dpm were corrected back to the original volume of synaptosomes filtered and expressed as nmole $^{45}Ca^{2+}$ / μg protein (Lowry *et al.*, 1951).

6. Measurement of Lactate Dehydrogenase (LDH) Activity

LDH release was quantified by the method of Bergmeyer and Bernt (1974). Synaptosomes were incubated with varying concentrations of control and LEMS IgG as described above. Immediately after the incubation period, synaptosomes were pelleted by centrifugation. The supernatant was decanted into a separate test tube. A 30 μl aliquot of supernatant was added to cuvettes containing 3 ml of the reaction mixture. LDH activity was quantified by the rate of oxidation of nicotinamide adenine dinucleotide (NADH), which was followed spectrophotometrically at 340 nM for 3 min. Activity was expressed as the percentage of LDH released into the supernatant. Total LDH activity (100 %) was determined by lysing an equal volume of synaptosomes in 0.1 % (v/v) Triton-X-100 followed by sonication.

7. Statisical Analysis

Data were analyzed via a randomized complete block analysis of variance followed by Dunnett's *t*-test or by Student's *t*-test for paired samples. Statistical significance was assessed at $p < 0.05$.

D. *Results*

1. Uptake of $^{45}Ca^{2+}$

Many LEMS patients, including patients used in this study, have SCC. Uptake of $^{45}Ca^{2+}$ was determined following incubation of synaptosomes with SCC serum. Serum from four patients with SCC in which no neurological involvement was observed produced no substantial reduction in depolarization-dependent uptake of $^{45}Ca^{2+}$ into rat forebrain synaptosomes (Fig. 3.1).

The effect of both control and LEMS patient sera on $^{45}Ca^{2+}$ uptake into synaptosomes during 10 sec K^+-stimulation is depicted in Figure 3.2. Concentrations of 6 and 12% (v/v) control sera and plasma, had little effect on depolarization-induced uptake of $^{45}Ca^{2+}$ into synaptosomes when compared to uptake into synaptosomes with no added sera/plasma. However, incubation with 24% (v/v) control plasma/sera significantly reduced uptake compared to incubations with no sera/plasma (Fig. 3.2).

Conversely, incubation of synaptosomes with plasma or sera from five LEMS patients at concentrations of 6 and 12% of the total incubation volume resulted in significant reductions in net K^+-stimulated influx (Fig 3.2). Inhibition of $^{45}Ca^{2+}$ uptake ranged from 0 to 20% (10.6 ± 4.2%) and 14 to 30% (20.4 ± 2.6%) at concentrations of 6 and 12%, (v/v) respectively. At a concentration of 24% (v/v), LEMS sera/plasma reduced influx by 0 to 61.8% (22.4 ± 10.8%), but this was not significantly less than its collective paired control. Incubation of synaptosomes with serum from a sixth patient with LEMS reduced uptake of $^{45}Ca^{2+}$ by 20.2 ± 18.1%,

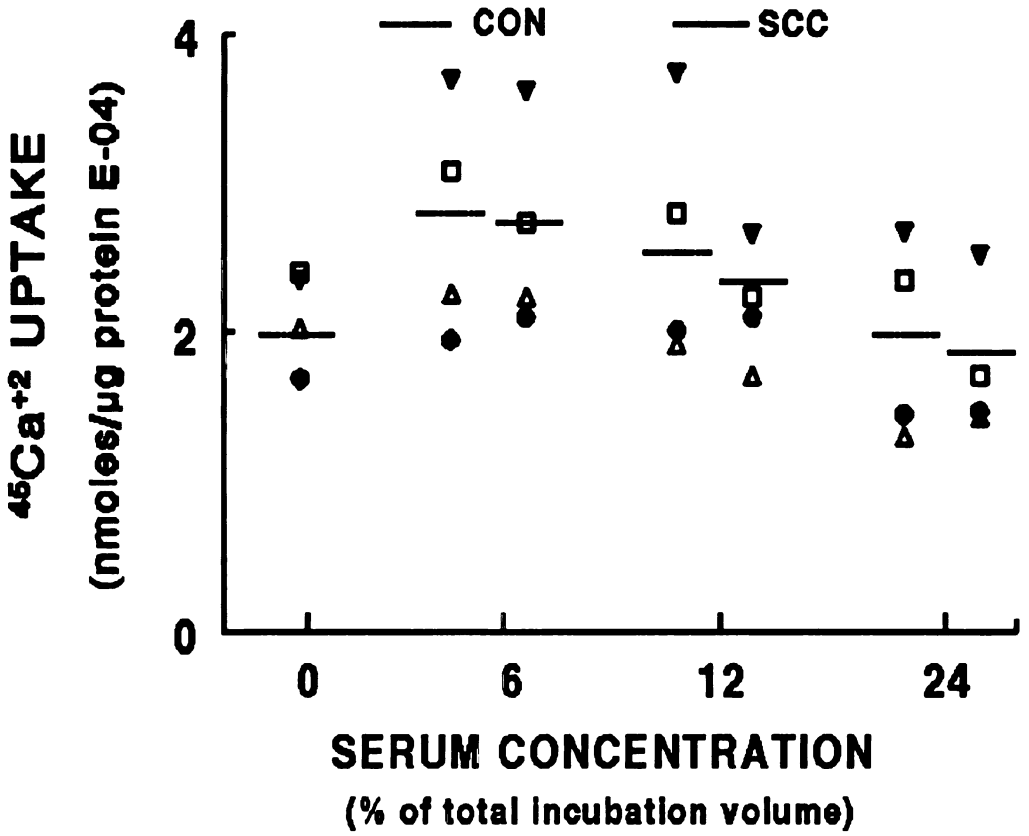


Figure 3.1. Effect of sera from 4 patients with SCC and no evidence of neurological involvement on $^{45}Ca^{2+}$ uptake into rat forebrain synaptosomes during 10 sec of depolarization with 42.5 mM KCl. Prior to uptake, synaptosomes were incubated for 1 hr with SCC or control serum. Uptake in 5 mM KCl was subtracted from that in 42.5 mM KCl as depolarization-independent background. Each symbol represents the mean of at least 3 experiments per patient. Paired experiments are denoted by identical symbols in the control and SCC group. The horizontal bar represents the mean of all 4 patients tested.

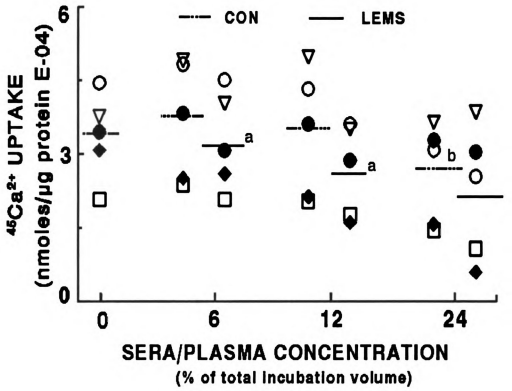


Figure 3.2. Effect of plasma and sera from 5 patients with LEMS on uptake of $^{45}Ca^{2+}$ into rat forebrain synaptosomes during a 10 sec depolarization. Synaptosomes were incubated for 1 hr with either LEMS serum or plasma or control serum or plasma, respectively. Uptake was measured as described in Figure 1.1. Each symbol represents the mean of at least three experiments per patient. Paired experiments are denoted by identical symbols in both the control and LEMS-treated group. The horizontal bar denotes the mean of all 5 patients tested. [a] Indicates values which are significantly less than its corresponding paired control value ($p < 0.05$). [b] Indicates a value that is significantly less than uptake into synaptosomes that received no serum or plasma ($p < 0.05$).

52.2 ± 18.4% and 43.8 ± 9.4% at 10, 30 and 50% of the total incubation volume, respectively (Fig 3.3).

2. Synaptosomal Membrane Integrity

To rule out the possibility that the effect of LEMS sera/plasma was a result of disruption of the synaptosomal membrane integrity, synaptosomal viability was assessed by measuring lactate dehydrogenase (LDH) released into the extrasynaptosomal fluid following incubation with plasma and sera. Neither incubation with LEMS serum nor LEMS plasma increased LDH release compared to incubation with control serum or plasma (Figs. 3.4, 3.5).

E. *Discussion*

The aim of the present study was to test whether acute application of plasma and serum from LEMS patients could disrupt synaptosomal Ca^{2+} channel activity. Our results indicate that serum and plasma from patients with LEMS act on presynaptic nerve terminal Ca^{2+} channels in the CNS to reduce depolarization-induced uptake of $^{45}Ca^{2+}$. These deficits are not due to a decrease in synaptosomal viability as LDH release was not increased over that released by control sera/plasma. These studies, to my knowledge, are the first to examine specifically the ability of a circulating humoral factor in serum from patients with SCC and LEMS to interact with nerve terminal Ca^{2+} channels. This is important as the putative site of action for the LEMS autoantibody is believed to be Ca^{2+} channels on motor nerve terminals.

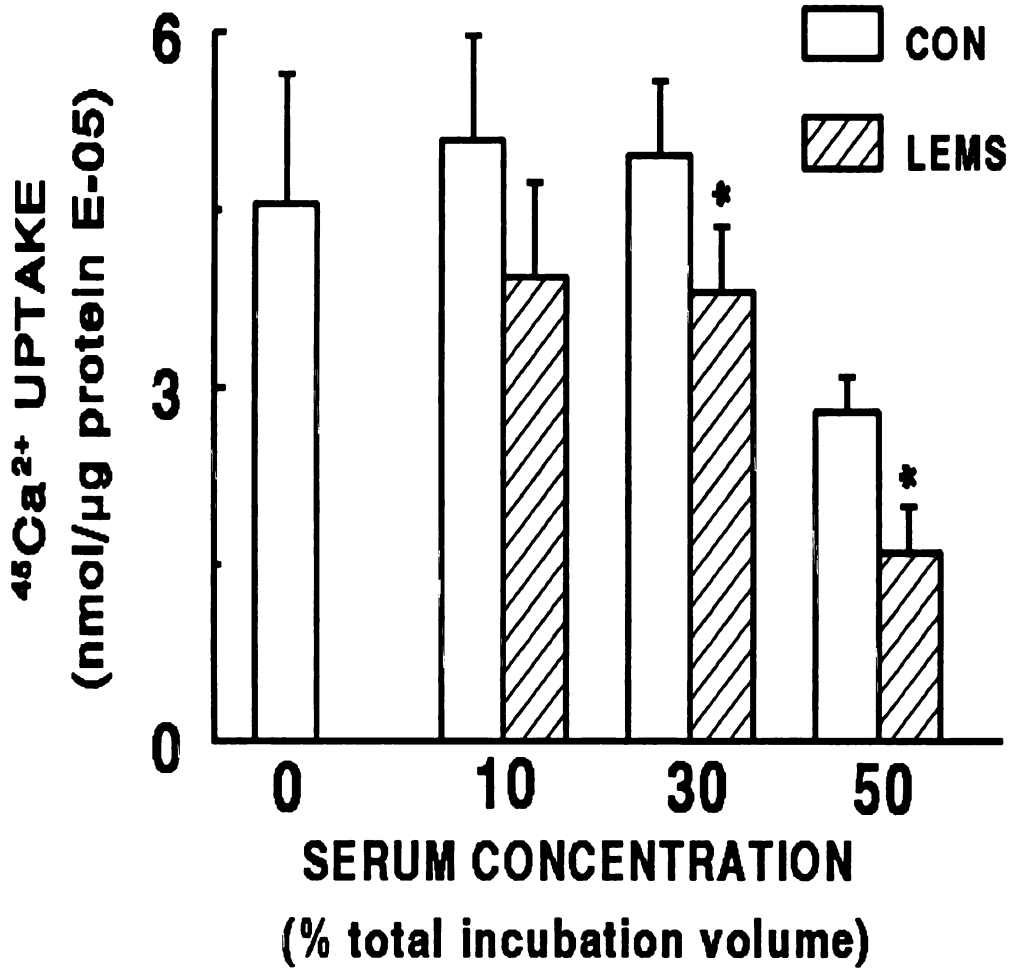


Figure 3.3. Effect of serum from one patient with LEMS on $^{45}Ca^{2+}$ uptake into synaptosomes. Synaptosomes were incubated with serum for 1 hr and uptake measured as described in Fig. 1.1. The asterisk ,*, indicates uptake that is significantly smaller than its respective control ($p < 0.05$).

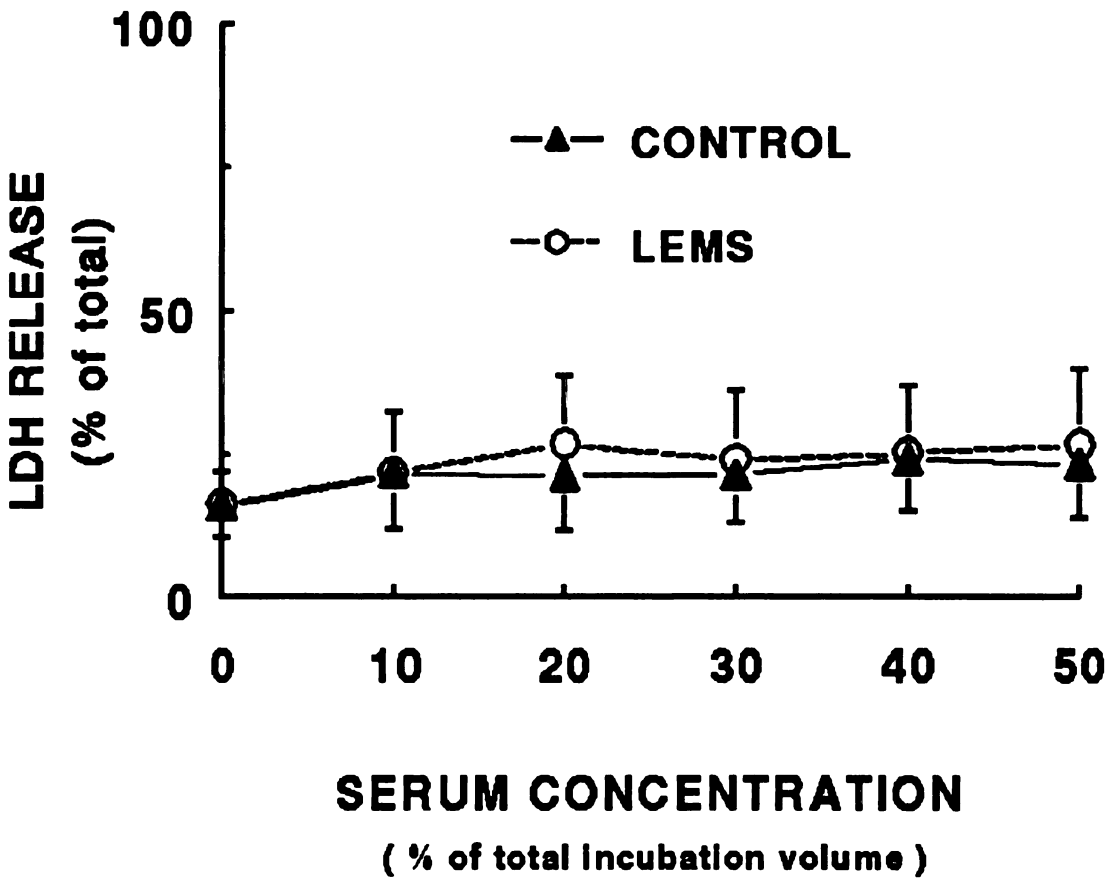


Figure 3.4. Effect of LEMS sera on synaptosomal viability. LDH activity was measured from the supernatant of synaptosomes treated for 1 hr with either LEMS or control sera. LDH activity was quantified by the rate of oxidation of NADH at 340 nm. The quantity of enzyme is expressed as the percent release of the total enzyme content of the synaptosomes, which was determined by lysis of an equal volume of synaptosomes with 0.1% (v/v) Triton-X-100 followed by sonication. Values are the mean ± SEM of 3 experiments.

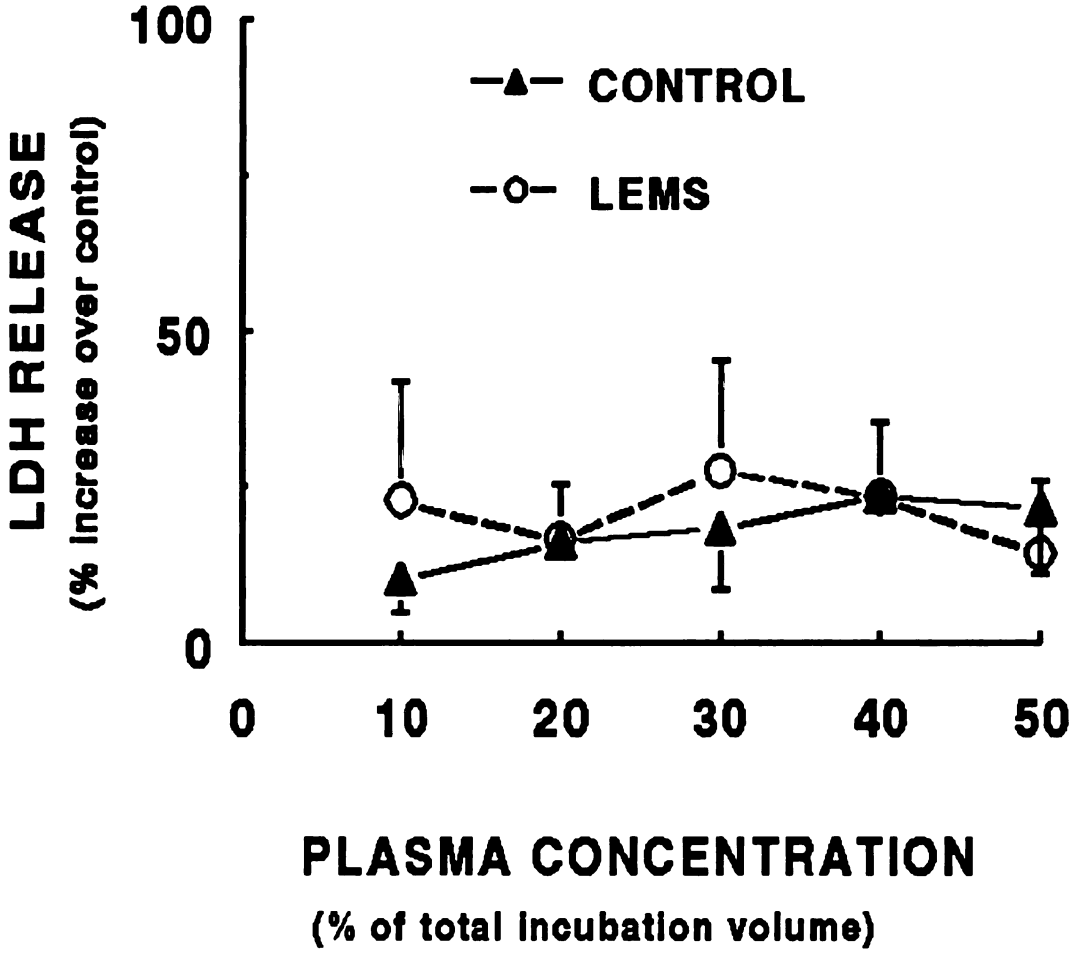


Figure 3.5. Effect of LEMS plasma on synaptosomal viability. LDH activity was measured from the supernatant of synaptosomes treated for 1 hr with either LEMS plasma, control plasma or physiological saline. LDH activity was quantified by the rate of oxidation of NADH at 340 nm. The quantity of LDH is expressed as the percent over release in physiological saline only. All values are the mean ± SEM of 5 experiments.

Serum from SCC patients lacking neurological symptoms did not reduce depolarization-dependent uptake of $^{45}Ca^{2+}$ into synaptosomes. This study controlled for possible effects associated with neoplastic disease, since approximately 60% of patients with LEMS also have SCC of the lung (Eaton and Lambert, 1957; Wise and MacDermot, 1962; Elmqvist and Lambert, 1968). Oat cells (small cells) possess several neuronal properties and are possibly of neural origin (Gadzar *et al.*, 1981; Moody *et al.*, 1981; Tapia *et al.*, 1981). They are electrically excitable, and action potentials in oat cells may be calcium channel-mediated as they are inhibited by Co^{2+} and Mn^{2+}, known calcium channel blockers (McCann *et al.*,1981; Roberts *et al.*, 1985). It has been suggested that LEMS may arise as an immune response to SCC and that cholinergic neurons may have similar epitopes leading to cross-reactivity between tumor and neuron (Newsom-Davis, 1982; Lennon *et al.*, 1982; Vincent *et al.*, 1989). LEMS, however, also exists in the non-neoplastic form and autoantibodies antagonistic to SCC Ca^{2+} channel activity were found in patients with LEMS independent of cancer status (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988). Since sera from SCC patients failed to inhibit calcium flux at any concentration tested, this suggests that the existence of SCC neoplastic disease is insufficient to trigger an autoimmune response in all patients. These data also demonstrate that interpretation of data derived from LEMS patients with SCC would not be complicated by the presence of carcinoma.

Incubation of synaptosomes with control plasma/sera at a concentration of 24% (v/v) significantly reduced $^{45}Ca^{2+}$ uptake compared to uptake in

synaptosomes which were incubated without sera/plasma. This may be explained simply by increased serum binding of $^{45}Ca^{2+}$ thereby effectively decreasing the concentration of radioactivity available for uptake by synaptosomes. This needs to be tested further.

It is evident from the variability in the data that the circulating factor found in different LEMS patients sera/plasma is not equieffective in disrupting $^{45}Ca^{2+}$ influx into synaptosomes. This is most likely due to differences in antibody titer between patients. Nevertheless, a modest yet significant decrease in uptake mediated largely by voltage-dependent Ca^{2+} channels was observed in synaptosomes treated with 6 and 12% sera or plasma from five LEMS patients as compared to their paired controls. A striking decrease in synaptosomal $^{45}Ca^{2+}$ uptake was observed in one patient studied in isolation.

Neither incubation with LEMS plasma nor LEMS serum increased LDH release compared to incubation with control serum or plasma. Thus, it is unlikely that the decrease in calcium uptake is due to decreased synaptosomal viability, but rather suggests block or disruption of presynaptic voltage-dependent calcium channels in the nerve terminal.

It is well established that nerve-evoked quantal release of neurotransmitter is dependent upon the influx of extracellular Ca^{2+} through membrane ionic channels (Katz and Miledi, 1965; Katz and Miledi, 1967*a*; Dodge *et al.*, 1969). In the absence of Ca^{2+}, the nerve action potential invades the nerve terminal but fails to evoke transmitter release (Katz and Miledi, 1965; Dodge and Rahamimoff, 1967).

Transmission at many synapses has a steep dependence on extracellular Ca^{2+} concentration (Dodge and Rahamimoff, 1967; Katz and Miledi, 1970; Drapeau and Blaustein, 1983; Smith *et al.*, 1985; Zucker and Fogelson, 1986). These observations have led to the hypothesis that Ca^{2+} ions act in a cooperative, exponential manner at intracellular sites to trigger secretion of transmitter quanta. The power index appears to vary considerably at different synapses and it has been reported that transmitter release following a nerve impulse may increase with as much as the fourth or even higher power of $[Ca^{2+}]_e$ (Dodge and Rahamimoff, 1967; Katz and Miledi, 1970; Zucker and Fogelson, 1986). This power relationship may be of functional importance in controlling transmitter release. Changes in the magnitude of presynaptic Ca^{2+} entry could have an amplifying effect on the amount of neurotransmitter secreted (Smith *et al.*, 1985). Thus, if transmitter secretion at the neuromuscular junction and/or in synaptosomes follows this power relationship then modest changes in Ca^{2+} influx, such as those in this study, could conceivably result in large changes in neurotransmitter secretion.

Rat cortical synaptosomes are neurochemically heterogeneous with only 10% percent of the suspension thought to be cholinergic (Gray and Whittaker, 1962). Incubation with LEMS sera/plasma resulted in decreases of $^{45}Ca^{2+}$ uptake which ranged from 0 to 60%. These decreases suggest that the autoantibody must interact with nerve terminal Ca^{2+} channels which secrete different neurotransmitters. These data also provide further evidence for the similarity between somatic motor nerve terminal and central nerve terminal Ca^{2+} channels.

Reduction in 10 sec depolarization-dependent $^{45}Ca^{2+}$ uptake into synaptosomes was evident following 1 hr of incubation of plasma/sera. Thus, acute exposure to LEMS autoantibody was sufficient to induce a change in activity associated with Ca^{2+} channels. This is in contrast to other models of the disease which have utilized chronic exposure. Using this acute, *in vitro* system may allow for more efficient use of the isolated immunoglobulin fraction from patients sera, in subsequent studies. This is desirable as patient sera is often difficult to acquire as the incidence of the disorder is low.

In conclusion, incubation of rat cortical synaptosomes with sera/plasma from patients with LEMS but not SCC reduced depolarization-dependent uptake of $^{45}Ca^{2+}$ This was not due to decreased synaptosomal viability. The results from this study are significant as they demonstrate that acute rather than chronic exposure to LEMS sera/plasma is sufficient to inhibit Ca^{2+} channel activity in isolated nerve terminals; that data obtained from patients with LEMS with and without SCC can be analyzed identically; and that Ca^{2+} channels of nerve terminals secreting different transmitters may share common epitopes recognized by the LEMS autoantibody. Thus, the synaptosomal preparation may a useful model in which to study, in more detail, the potential interaction of the autoantibody in LEMS with calcium channels involved in transmitter release.

CHAPTER FOUR

SPECIFICITY OF LAMBERT-EATON MYASTHENIC SYNDROME IMMUOGLOBULIN FOR NERVE TERMINAL CALCIUM CHANNELS

A. *Summary*

Data from Chapter Three provide evidence which suggests that synaptosomal Ca^{2+} channel function is altered by incubation with serum/plasma from patients with LEMS. The goal of the present study was to examine the specificity of LEMS autoantibodies for nerve terminal Ca^{2+} channels compared to other voltage-sensitive ion channels in nerve terminals, and to determine if non-specific membrane damage contributed to the pathogenesis of LEMS. The ion channel specificity of LEMS autoantibody was assessed by comparing the ability of acute application of IgG isolated from the plasma of a patient with LEMS to reduce depolarization-dependent uptake of $^{45}Ca^{2+}$ and $^{22}Na^{+}$ into or efflux of $^{86}Rb^{+}$ from rat forebrain synaptosomes. The clinical diagnosis of LEMS was confirmed electrophysiologically by treatment of mice for 30 days with plasma (1.5 ml/day) taken from this patient. Characteristic reduction of quantal content elicited at 1 Hz and facilitation at 20 Hz was observed in mice treated with LEMS plasma compared to those treated with control plasma. One sec, K^{+}-stimulated $^{45}Ca^{2+}$ uptake was inhibited 36.5 ± 14.5% and 44.5 ± 9.8% by acute application of 2 and 4 mg/ml LEMS IgG, respectively; IgG from patients with SCC had no effect on $^{45}Ca^{2+}$ entry. The same concentrations of LEMS IgG affected neither voltage-dependent uptake of $^{22}Na^{+}$ into veratridine-depolarized synaptosomes nor $^{86}Rb^{+}$ efflux from K^{+}-depolarized synaptosomes. LDH release from synaptosomes incubated with varying concentrations LEMS IgG was not increased over control indicating the reduction in $^{45}Ca^{2+}$ uptake was not a result of disruption of synaptosomal

membrane integrity. Likewise, normal activation of Ca^{2+} channels was not prevented by IgG-induced alterations in synaptosomal membrane potentials as the fluorescence of a membrane potential sensitive carbocyanine dye (diS-C2(5)) was unaltered following acute application of 2 mg/ml LEMS or control IgG. These results indicate that immunoglobulins isolated from a patient with LEMS alter presynaptic nerve terminal Ca^{2+} channel activity. These deficits are not due to decreases in synaptosomal viability nor to IgG-induced alterations in the synaptosomal membrane potential but appear to be a direct block or modulation of voltage-dependent Ca^{2+} channels. The antibody from this patient appears to be specific for nerve terminal Ca^{2+} channels as it did not affect flow through voltage-dependent Na^{+} or K^{+} channels.

B. *Introduction*

The electrophysiological features of LEMS include reduced quantal content at low frequencies of nerve stimulation with marked facilitation at high frequencies of stimulation (Elmqvist and Lambert, 1968; Lambert and Elmqvist, 1971; Cull-Candy *et al.*, 1980). Morphologic data suggest that AZPs represent the target of the pathogenic autoantibodies in LEMS (Fukunaga *et al.*, 1982; 1983; Fukuoka *et al.*, 1987). Mice injected with either plasma or immunoglobulin derived from LEMS patients (Kim, 1985;1986; Lang *et al.*, 1983) reproduce both the electrophysiological and morphological features of LEMS.

It has been proposed that AZPs correspond to voltage-sensitive Ca^{2+}

channels of the presynaptic membrane (Pumplin *et al.*, 1981; Robitaille *et al.*, 1990; Tarelli *et al.*, 1991; Cohen *et al.*, 1991). Thus, the reduced transmitter release in LEMS could be related to a reduced entry of Ca^{2+} into the nerve terminal. In support of this contention, LEMS autoantibodies appear to interact with voltage-dependent Ca^{2+} channels in several cell lines of diverse origin (Roberts *et al.*, 1985; Login *et al.*, 1987; De Azipurua *et al.*, 1988*a*, 1988*b*; Kim and Neher, 1988; Lang *et al.*, 1989*a*; Peers et al., 1990). Although these studies have proven useful, several important deficiencies still remain. First, the identity of Ca^{2+} channels in motor nerve terminals remains unclear. Ca^{2+} channel subtypes in nerve terminals or populations of nerve terminals may differ from those in the cell body (Suszkiw *et al.*, 1986; Charlton and Augustine, 1990). Second, the function and number of ion channels could be affected by damage to the membrane or membrane depolarization. Third, no assessment has been made of the Ca^{2+} channel specificity of the LEMS autoantibody as opposed to other voltage-gated channels present in nerve terminal. An antibody directed at an epitope on voltage-dependent Ca^{2+} channels might also affect other voltage-dependent ion channels if the IgG recognizes portions of the channel proteins common to all ion channels.

Data from the previous chapter indicates that serum and plasma from patients with LEMS reduces depolarization-dependent uptake of $^{45}Ca^{2+}$ into synaptosomes (Hewett and Atchison, 1991). As an IgG autoantibody is presumed to mediate the pathogenesis of LEMS, the present study was designed to answer the following three questions: does IgG isolated from a patient with LEMS reduce

depolarization-dependent $^{45}Ca^{2+}$ into nerve terminal Ca^{2+} channels?; if so, does the IgG cause Ca^{2+} channel dysfunction secondary to nonspecific membrane damage or alterations of membrane potential?; and are other voltage-dependent ion channels in the terminal also affected?

C. *Materials and Methods*

1. Materials

Dimethyl sulfoxide (DMSO) was obtained from EM Science (Gibbstown, NJ). Tetraethylammonium chloride (TEA), tetrabutylammonium chloride (TBA), tetrodotoxin (TTX), bovine serum albumin (BSA) cyclophosphamide, oligomycin, nickel chloride ($NiCl_2$), ethyleneglycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO.) Sodium cyanide (NaCN) was obtained from Mallinckrodt Chemical Works (St. Louis, MO). Triton-X-100 was obtained from Research Products International Corp. (Elk Grove, IL). The radiolabels $^{45}Ca^{2+}$ (15-50 mCi/mg), $^{86}Rb^{+}$ (1-10 mCi/mg), and $^{22}Na^{+}$ (800-1000 mCi/mg) were purchased from New England Nuclear (Boston, MA). 3,3'-Diethyldicarbocyanine iodide [diS-C_2(5)] was obtained from Molecular Probes (Eugene, OR). Disease-free control human plasma was purchased from the American Red Cross (Lansing, MI). Control human serum was donated by a healthy volunteer. Plasma from patients with the clinical and electromyographic characteristics of LEMS was provided by Drs. Mark Glasberg, Eva Feldman, Andrew Massey, and James Albers. Serum from patients with SCC was provided by Dr. David Johnson.

2. Solutions

For electrophysiological measurements of neuromuscular function, solutions used for both bathing and recording contained (mM): NaCl, 137.5; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 2; *d*-glucose, 11; and HEPES, 14. The solutions used in the $^{45}Ca^{2+}$ uptake experiments were identical to those described in Chapter Three.

For experiments using $^{22}Na^+$, *incubation buffer* contained (mM): KCl, 5; $MgCl_2$, 1; choline chloride, 145; *d*-glucose, 10; HEPES, 10; veratridine (VER), 0.15; ouabain, 0.1; BSA, 1 mg/ml; ± TTX, 0.001. *Uptake solutions* were identical to the incubation buffer except that 2 mM NaCl and $^{22}Na^+$ (2 μCi/ml) were added and the ouabain concentration was increased to 1 mM. Cold *quench solution* was identical to that described in Chapter Three. Veratridine was used in lieu of K^+-depolarization because voltage-dependent Na^+ channels inactivate rapidly in response to normal depolarizing stimuli (Tamkun and Catterall, 1981).

The *wash buffer* used in the $^{86}Rb^+$ efflux experiments contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 2; RbCl, 0.1; *d*-glucose, 10; and HEPES, 10. *Nondepolarizing efflux buffer* was identical to the wash buffer. In the *depolarizing efflux buffer*, NaCl was replaced by KCl for a final $[K^+]_o$ of 150 mM. Cold *stop solution* contained (mM); TEA, 70; TBA, 10; $NiCl_2$, 10; RbCl, 5; $MgCl_2$, 1; *d*-glucose, 10; HEPES, 10; and N-methyl-*d*-glucamine, 50.

Stock solutions of diS-C_2(5) for fluorescence measurements were dissolved in DMSO. Working solution were made daily by dilution of the stock into physiological saline buffer (final concentration, 0.5 μM). The final concentration of DMSO

after addition of dye to synaptosomes was 0.05% (v/v).

3. Immunoglobulin Isolation

Control, SCC and LEMS sera and/or plasma were stored at -20°C until used. After thawing, fibrin was removed from the plasma samples by clotting which was facilitated by the addition of 1 M $CaCl_2$. Total immunoglobulin (Ig's) were isolated by serial precipitations of serum in 30% and 50% saturated ammonium sulfate at 4°C as described by Heide and Schwick (1978). Each precipitate was pelleted by centrifugation at 1000xg for 15 min and redissolved in 0.85% (w/v) saline. The final precipitate was redissolved to 1/2 the original volume and dialyzed at 4°C against saline using Spectrapor 2 dialysis tubing (15-25000 m.w. cutoff, Spectrum Medical Ind., Los Angelese, CA) until the sulfate ions were no longer detectable (≈ 72 hr) as discerned by the barium chloride test (Garvey *et al.*, 1977). Flocculent material formed during dialysis was removed by centrifugation at 1000xg for 15 min at 4°C. Ig's were quantitated by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). The IgG concentration was presumed to be 80% of the total protein (Kimbal, 1986).

The LEMS IgG used in the synaptosomal studies was isolated from a patient with SCC. We show here that this patients' plasma caused the characteristic reduction of evoked release of ACh at the neuromuscular junction (Patient 3; Fig. 4.2).

4. Measurement of Quantal Content (*m*)

Plasma was used from three patients with typical clinical and electromyographic features of LEMS. One LEMS patient (Patient 3) has SCC. Plasma fractions (1.5 ml) from these patients and from disease-free human controls were injected daily intraperitoneally into Swiss Webster female mice (15-20 g) (Kim, 1985). In order to induce immune tolerance to the foreign protein, animals were given cyclophosphamide, 300 mg/kg (i.p.), one day following the first plasma injection (Lang *et al.*, 1983). On day 30, the diaphragm with phrenic nerve intact was isolated and was perfused continuously with a low K^+ (2.5 mM) saline solution continually aerated with 100% O_2. Muscle fibers were transected approximately 4 mm on either side of the main intramuscular nerve branch to prevent contractions due to stimulation of the phrenic nerve (Barstad and Lilleheil, 1968; Glavinovic, 1979). Preparations were pinned out in a Sylgard-coated plexiglass chamber and perfused with low [K^+] saline. All experiments were performed at room temperature (≈25°C) and replicated in a minimum of four animals.

The setup for recording under current clamp is depicted schematically in Figure 4.1. Intracellular microelectrode recordings of synaptic potentials were made using conventional techniques and borosilicate glass microelectrodes (1.0 mm, WPI, New Haven, CT) having impedences of 15-30 meg Ω when filled with 3 M KCl. Prior to nerve stimulation, an average MEPP amplitude was obtained by recording 5 min of MEPPs on magnetic tape using an FM instrumentation tape recorder. EPPs were elicited by single shock rectangular voltage stimuli of 0.07 msec

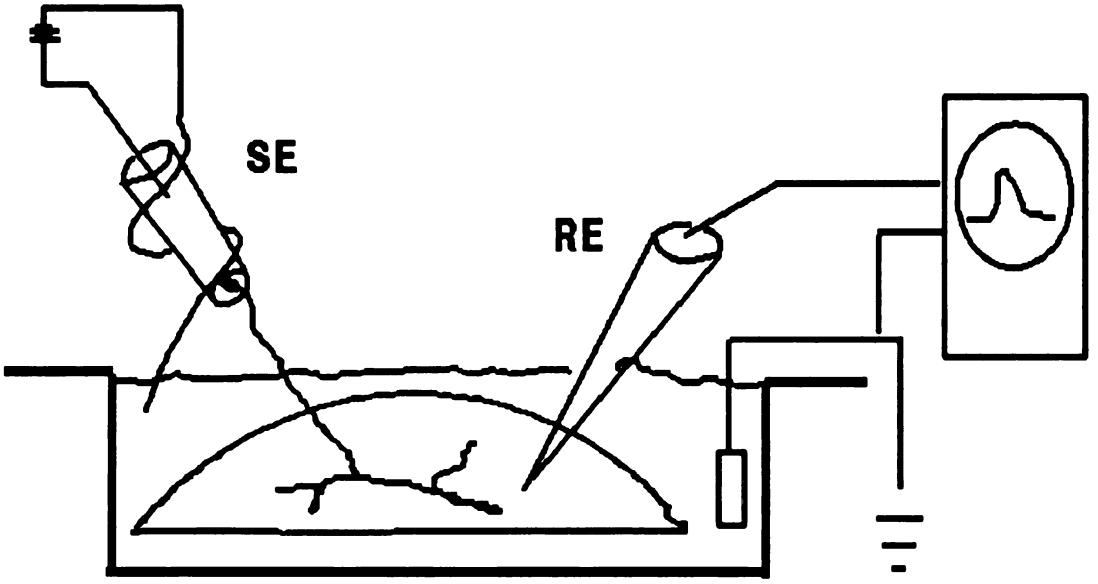


Figure 4.1. Setup for recording under current clamp. Hemidiaphragm preparations with phrenic nerve intact were removed from control- and LEMS-treated mice. Preparations were transected to prevent contraction following stimulation of the nerve. Muscles were pinned down in a Sylgard-coated plexiglas chamber and perfused with Low K^+ buffer. End-plate regions were impaled with a KCL-filled microelectrode and EPPs and MEPPs recorded.

Figure modified from that contained in the dissertation of John M. Spitsbergen

duration applied to the phrenic nerve at a frequency of 1, 20, or 40 Hz (200 stimuli) allowing 5 min of rest between pulse protocols for redistribution of Ca^{2+}. Quantal content was determined off-line from the amplitudes and variances of the EPPs (variance method) and by dividing the average amplitude of EPPs by the average MEPP amplitude (mean value method). EPP amplitudes were standardized to a membrane potential of -40 mV (Katz and Theseleff, 1967). Only those EPPs and MEPPs with rise time ≤ 1 msec were used for subsequent analysis.

5. Preparation of Synaptosomes

Synaptosomal preparation was identical to that previously described in Chapter Three. In all experiments except $^{86}Rb^{+}$ efflux, synaptosomes were incubated at 4°C under 100% O_2 for 60 min with 10% (v/v) control human serum and varying concentrations of IgG from patients with no known neurological or immunological illness (control), SCC or LEMS. The rationale for using control human serum will be discussed in Chapter Five. The proper volume of physiologic saline buffer was added to maintain a constant incubation volume. Prior to uptake measurements, synaptosomes were allowed to equilibrate for 30 min at 37°C.

6. Measurement of $^{45}Ca^{2+}$ Uptake

Uptake of $^{45}Ca^{2+}$ was measured as described in detail in Chapter Three. Readings of dpm were corrected back to to the original volume of synaptosomes filtered and expressed as nmole $^{45}Ca^{2+}$ /μg protein or as % of disease-free control.

7. Measurement of $^{22}Na^+$ Uptake

The time course of uptake of $^{22}Na^+$ into rat forebrain synaptosomes was measured by the addition of 50 μl of synaptosomes to 50 μl of incubation buffer containing 150 μM VER with or without TTX (1 μM) for varying periods of time. VER is used to activate synaptosomal Na^+ channels as these channels inactivate rapidly in response to K^+-depolarization (Kreuger and Blaustein, 1980; Tankum and Catterall, 1981). NaCl was replaced isotonically with choline chloride in all solutions. Ouabain was present during both the incubation and uptake periods to prevent $^{22}Na^+$ extrusion by blocking the Na^+/K^+ ATPase. Following this incubation, a 100 μl of uptake solution containing $^{22}Na^+$ (2 μCi/ml) was added. After 5 sec of incubation, 2 ml of cold quench solution was added followed by suction-filtration. Uptake of $^{22}Na^+$ due to activation of voltage-dependent Na^+ channels was determined as the difference of uptake in the presence and absence of TTX. Readings of dpm were corrected back to the original volume of synaptosomes filtered and expressed as nmole $^{22}Na^+/\mu$g protein.

$^{22}Na^+$ uptake following incubation with control and LEMS IgG (2 and 4 mg/ml) was measured as described above except that the synaptosomal suspension was added to the incubation buffer for 10 min only prior to the addition of uptake solution.

8. Measurement of $^{86}Rb^+$ Loading and Efflux

Loading of synaptosomes with $^{86}Rb^+$ and efflux under depolarizing conditions were done using a modification of the method of Bartschat and

Blaustein (1985*a*). Synaptosomes were first incubated for 45 min at 4°C with the following: (1) $^{86}Rb^+$ (20 μCi/ml) alone; (2) $^{86}Rb^+$ plus 10% (v/v) control human serum; (3) $^{86}Rb^+$, 10% (v/v) control human serum and control IgG (4 mg/ml); or (4) $^{86}Rb^+$, 10% (v/v) control human serum and LEMS IgG (4 mg/ml). Prior to the initiation of $^{86}Rb^+$ release, synaptosomes were incubated for an additional 45 min at 37°C. It is during this time that the synaptosomes became loaded with the radiolabel. Next, 100 μl of labeled synaptosomes were placed on a filtration apparatus containing 2 ml of a wash solution and suction applied. Synaptosomes were washed in this manner with a total of 10 ml of washing solution to remove any extracellular $^{86}Rb^+$. Following the final wash, the filter was removed to a second filtration apparatus and 2 ml of depolarizing (77.5 mM) or resting (5 mM) K^+ solution was added. Five sec later, 2 ml of stop solution was added and suction applied. The filtrate was collected directly. Radioactivity in the filtrate and that remaining on the filter was estimated by gamma counting. Efflux in low $[K^+]_o$ was subtracted from that in high $[K^+]_o$ solutions as depolarization-independent background. In order to control for possible differences in $^{86}Rb^+$ loading, efflux is expressed as a % of total.

9. Measurement of Lactate Dehydrogenase (LDH) Activity

LDH release was quantified by the method of Bergmeyer and Bernt (1974), as described in detail in Chapter Three. Activity was expressed as the percentage of LDH released into the supernatant. Total LDH activity (100%) was determined by lysing an equal volume of synaptosomes in 0.1% (v/v) Triton-X-100.

10. Fluorescence Measurements

Measurements of diS-C_2(5) fluorescence as an index of synaptosomal membrane potential were made as described by Hare and Atchison (1992). Briefly, a 50 μl aliquot of prewarmed synaptosomal suspension was added to 2 ml physiologic saline buffer containing 0.5 μM diS-C_2(5) and maintained at 37°C for 10 min. Determination of diS-C_2(5)-mediated fluorescence in synaptosomes was made using a SPEX fluorolog spectrofluorometer (SPEX Industries, Edison, NJ) at excitation and emission wavelengths of 649 and 682 nm respectively. In order to measure plasma membrane potential fluorescence independent of mitochondrial fluorescence, NaCN (2 mM) and oligomycin (4 μg/ml) were added to the suspension to collapse the mitochondrial membrane potential(Heinonen *et al.*, 1985; Hare and Atchison, 1992). Once baseline fluorescence was judged to be stable, KCl (40 mM) was added to depolarize the plasma membrane. Data are expressed as the percent change from baseline fluorescence.

11. Statistical analysis

Differences in quantal content in animals receiving control or LEMS plasma were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's *t*-test. Uptake of $^{45}Ca^{2+}$ and $^{22}Na^{+}$ were analyzed using Student's *t*-test for paired samples. Efflux of $^{86}Rb^{+}$ and fluorescence measurements were analyzed by a completely blocked ANOVA. The LDH experiments were analyzed by split-plot design ANOVA (2X5). Difference were considered significant at $p < 0.05$.

D. *Results*

1. Quantal Content

To verify experimentally the clinical diagnosis of LEMS, and thus correlate potential actions on synaptosomal Ca^{2+} channels with clinical neuromuscular effects, the ability of LEMS patient plasma to reduce quantal content when given chronically was examined. Quantal content of nerve-evoked EPPs was reduced significantly in mice receiving chronic injection of plasma from three patients clinically diagnosed with LEMS. Mean quantal content as determined by the variance method was reduced from ≈ 70 in diaphragms from control mice to 28-19 in diaphragms from mice receiving LEMS plasma (Fig. 4.2). This represents a reduction of approximately 60 to 70%. Similar percent reductions were observed using the mean value method (Table 4.1). MEPP amplitude was unaffected by treatment with LEMS plasma (Table 4.1). Cyclophosphamide alone had no effect on quantal content (results not shown).

Facilitation of quantal content at 20 Hz was observed in diaphragms from mice treated with LEMS plasma from all three patients. Quantal content in mice treated with plasma from two of three patients facilitated at 40 Hz. Facilitation was not observed in mice treated with control plasma (Fig. 4.3).

2. $^{45}Ca^{2+}$ Uptake

The effect of LEMS IgG from Patient 3 on uptake of $^{45}Ca^{2+}$ during 1 sec of K^+-depolarization into rat forebrain synaptosomes is depicted in Figure 4.4. Depolarization-induced uptake was inhibited 36.5 ± 14.5%, 44.5 ± 9.8% and 34.6

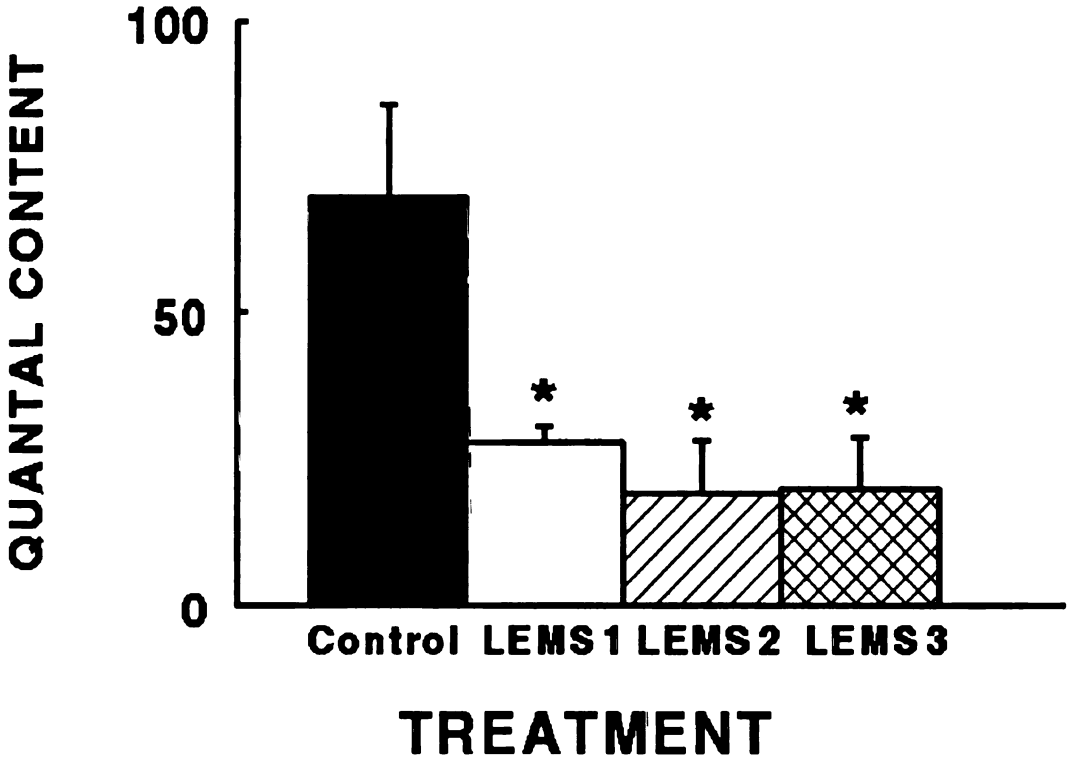


Figure 4.2. Effects of chronic treatment of mice with LEMS plasma on quantal content. Mice were treated for 30 days with 1.5 ml/day (i.p.) of plasma from control or one of 3 different patients with the clinical diagnosis of LEMS. Neuromuscular transmission was examined using conventional intracellular recordings of MEPPs and EPPS elicited at 1 Hz from isolated hemidiaphragm preparations. Contractions evoked by phrenic nerve stimulation were prevented by transsection of the myofibers. Quantal content (*m*) was determined from the amplitudes and variances of the EPPs. At least 200 EPPs were used in the calculation of *m*. Values are the mean ± SEM of at least four animals. Asterisks indicate values of quantal content which are significantly lower than control ($p < 0.05$).

Table 4.1
Determination of Quantal Content via Direct Method

	m	MEPP (mV)
Control	9.75 ± 2.2	0.353 ± 0.05
Patient 1	3.92 ± 1.7*	0.335 ± 0.14
Patient 2	5.78 ± 1.2*	0.328 ± 0.04
Patient 3	3.33 ± 0.51*	0.350 ± 0.06

Mice were treated for 30 days with 1.5 ml/day (i.p.) of plasma from control or one of 3 different patients with the clinical diagnosis of LEMS. Neuromuscular transmission was examined using conventional intracellular recordings of MEPPs and EPPS elicited at 1 Hz from isolated hemidiaphragm preparations. Quantal content (*m*) was determined by dividing the average amplitude of EPPs by the average amplitude of the MEPPs. Values are the mean ± SEM of at least four animals. Asterisks indicate values of quantal content which are significantly lower than control ($p < 0.05$).

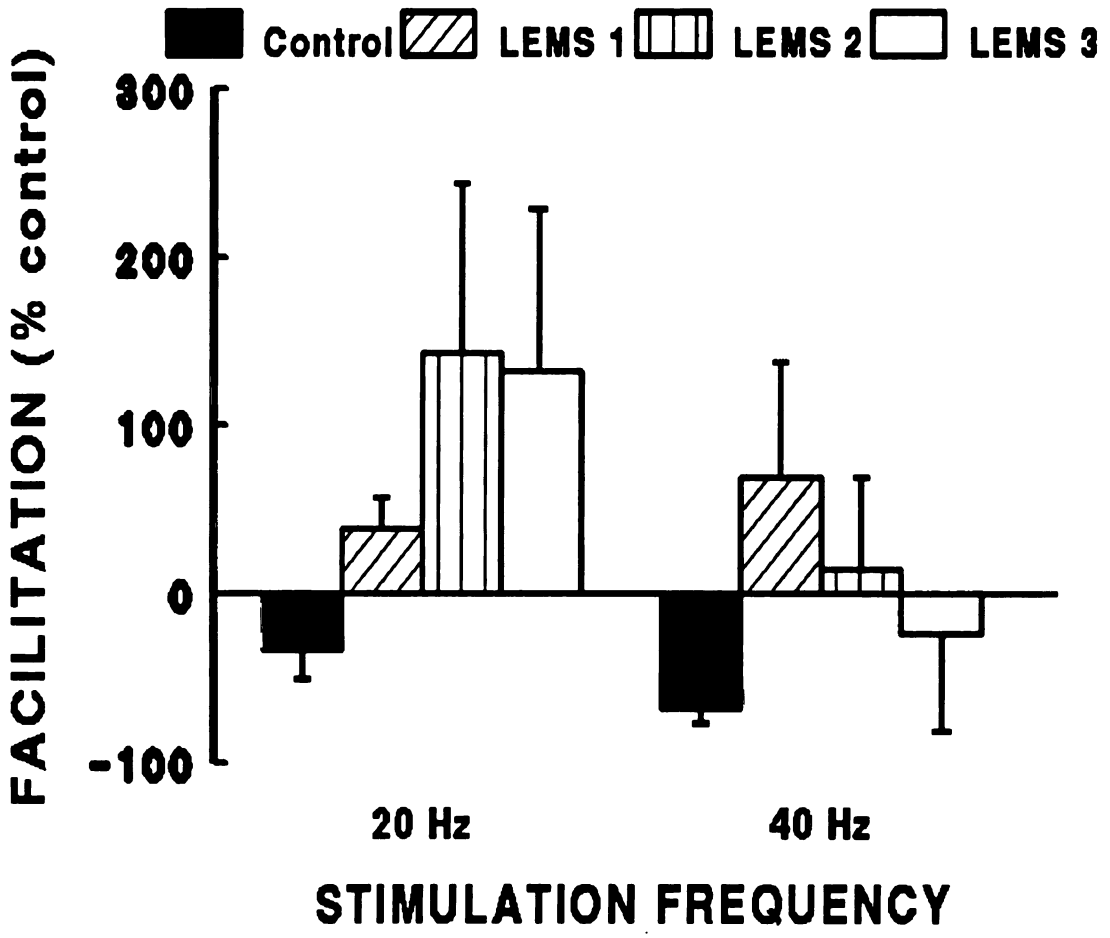


Figure 4.3. Frequency-dependent facilitation of neuromuscular transmission in mice treated chronically with LEMS plasma. Treatments and recording procedures were as described in Figure 4.2. EPPs were elicited at the indicated frequencies of stimulation. Facilitation is defined as an increase in quantal content compared to that obtained at 1 Hz.

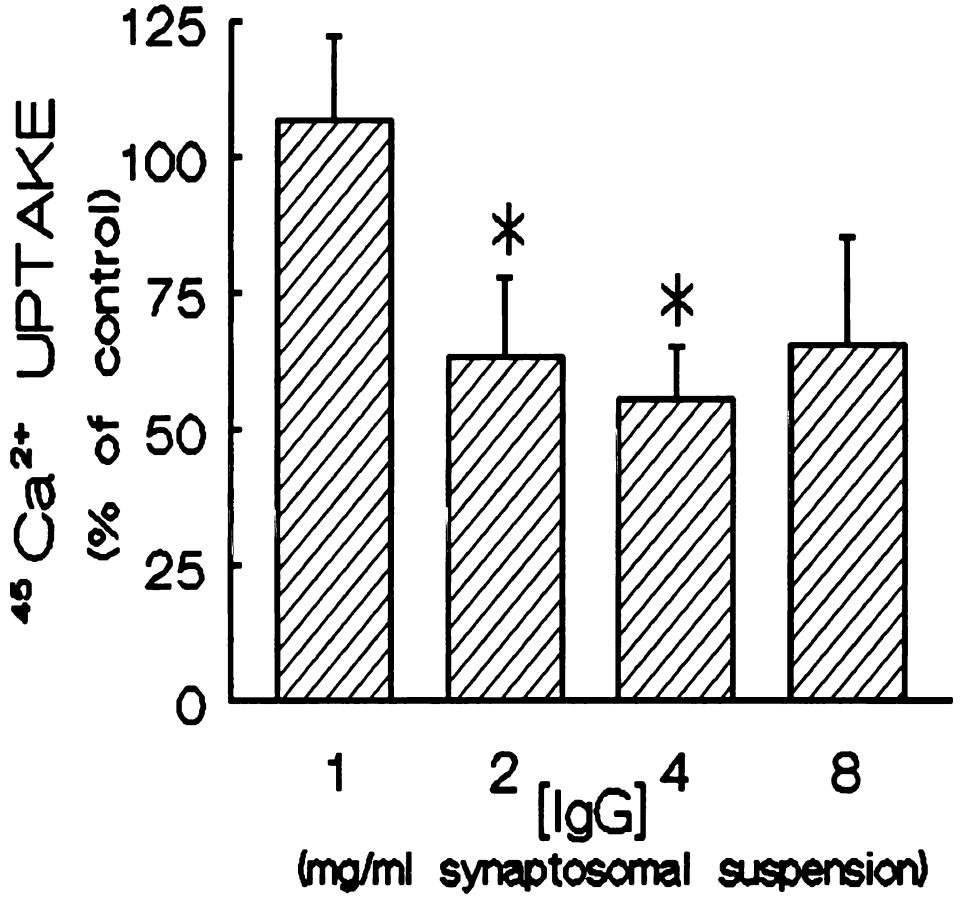


Figure 4.4. Effect of LEMS IgG on uptake of $^{45}Ca^{2+}$ into rat forebrain synaptosomes. Synaptosomes were incubated for 1 hr at 4°C with varying concentrations of LEMS or control IgG and 10 % control human serum. Prior to initiation of uptake, synaptosomal suspensions were equilibrated at 37°C for 30 min. One sec uptake in 5 mM K^+ was subtracted from that in 42.5 mM K^+ as depolarization-independent background. $^{45}Ca^{2+}$ uptake is expressed as % of control. All values are the mean ± SEM of 7 experiments with each individual experiment being the average of 3 measurements. Asterisks indicate values which are significantly lower than their respective paired control ($p < 0.05$).

± 19.8% by 2, 4 and 8 mg/ml LEMS IgG respectively, as compared to synaptosomes incubated with equivalent concentrations of control IgG. Depolarization-independent uptake was unaffected (results not shown).

A number of LEMS patients, including the patient used in the previous and proceeding studies have SCC. Uptake of $^{45}Ca^{2+}$ was determined following incubation with SCC IgG pooled from a number of patients in which no neurological involvement was observed to control for possible effects associated with neoplastic disease. SCC IgG (2 and 4 mg/ml) produced no reduction in depolarization-dependent uptake of $^{45}Ca^{2+}$ (Fig. 4.5).

3. Synaptosomal Membrane Integrity

To rule out the possibility that the effect of LEMS IgG was a result of nonspecific disruption of the synaptosomal membrane, LDH release following incubation with varying concentrations of LEMS and control IgG was measured. LDH released into the extrasynaptosomal fluid following incubation with LEMS IgG was not increased over that which occurred with control IgG (Fig. 4.6). Thus, it is unlikely that decreased Ca^{2+} uptake is due to disruption of synaptosomal membrane integrity.

4. Potentiometric Dye Fluorescence

Alterations in the synaptosomal membrane potential by IgG could prevent normal activation of the Ca^{2+} channels. We tested for this possibility using the fluorescent, potentiometric carbocyanine dye diS-C_2(5). NaCN (2 mM) and

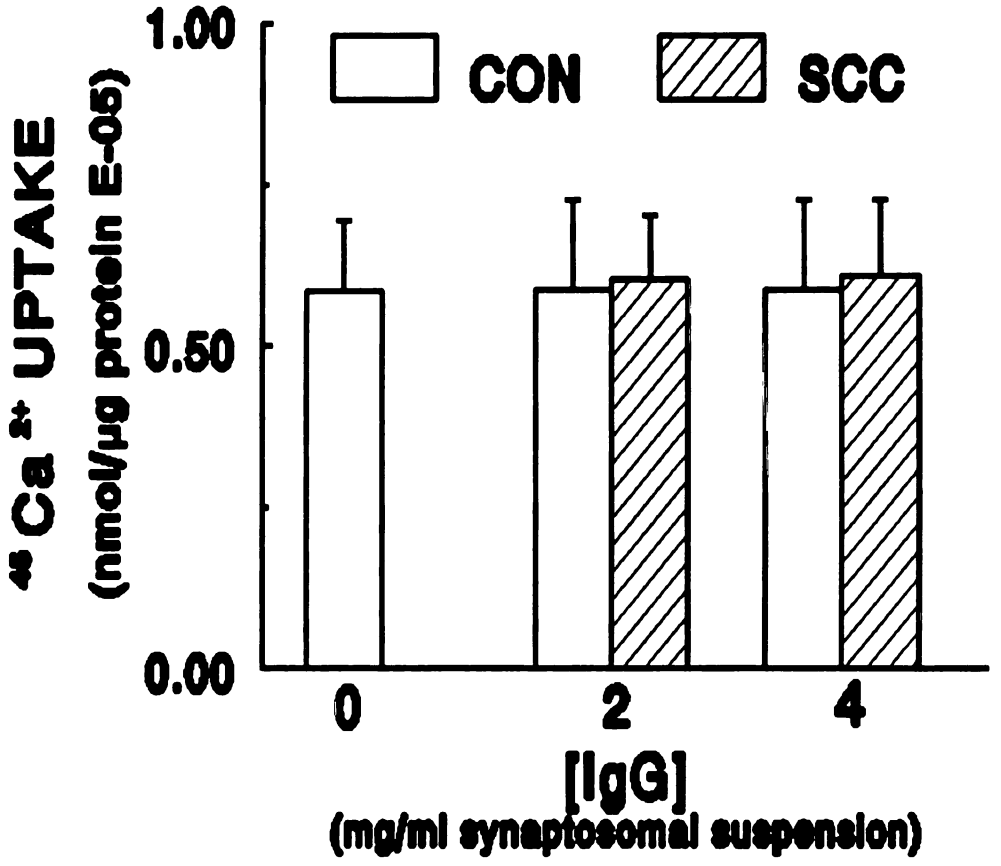


Figure 4.5. Effect of IgG from patients with small cell carcinoma (SCC) on uptake of $^{45}Ca^{2+}$ into rat forebrain synaptosomes during 1 sec of depolarization by 41.25 mM KCl. Synaptosomes were incubated for 1 hr at 4°C with 10% control human serum and control or SCC IgG. Prior to initiation of uptake, synaptosomal suspensions were equilibrated for 30 min at 37°C. Uptake in 5 mM K^+ was subtracted from that in 42.5 mM K^+ as depolarization-independent background. Data are expressed as nmol $^{45}Ca/\mu g$ protein. All values are the mean ± SEM of 4 experiments with each individual experiment being the average of 3 measurements.

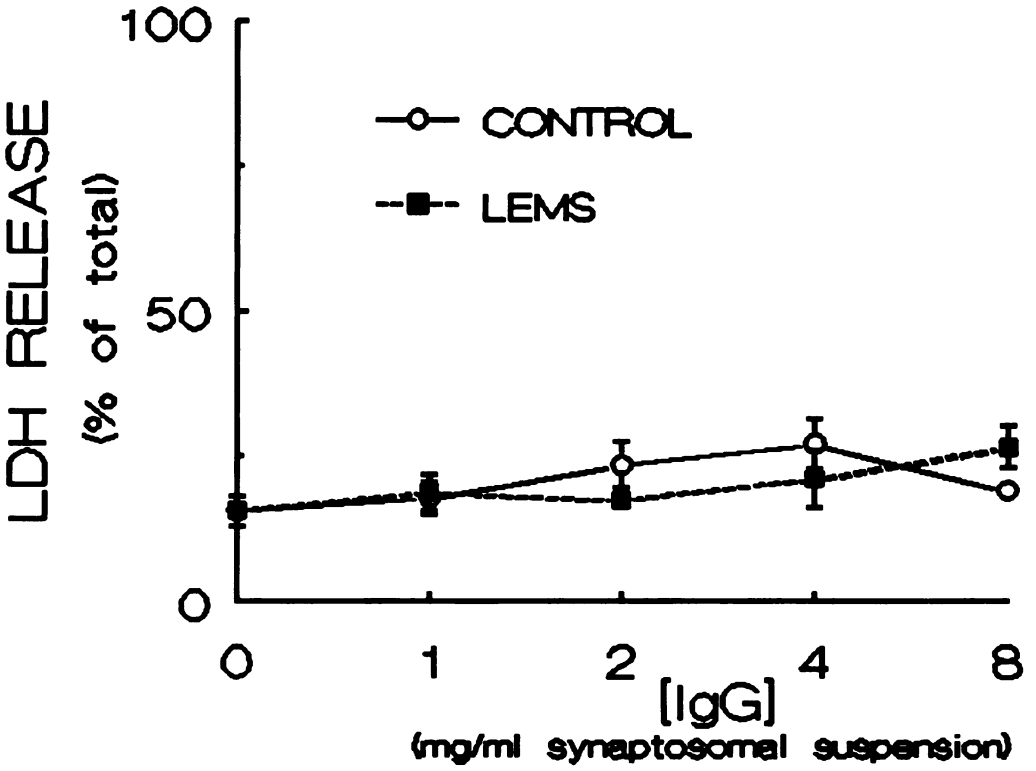


Figure 4.6. Effect of LEMS IgG on synaptosomal membrane integrity. LDH release was measured from the extrasynaptosomal fluid following treatment for 90 min with 10 % control human serum and varying concentrations of LEMS or control IgG. LDH activity was quantified by the rate of NADH oxidation at 340 nM. The quantity of the enzyme is expressed as the percent of release of the total enzyme content of the terminals, which was determined by lysis of equal volume of synaptosomes with 0.1% triton-X-100 followed by sonication. Values are expressed as the mean ± SEM of 4 experiments.

oligomycin (4 μg/ml) were first added to the suspension to collapse the mitochondrial membrane potential (Fig. 4.7). LEMS IgG caused no alterations in dye fluorescence following addition of KCl (40 mM) (Fig. 4.8).

5. $^{22}Na^+$ Uptake

In order to determine the length of time the synaptosomes should be incubated with VER, a time course of $^{22}Na^+$ uptake was performed. The reults are depicted in Figure 4.9. Five sec $^{22}Na^+$ uptake into synaptosomes is maximal following 10 min incubation with incubation buffer containing 150 μM VER. These results are similar to those of Tankum and Catterall (1981).

Incubation of synaptosomes with concentrations of LEMS IgG which significantly altered uptake of $^{45}Ca^{2+}$ during K^+-evoked depolarization had no effect on depolarization-induced uptake of $^{22}Na^+$ into VER-treated synaptosomes (Fig. 4.10).

6. $^{86}Rb^+$ Loading and Efflux

Treatment of synaptosomes with 10 % (v/v) control human serum or serum and either control or LEMS IgG (4 mg/ml) did not alter loading of $^{86}Rb^+$ into rat forebrain synaptosomes (Fig. 4.11). Likewise, incubation of synaptosomes with concentrations of LEMS IgG which significantly altered uptake of $^{45}Ca^{2+}$ during K^+-evoked depolarization had no effect on $^{86}Rb^+$ efflux from K^+-depolarized synaptosomes (Fig. 4.12).

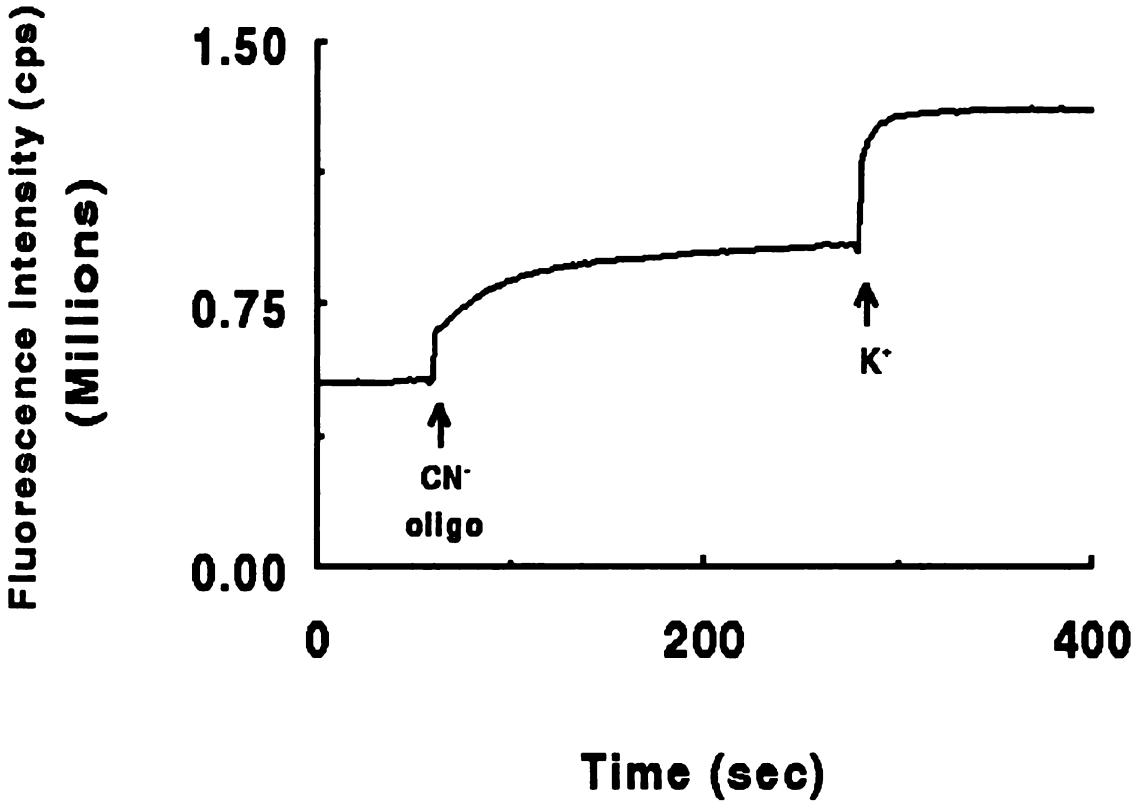


Figure 4.7. Representative graph depicting typical fluorescent dye experiment. An aliquot of untreated synaptosomal suspension was added to 2 ml of physiologic saline buffer containing 0.5 μM diS-C2(5) and maintained at 37°C for 10 min. In order to measure fluorescence derived from the plasma membrane independent of mitochondrial fluorescence, NaCN (2 mM) and oligomycin (4 μg/ml) were added to the synaptosomal suspension to collapse the mitochondrial membrane potential. Once baseline was judged to be stable (visibly), KCl was added to depolarize the plasma membrane. Data expressed in counts per second.

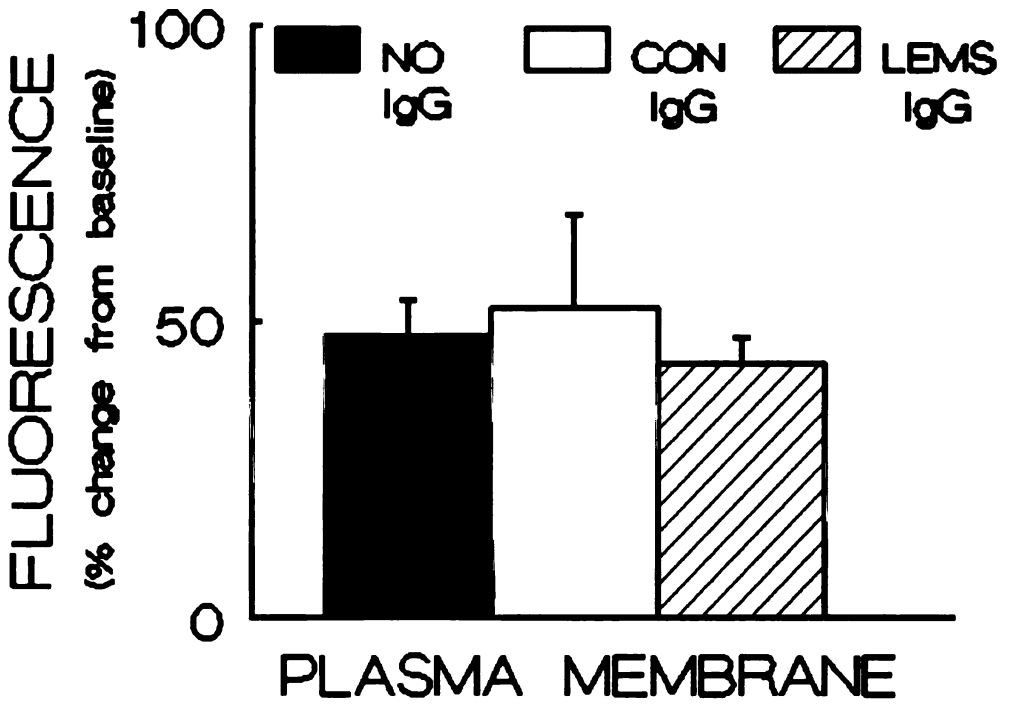


Figure 4.8. Effect of LEMS IgG on synaptosomal membrane potentials. Synaptosomes were incubated with 2 mg/ml of control or LEMS IgG, or no IgG for 90 min. Following incubation, synaptosomes were combined with a fluorescent, potentiometric dye, diS-C_2(5). Initially, NaCN (2 mM) and oligomycin (4 μg/ml) were added to collapse the mitochondrial membrane potential. Addition of 40 mM KCl depolarized the plasma membrane potential. Data are expressed as % change in baseline fluorescence. Values are the mean ± SEM of 3 experiments.

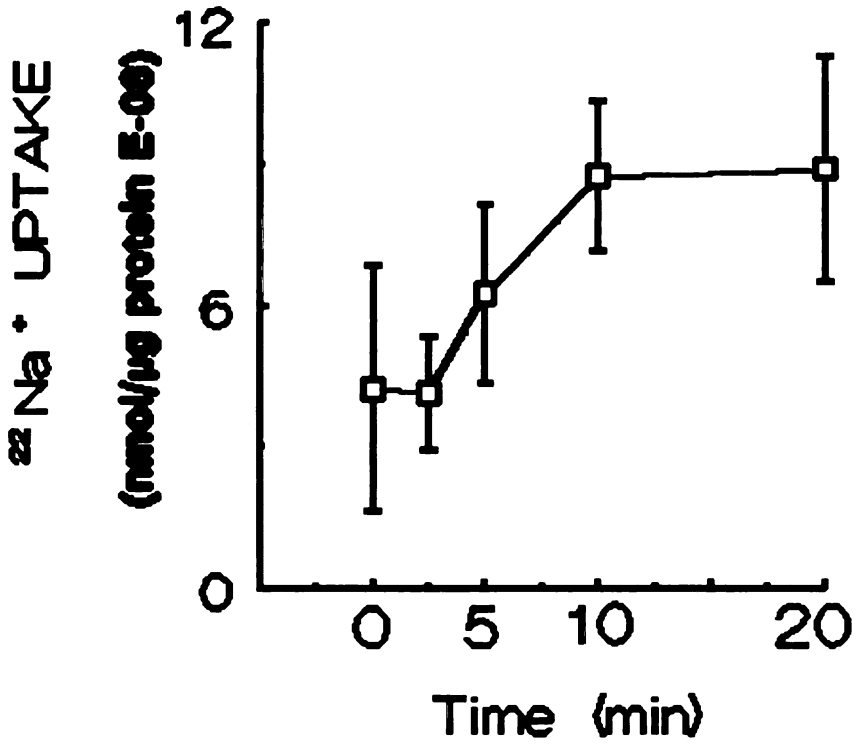


Figure 4.9. Time course of $^{22}Na^+$ uptake into rat forebrain synaptosomes. Synaptosomes were added to incubation buffer which contained no added Na^+, 150 μM VER, 100 μM ± TTX (1 μM) for varying periods of time. After incubation, uptake solution containing $^{22}Na^+$ was added and allowed to mix for 5 sec subsequent to addition of cold quenching solution. Uptake is expressed as nmol $^{22}Na^+$/μg protein. Values are the mean ± SEM of 4 experiments.

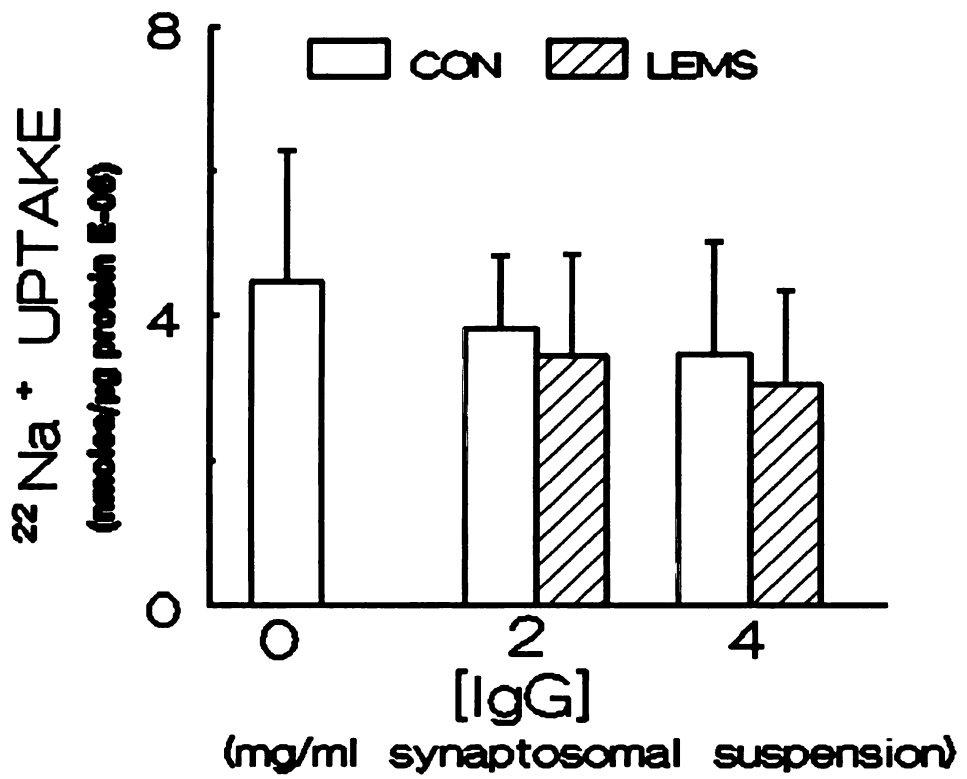


Figure 4.10. Effect of LEMS IgG on uptake of $^{22}Na^+$ into rat forebrain synaptosomes depolarized with veratridine. Synaptosomes were incubated with IgG and serum as in Fig. 4.4 after which they were combined with veratridine (150 μM) for 10 min in a solution in which the NaCl had been replaced with choline chloride. Synaptosomes were then incubated with $^{22}Na^+$ for 5 sec. Uptake of $^{22}Na^+$ due to activation of voltage-dependent Na channels was determined as the difference of uptake in the presence and absence of tetrodotoxin (TTX; 1 μM). Values are the mean ± SEM of 4 separate experiments.

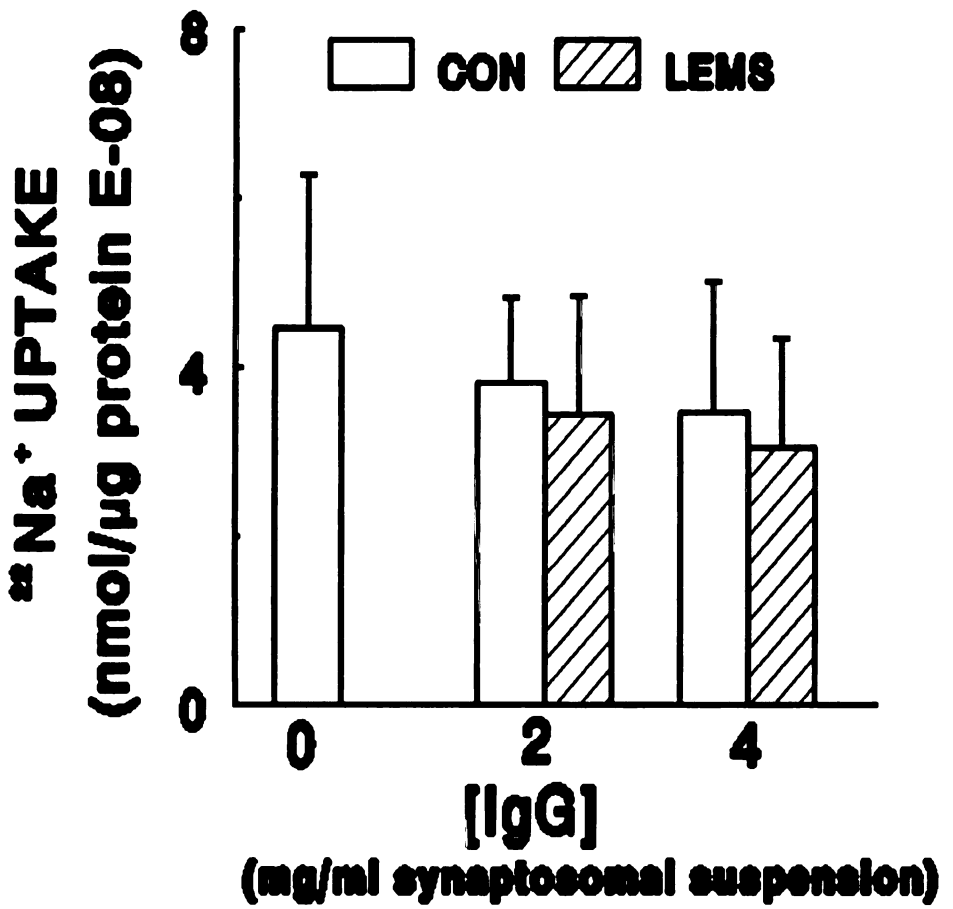
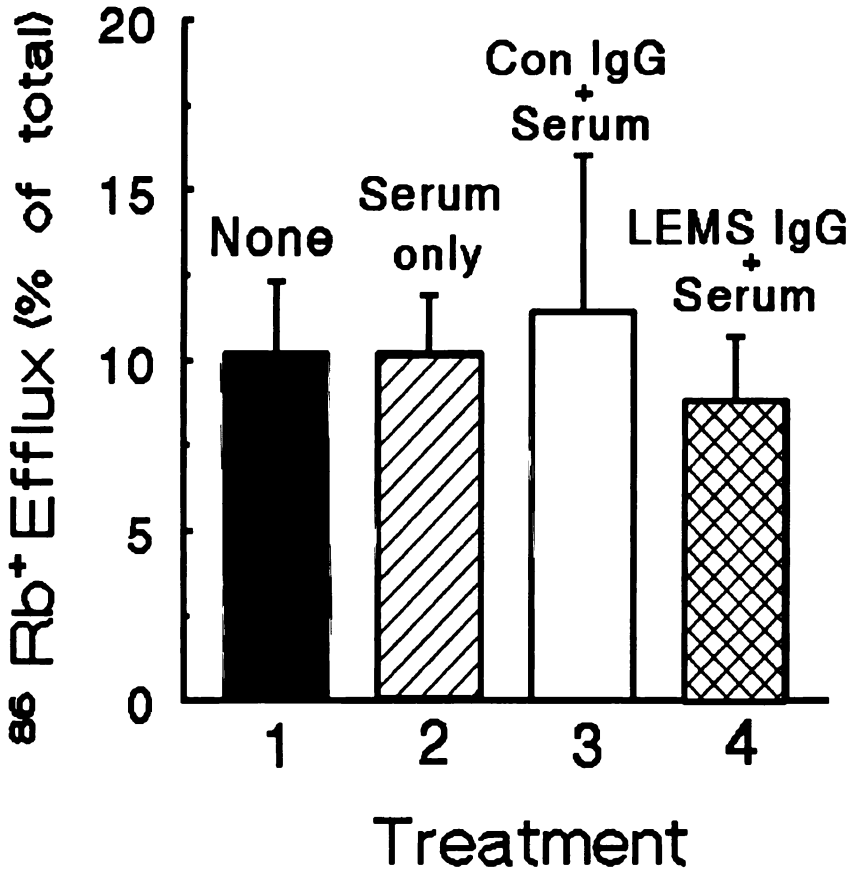


Figure 4.11. Time course of $^{86}Rb^{+}$ loading into synaptosomes. Synaptosomes were treated for 1 hr as described in methods. Prior to addition of radiolabel, synaptosomes were allowed to equilibrate at 37°C for 30 min. $^{86}Rb^{+}$ (20 μCi/ml) was added to the synaptosomal suspension and loading allowed to proceed for varying periods of time. After the specified time, 2 ml of cold quench solution was added to terminate loading. Uptake is expressed as nmol $^{86}Rb^{+}/\mu$g protein.



Figue 4.12. Effect of LEMS IgG on $^{86}Rb^+$ efflux from rat forebrain synaptosomes. Following incubation and removal of residual extracellular $^{86}Rb^+$, efflux of $^{86}Rb^+$ was measured by addition of depolarizing and nondepolarizing K^+ solutions to the filter well. After 5 sec, cold quenching solution was added followed by suction filtration. Radioactivity in the filtrate and that remaining on the filter was determined. Efflux in low $[K^+]_e$ solutions was subtracted from that in high $[K^+]_e$ solutions as depolarization-independent background. Efflux is expressed as % of total. Values are the mean ± SEM of 4 separate experiments.

E. *Discussion*

The aim of the present study was to test explicitly whether LEMS autoantibody affects nerve terminal Ca^{2+} channels and whether this action was direct and/or specific. In particular, we sought to determine if effects of LEMS IgG on nerve terminal function were related to a generalized membrane disturbance, and if not, whether the IgG recognized other voltage-dependent ion channels in axon terminals. This is important as nonspecific membrane alterations and/or alteration in function of other ion channels could result indirectly in Ca^{2+} channel dysfunction. Our results indicate that IgG from a patient with LEMS acts on presynaptic nerve terminal Ca^{2+} channels in the CNS to reduce depolarization-dependent uptake of $^{45}Ca^{2+}$. The antibody from this patient appears to be specific for Ca^{2+} channels as it is unable to disrupt ion flow through voltage-dependent Na^{+} or K^{+} channels. These deficits are not due to decreases in synaptosomal viability nor to IgG-induced alterations in the synaptosomal membrane potential but appear to be a direct block of or alteration in voltage-dependent Ca^{2+} channel function.

Passive transfer experiments demonstrated that the plasma from the three patients studied produced the electrophysiological characteristics associated with LEMS. Quantal content of the EPP was decreased when the nerve was stimulated at low frequencies of stimulation, but was facilitated at high frequencies of stimulation. MEPP amplitude was unaffected. These transmission characteristics are qualitatively similar whether the disease is transferred passively by plasma, crude

Ig's or purified IgG (Newsom-Davis *et al.*, 1982; Lang *et al.*, 1983; Kim, 1985; 1986; Lennon and Lambert, 1988). Passive transfer experiments were useful in determining that a presynaptic abnormality existed in LEMS. However, they represent an indirect measurement of nerve terminal Ca^{2+} channel activity as the postsynaptic response measured results from many processes ongoing in the presynaptic nerve terminal.

Likewise, Meyer *et al.* (1986) and Login *et al.*(1987) showed that serum and serum fractions from patients with LEMS reduced ACh release from synaptosomes and growth hormone and prolactin release from rat anterior pituitary cells respectively. However, no measurement of ion channel activity was made (Meyer *et al.*, 1986; Login *et al.*, 1987). Still, others have shown that LEMS IgG reduced the function of somal Ca^{2+} channels in human SCC (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988*a,b*), rodent neuroblastoma X glioma hybrid (Peers *et al.*, 1987) , bovine adrenal chromaffin (Kim and Neher, 1988), and rat pheochromocytoma (PC12) cells (Lang *et al.*, 1989*a,b*). It is quite possible, however, that the type of Ca^{2+} channel in the terminal may differ from that in the cell body (Nachshen and Blaustein, 1979; Suszkiw *et al.*, 1989; Charlton and Augustine, 1990; Utchitel *et al.*, 1992). Also, the binding of ω-CgTx to somal Ca^{2+} channels has been used as an index of localization of LEMS autoantibodies (Sher *et al.*, 1989), although ω-CgTx does not block mammalian neuromuscular transmission (Anderson and Harvey, 1987; Protti *et al.*, 1991) whereas the LEMS autoantibody clearly does (Lambert and Elmqvist, 1971; Lang *et al.*, 1983; Kim, 1985;1986).

We demonstrated previously that serum and plasma from patients with LEMS reduced depolarization-dependent uptake of $^{45}Ca^{2+}$ (10 sec) into synaptosomes (Hewett and Atchison, 1991; Chapter Three). In the present study the interaction of isolated LEMS IgG with nerve terminal Ca^{2+} channels thought to be involved in neurotransmitter release was examined directly. Reduction in depolarization-dependent uptake of $^{45}Ca^{2+}$ during 1 sec was evident following 90 min incubation with LEMS IgG. This "fast" phase of uptake (1 sec) into synaptosomes has been consistently correlated with neurotransmitter release from synaptosomes (Drapeau and Blaustein, 1983; Floor, 1983; Leslie *et al.*, 1985).

IgG from SCC patients lacking neurological symptoms did not reduce depolarization-dependent uptake of $^{45}Ca^{2+}$ into synaptosomes. Thus, the presence of a tumor alone is not sufficient to result in nerve terminal Ca^{2+} channel dysfunction. This is not unexpected, as many patients with SCC do not have the manifestation of LEMS. Nonetheless, interpretation of data obtained by using IgG from a LEMS patient with SCC is not complicated by actions of Ig's in the plasma of patients with SCC.

The decrease in $^{45}Ca^{2+}$ uptake into synaptosomes was not a result of disruption of synaptosomal membrane intergrity. LDH is a cytosolic enzyme that can be released into the extrasynaptosomal fluid if the plasma membrane is permeabilized. Its release following incubation with LEMS IgG was not increased over that of paired controls, so it is unlikely that the decrease in Ca^{2+} uptake was due to nonspecific membrane damage. It remains possible that the LEMS IgG causes

membrane damage in only a fraction of the terminals and the release of LDH from the whole population may not be sufficiently sensitive to detect this. However, in view of the lack of discrimination of the LEMS IgG for L- and N-type channels in other systems, and the magnitude of decreases seen with both serum/plasma (Hewett and Atchison, 1991) and IgG (present study) in synaptosomes, it seems unlikely that a specific population of terminals would be targeted.

IgG-induced alterations in the synaptosomal membrane potential could reduce $^{45}Ca^{2+}$ uptake indirectly. If IgG depolarized the synaptosomal membrane, a greater fraction of Ca^{2+} channels might be inactivated and unable to respond to subsequent K^+-depolarization. Alternatively, hyperpolarization of the synaptosomal membrane would decrease uptake of Ca^{2+} by reducing the probability of opening of channels for a given depolarizing pulse of K^+. Changes in the fluorescence of carbocyanine dyes as an indicator of alterations in membrane potentials have been studied in a variety of cell types and in synaptosomes (Blaustein and Goldring, 1975; Heinonen *et al.*, 1985; Hare and Atchison, 1992). Multiple dye compartmentation is avoided by elimination of the mitochondrial membrane potential by depolarization (Heinonen *et al.*, 1985; Hare and Atchison, 1992). Neither plasma membrane depolarization nor hyperpolarization is involved in the reduction of $^{45}Ca^{2+}$ uptake since LEMS IgG incubation caused no alterations in dye fluorescence following addition of KCl (40 mM). Thus, LEMS IgG induces a direct functional disruption of nerve terminal Ca^{2+} channels rather than indirectly by altering the membrane potential.

To determine if the LEMS antibody was specific for Ca^{2+} channels, we examined its potential to interact with other voltage-dependent channels. If the antibody were not specific for Ca^{2+} channels, it may indicate the IgG could recognize a portion of the channel molecule common to all ion channels. This possibility had not been previously tested directly.

Synaptosomes contain voltage-dependent Na^+ channels. Maximum uptake of $^{22}Na^+$ into synaptosomes occurs following 10 min incubation with 150 μM VER. VER is used to activate synaptosomal Na^+ channels as these channels inactivate rapidly in response to K^+-depolarization (Kreuger and Blaustein, 1980; Tankum and Catterall, 1981).

There is considerable homology between the α subunit of the voltage-dependent Na^+ channel and α_1 subunit of the dihydropyridine (DHP)-sensitive (L-type) voltage-dependent Ca^{2+} channel (Ellis *et al.*, 1988). LEMS IgG affects L-type Ca^{2+} channels in several cell lines (Login *et al.*, 1987; Peers *et al.*, 1987; Kim and Neher, 1988). Since the α subunits contain the binding site for pharmacological antagonists, are the pore forming subunits and are indispensable for the function of the channel(Catterall, 1988), they are potential candidates as antigenic determinants for autoantibodies. Therefore, LEMS antibodies directed against the α_1 subunit of the Ca^{2+} channel might cross-react with the α subunit of Na^+ channels as well. Incubation of synaptosomes with concentrations of LEMS IgG that significantly altered uptake of $^{45}Ca^{2+}$ into K^+-depolarized synaptosomes had no effect on uptake of $^{22}Na^+$ into veratridine-depolarized synaptosomes. This is

consistent with the work of Pascuzzi and Kim (1988) in which functional *in vivo* measurements of nerve conduction velocity made from animals chronically treated with LEMS IgG demonstrated that conduction was normal.

In some cells, Ca^{2+} and K^+ channels may be structurally similar (Hume, 1985; Nerbonne *et al.*, 1987). Also, some patients with MG, another autoimmune, neuromuscular disease, have antibodies against a β-bungarotoxin binding protein (Lu *et al.*, 1991). This protein has been demonstrated to belong to a class of voltage-gated K^+ channels (Rehn and Betz, 1984). If patients with LEMS have a similar antibody, alterations in K^+ channel conductance could indirectly alter Ca^{2+} channel conductance. Treatment of synaptosomes with serum or serum and IgG did not alter the loading of $^{86}Rb^+$ into synaptosomes by the Na^+/K^+ ATPase. Likewise, concentrations of LEMS IgG that significantly altered uptake of $^{45}Ca^{2+}$ into K^+-depolarized synaptosomes had no effect on efflux of $^{86}Rb^+$ following K^+-depolarization. This provides further evidence that the LEMS autoantibody recognizes an epitope which is specific for voltage-dependent Ca^{2+} channels.

In summary, LEMS IgG from Patient 3 acts on central nerve terminal Ca^{2+} channels to reduce depolarization-dependent uptake of $^{45}Ca^{2+}$. The antibody appears specific for Ca^{2+} channels as it is unable to disrupt ion flow through voltage-dependent Na^+ or K^+ channels. These deficits are not due to indirect effects on membrane integrity or synaptosomal membrane potential but appear to result from a direct block of or alteration in Ca^{2+} channel function. The preferential action of the LEMS antibody for nerve terminal Ca^{2+} channels suggests that there

is no common determinant or epitope on different voltage-gated channel proteins to which the antibody can bind. We cannot exclude, however, the possibility that some patients with LEMS may synthesize antibodies with different specificities. These data also suggest antigenic similarities exist between somatic motor nerve terminal and central nerve terminal Ca^{2+} channels. These findings are important as the existence of autoantibodies which are specific for a component of the neurosecretory process may serve as molecular tools for studying the steps involved in quantal transmitter release both in the peripheral and central nervous system.

CHAPTER FIVE

DISRUPTION OF SYNAPTOSOMAL Ca^{2+} CHANNELS BY LAMBERT-EATON MYATHENIC SYNDROME IMMUNOGLOBULIN: SERUM DEPENDENCY

A. *Summary*

Chapter Four demonstrated that in the presence of control human serum, immunoglobulins isolated from a patient with LEMS reduced flux of Ca^{2+} into isolated nerve terminals during depolarization. The objective of the present study was to determine the role of serum in reducing uptake of $^{45}Ca^{2+}$ into rat brain synaptosomes by LEMS IgG. Depolarization-dependent uptake of $^{45}Ca^{2+}$ through voltage-gated Ca^{2+} channels was determined using synaptosomes incubated with control (disease-free) and LEMS IgG with or without control human serum. In the absence of human serum, LEMS IgG did not reduce uptake of $^{45}Ca^{2+}$ into synaptosomes. However, in the presence of control human serum (10% of total incubation volume), $^{45}Ca^{2+}$ uptake was reduced significantly by LEMS IgG (2 and 4 mg/ml), but not by IgG from disease-free patients or by 10% (v/v) control human serum alone. This concentration of serum was found to be optimal; higher concentrations produced significant reductions in Ca^{2+} uptake, whereas at lower concentrations the serum/IgG combination was ineffective. The depressant effect of serum alone on $^{45}Ca^{2+}$ uptake was mimicked by bovine serum albumin (BSA) suggesting that deficits in $^{45}Ca^{2+}$ uptake produced by serum were the result of increased protein binding of the radiolabel. Heat-inactivating the serum abolished its ability to interact with LEMS IgG to depress $^{45}Ca^{2+}$ uptake. This suggested a role for complement in this effect. To test whether the membrane attack complex (MAC) or the alternative pathway of complement (APC) contributed to the alteration in Ca^{2+} channel function, $^{45}Ca^{2+}$ uptake into synaptosomes was measured

following incubation with LEMS IgG and C5- or Factor B- deficient serum, respectively. LEMS IgG reduced K^+-stimulated uptake in both cases. Thus, neither the MAC nor the APC contributed to disruption of Ca^{2+} channel function by LEMS IgG in synaptosomes. In contrast, incubation of synaptosomes with LEMS IgG and C3-deficient serum prevented the reduction in $^{45}Ca^{2+}$ uptake suggesting that the C3 component of the complement pathway contributes to Ca^{2+} channel dysfunction in synaptosomes following incubation with serum and LEMS IgG.

B. *Introduction*

Autoantibodies, such as those in LEMS, that interfere with the release process may either interrupt Ca^{2+} flux into the terminal or some intraterminal event critical to transmitter release. Results from several studies indicate that LEMS autoantibodies interact with voltage-dependent Ca^{2+} channels in cell lines of diverse origin (Peers *et al.*, 1987; De Azipurua, 1988*a*; Kim and Neher, 1988). LEMS serum also reduces depolarization-dependent Ca^{2+} entry into and ACh release from isolated nerve terminals of rat brain (Meyer *et al.*, 1986; Hewett and Atchison, 1991). Data in Chapter Four demonstrate that in the presence of control human serum, IgG isolated from a patient with LEMS reduced Ca^{2+} flux into synaptosomes as well.

Activation of the serum component complement plays a central role in mediating postsynaptic membrane damage in animal models of the autoimmune neuromuscular disorder MG, as well as in the human disease state (Engel *et al.*, 1977; Sahashi *et al.*, 1978). Attempts to clarify the participation of complement in

the pathogenesis of LEMS has so far yielded negative results (Prior *et al.*, 1987; Lennon and Lambert, 1988). As it is still possible that complement activation may be important in LEMS, the present study was undertaken to assess the role of serum in the pathogenesis of Ca^{2+} channel dysfunction induced by LEMS IgG in isolated nerve terminals from mammalian CNS.

C. *Materials and Methods*

1.Materials

N-methyl-*d*-glucamine, C5-, C3-and Factor B-deficient human sera were purchased from Sigma Chemical Co. (St. Louis, MO). HVLP (0.45 μM pore size; 25 mm diameter) filters were purchased from Millipore (Bedford, MA). HEPES was purchased from United States Biochemical Corp. (Cleveland, OH). The radiolabel $^{45}Ca^{2+}$ (15-50 mCi/mg) was purchased from New England Nuclear (Boston, MA). Disease-free control human plasma was purchased from the American Red Cross (Lansing, MI). Control human serum was donated by a healthy volunteer. Plasma from a patient with the clinical and electromyographic features of LEMS was donated by Mark Glasberg, M.D., Henry Ford Hospital, Detroit, MI.

2. Immunoglobulin Isolation

Immunoglobulin from control and LEMS patients was isolated and quantitated as described in detail in Chapter Four. The LEMS IgG used in these studies was identical to that used in Chapter Four as well.

3.Preparation of Synaptosomes

Synaptosomes were prepared according to a modification of the method of Gray and Whittaker (1962), as described in detail in Chapter Three. Equal aliquots of the synaptosomal suspension were incubated at 4°C under 100% O_2 for 60 min with either no serum or 10% (v/v) control, C5-, C3- or Factor B-deficient serum and varying concentrations of IgG from patients with no known neurological or immunological illness (control) or LEMS. Alternatively, one concentration of IgG and varying concentrations of human serum were added to the suspension to test effects of serum on $^{45}Ca^{2+}$ uptake into synaptosomes. Prior to uptake measurements, synaptosomes were allowed to equilibrate for 30 min at 37°C.

4. Solutions and Measurement of $^{45}Ca^{2+}$ Uptake

The solutions for and the measurement of $^{45}Ca^{2+}$ are described in detail in Chapter Three. Uptake is expressed as nmole $^{45}Ca^{2+}/\mu g$ protein or as a percentage of disease-free control.

5. Statistical Analysis

Data were analyzed by randomized complete block analysis of variance followed by Dunnett's *t*-test or Students *t*-test for paired samples. Differences were considered significant at $p < 0.05$.

D. *Results*

1. LEMS IgG in the Presence and Absence of Control Human Serum

Uptake of $^{45}Ca^{2+}$ into synaptosomes during 1 sec of K^+-depolarization was unaffected by 90 min incubation with LEMS IgG alone (Fig. 5.1). However, in the presence of control human serum (10% of the total incubation volume) 2 and 4 mg/ml LEMS IgG inhibited uptake by 28.3 ± 10.0% and 39.1 ± 21.3%, respectively, as compared to their paired controls incubated with equivalent concentrations of control (disease-free) IgG (Fig. 5.1). This concentration of serum (10 %; v/v) is the concentration used in standard tissue culture procedures.

2. Control Human Serum

Since the presence of serum with LEMS IgG is important in reducing $^{45}Ca^{2+}$ uptake, the effect of control (disease-free) human serum on uptake of $^{45}Ca^{2+}$ into synaptosomes was examined (Fig. 5.2). Synaptosomes were incubated with 2 mg/ml IgG from disease-free patients and varying concentrations of control (disease-free) human serum. A concentration-dependent suppression of uptake is evident, with uptake at concentrations ≥ 15 % (v/v) being significantly lower than those in the absence of serum. No statistically significant reduction in uptake was observed with a serum concentration of 10 % (v/v). Similar results were obtained when bovine serum albumin was substituted for serum (Fig. 5.3).

Synaptosomes were incubated with 2 mg/ml control or LEMS IgG and varying concentrations of disease-free human serum to determine the optimal concentration of serum needed in the reaction mixture. Again, suppression of

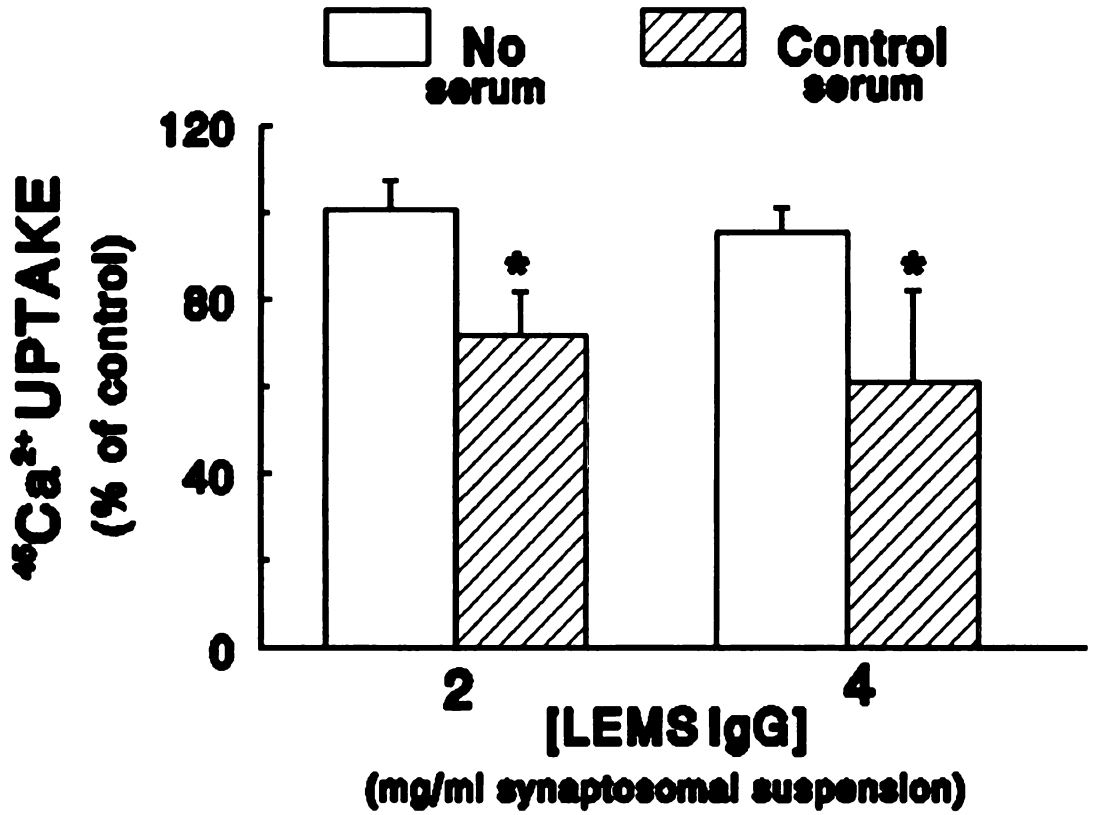


Figure 5.1. Effect of LEMS IgG in the presence and absence of control human serum on uptake of $^{45}Ca^{2+}$ into rat forebrain synaptosomes. Synaptosomes were incubated for 60 min at 4°C with 2 and 4 mg/ml LEMS or control IgG ± control human serum. Prior to the initiation of uptake, synaptosomal suspensions were equilibrated at 37°C for 30 min. Uptake in 5 mM K^+ was subtracted from that in 42.5 mM K^+ as depolarization-independent background. $^{45}Ca^{2+}$ uptake is expressed as % of paired control, control being synaptosomes incubated with equivalent concentrations of control IgG. All values are the mean ± SEM of 5 experiments. Asterisks indicate values that are significantly lower than their respective paired control ($p < 0.05$).

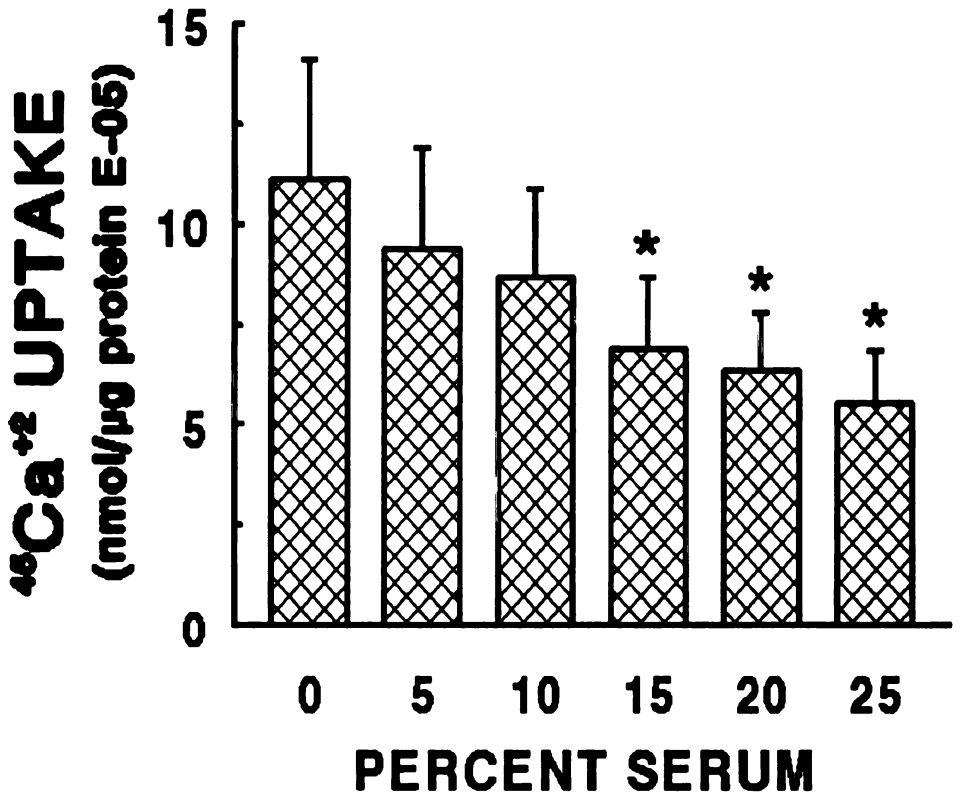


Figure 5.2. Effect of control human serum on $^{45}Ca^{2+}$ uptake into rat forebrain synaptosomes during 1 sec depolarization by 41.25 mM KCl. Synaptosomes were incubated with 2 mg/ml control IgG and varying concentrations of control human serum for 60 min at 4°C. Prior to the initiation of uptake, synaptosomes were allowed to equilibrate for 30 min at 37°C. Uptake in 5 mM K^+ was subtracted from uptake in 42.5 mM K^+ as depolarization-independent background. $^{45}Ca^{2+}$ uptake is expressed as nmol/μg protein. All values are the mean ± SEM of 3 experiments. Asterisks indicate values that are significantly lower than uptake into synaptosomes which had been incubated with no serum.

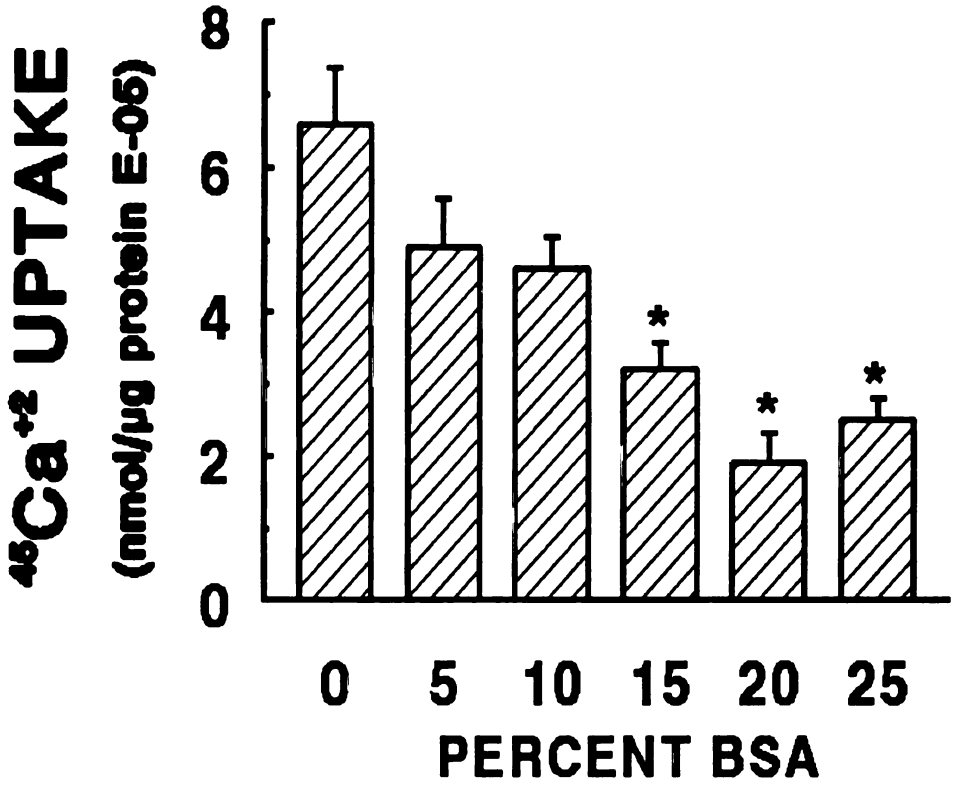


Figure 5.3. Effect of BSA on $^{45}Ca^{2+}$ uptake into rat forebrain synaptosomes during 1 sec of depolarization. A BSA solution containing the approximate protein concentration of serum was made in deionized water. Synaptosomes were incubated with 2 mg/ml control IgG and varying concentrations of BSA as in Figure 5.2. All values are the mean ± SEM of 5 experiments. Asterisks indicate values that are significantly lower than uptake into synaptosomes which had been incubated with no BSA ($p < 0.05$).

uptake by serum alone is evident (Fig. 5.4). $^{45}Ca^{2+}$ uptake was reduced significantly only by the combination of 2mg/ml LEMS IgG and 10% (v/v) serum when compared to its paired control (2 mg/ml control IgG and 10% serum).

3. LEMS IgG and Complement

Control human serum was incubated for 45 min at 57°C to inactivate complement. This prevents the reduction in uptake of $^{45}Ca^{2+}$ into the synaptosomes by LEMS IgG (2 and 4 mg/ml) and control human serum (10%) (Fig. 5.5).

Two crucial components of the complement cascade are C3 and C5. Removal of C5 prevents the assembly of the membrane attack complex (MAC). The ability of LEMS IgG and control human serum to reduce $^{45}Ca^{2+}$ uptake into synaptosomes was unaltered when control human serum was replaced with C5-deficient human serum (Fig. 5.6). Conversely, removal of the C3 component of complement from control human serum prevented the action of the LEMS IgG (Fig. 5.7). Incubation of synaptosomes with 2 and 4 mg/ml of LEMS IgG and C3-deficient serum (10 %; v/v) abolished the reduction in uptake produced by the same concentrations of LEMS IgG in the presence of control human serum or C5-deficient serum.

To determine if the alternative pathway of complement (APC) played a role in mediating the dysfunction of synaptosomal Ca^{2+} channels by LEMS IgG and control human serum, synaptosomes were incubated with 2 and 4 mg/ml control and LEMS IgG in the presence of either control human serum or Factor B-deficient serum (Fig. 5.8). Factor B is essential for activation of the APC. In the presence of

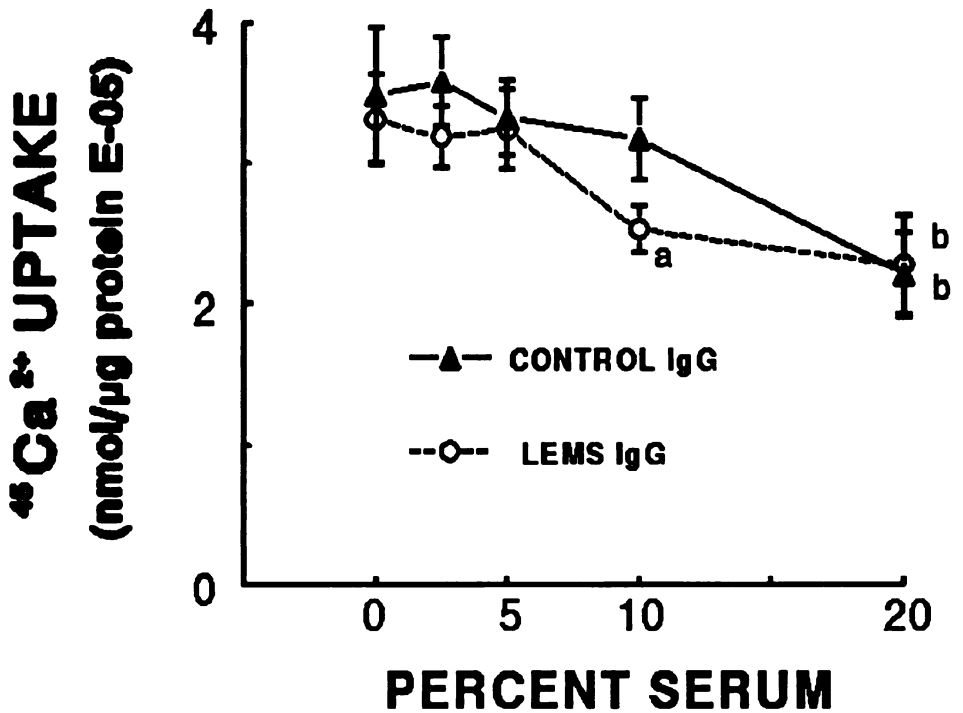


Figure 5.4. Effect of control and LEMS IgG and various concentrations of control human serum on 1 sec K^+-stimulated $^{45}Ca^{2+}$ uptake into rat forebrain synaptosomes. Synaptosomes were incubated with 2 mg/ml control and LEMS IgG and varying concentrations of control human serum as described in Figure 5.2. All values are the mean ± SEM of 5 experiments. [(a)], indicates values that are significantly different from their paired control. [(b)], indicates values that are significantly different from uptake into synaptosomes that were incubated with no serum. ($p < 0.05$)

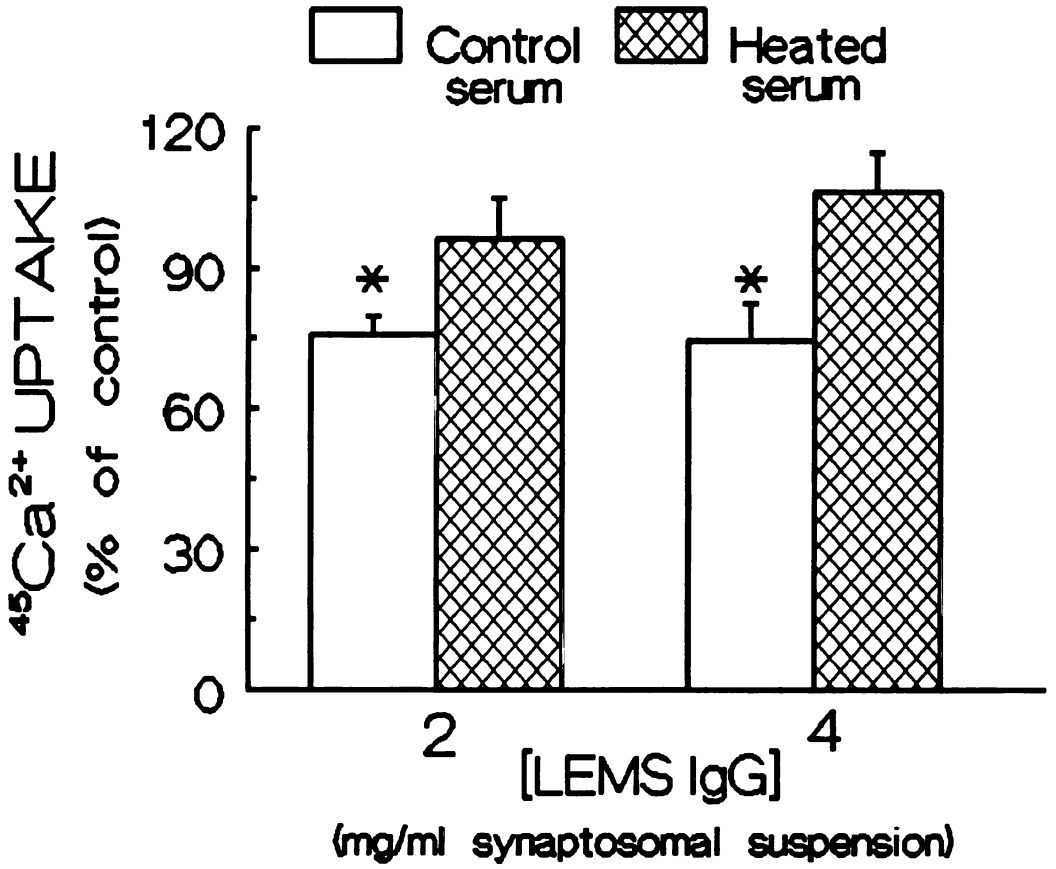


Figure 5.5. The effect of LEMS IgG in the presence of control or heat inactivated serum on uptake of $^{45}Ca^{2+}$ into rat synaptosomes. Synaptosomes were incubated for 60 min at 4°C with 2 & 4 mg/ml control or LEMS IgG and heat inactivated serum or control human serum. Prior to uptake, synaptosomal suspensions equilibrated for 30 min at 37°C. Synaptosomes were then incubated for one sec in a solution containing $^{45}Ca^{2+}$ and 42.5 mM KCl. Results are the mean ± the SEM of 8 experiments Asterisks indicate values that are significantly lower than their paired control, control being synaptosomes incubated with equivalent concentrations of control IgG ($p < 0.05$).

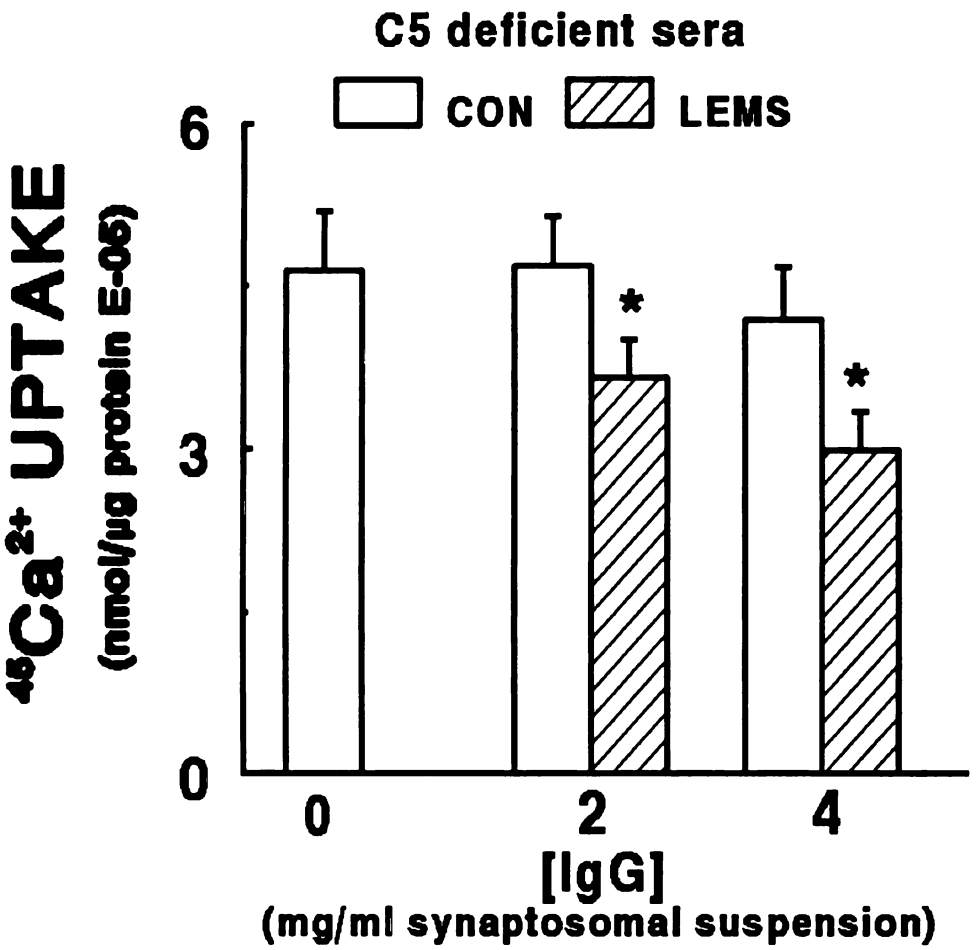


Figure 5.6. The effect of C5-deficient human serum on the LEMS IgG induced reduction of K^+-stimulated $^{45}Ca^{2+}$ uptake into rat synaptosomes. Synaptosomes were incubated with 2 and 4 mg/ml control and LEMS IgG in the presence of C5-deficient serum for 60 min at 4°C. Thirty min prior to the initiation of uptake, synaptosomes were allowed to equilibrate at 37°C. Uptake in 5 mM K^+ was subtracted from uptake in 42.5 mM K^+ as depolarization-independent background. Data are expressed as nmol $^{45}Ca^{2+}$ uptake/μg protein. Values are the mean ± SEM of 6 experiments. Asterisks indicate values that are significantly lower than their paired control, control being synaptosomes incubated with equivalent concentrations of control IgG ($p < 0.05$).

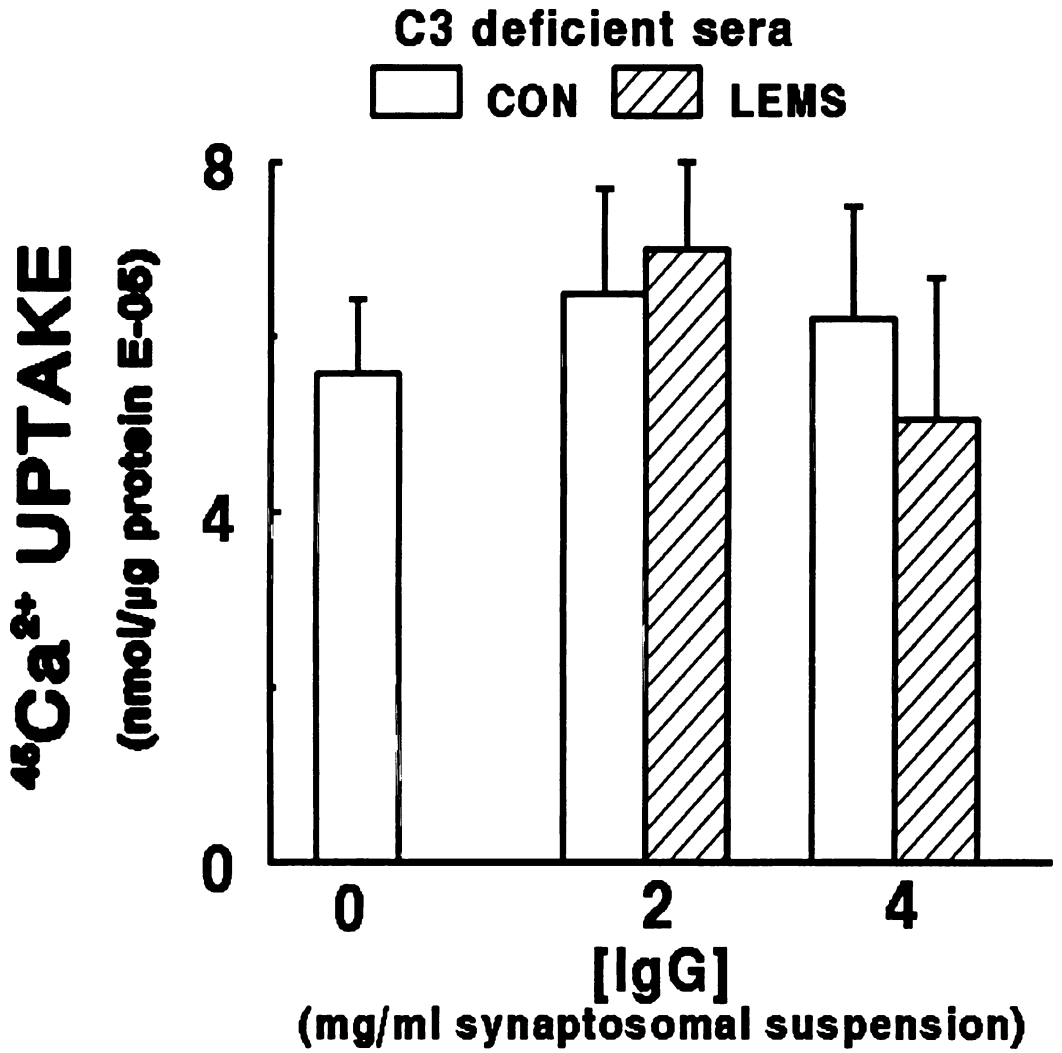


Figure 5.7. The effect of C3-deficient human serum on the LEMS IgG induced reduction in 1 sec K^+-stimulated $^{45}Ca^{2+}$ uptake into rat synaptosomes. Synaptosomes were incubated as described in Figure 5.6. Values are the mean ± SEM of 4 experiments.

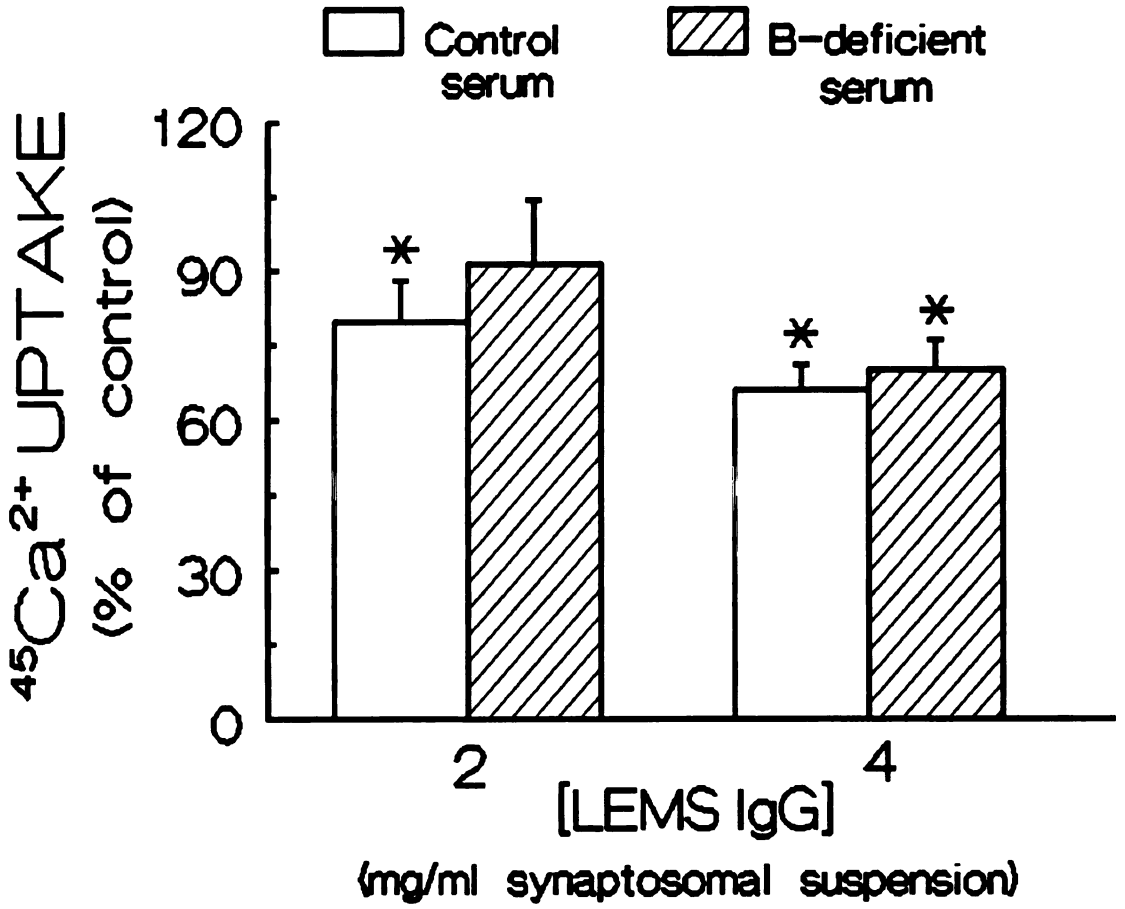


Figure 5.8. The effect of control and LEMS IgG in the presence of Factor B-deficient human serum on 1 sec K^+-stimulated $^{45}Ca^{2+}$ uptake into rat synaptosomes. Synaptosomes were incubated with 2 and 4 mg/ml control and LEMS IgG in the presence of control human serum or B-deficient serum as described in Figure 5.6. Values are the mean ± SEM of 5 experiments. Asterisks indicate values that are significantly lower than their paired control, control being synaptosomes incubated with equivalent concentrations of control IgG ($p < 0.05$).

control human serum, uptake was reduced 20.4 ± 8.4% and 34.1 ± 5.1% by 2 and 4 mg/ml LEMS IgG when compared to synaptosomes incubated equivalent concentrations of control IgG. Uptake in the presence of B-deficient human serum was inhibited 8.8 ± 13.0% and 29.9 ± 6.0% by 2 and 4 mg/ml LEMS IgG, when compared to the respective paired controls. At 4 mg/ml LEMS IgG this reduction was statistically significant.

E. *Discussion*

LEMS IgG in the presence of control human serum is able to reduce depolarization-dependent uptake of $^{45}Ca^{2+}$ into isolated central nerve terminals (Chapter Four). The aim of the present study was to assess the role of serum, in particular complement, in the pathogenesis of Ca^{2+} channel dysfunction in synaptosomes following incubation with LEMS IgG and human serum. Complement has been shown to be important in mediating other autoimmune, neuromuscular diseases (Engel *et al.*, 1987; Sahashi *et al.*, 1978; Toyka *et al.*, 1988).

In this study, the IgG from the LEMS patient that was studied in Chapter Four was used. The plasma preparation from this patient has been shown to transfer the electrophysiological features of LEMS to mice (Patient 3; Chapter Four), thus providing us with a positive functional control. IgG isolated from this patient only alters $^{45}Ca^{2+}$ flux into rat forebrain synaptosomes in the presence of control human serum. Incubation with IgG alone or heat-inactivating the serum abolishes this effect. The optimal concentration of serum appears to be 10% (v/v)

of the total incubation volume. At this concentration, serum alone caused no significant decreases in $^{45}Ca^{2+}$ uptake into synaptosomes. The MAC of complement does not appear to be involved in the reduction of uptake produced by LEMS IgG and serum since uptake is reduced when LEMS IgG is incubated with C5-deficient serum. Incubation of synaptosomes with 4 mg/ml LEMS and B-deficient serum showed similar reductions as those incubated with the same concentration of IgG but with control human serum. This suggests that at least at the higher concentration of IgG the alternative pathway does not appear to be involved. Our data suggests that it may play a role at lower IgG concentrations, however. In contrast, incubation of synaptosomes with C3-deficient serum and either 2 or 4 mg/ml LEMS IgG did not reduce K^+-stimulated $^{45}Ca^{2+}$ uptake.

LEMS IgG alone did not reduce Ca^{2+} flux into isolated nerve terminals following 90 min of incubation. Incubation of synaptosomes with LEMS IgG in the presence of control human serum, however, resulted in significant reductions in stimulated $^{45}Ca^{2+}$ uptake. In other studies, cells cultured with LEMS IgG for one to seven days reduced Ca^{2+} flux (Roberts *et al.*, 1985; Peers *et al.*, 1987). However the tissue culture media used in the above studies contained 10% (v/v) fetal calf or horse serum. As this is standard cell culture procedure, the importance of serum components with LEMS IgG may not have been apparent.

Serum alone decreases depolarization-dependent uptake of $^{45}Ca^{2+}$ in a concentration-dependent manner. This is likely due to increased binding of $^{45}Ca^{2+}$ to serum proteins thereby effectively decreasing the concentration of radiolabel

available for uptake, since equivalent concentrations of BSA alone reduce uptake. More notable is the observation that 10% (v/v) control human serum does not significantly alter uptake as compared to uptake measured in the absence of serum. This is important since 10% of the total incubation volume is the concentration of serum used in our experimental paradigm.

This concentration of serum appears to be optimal. It was the only serum concentration tested, which when combined with 2 mg/ml LEMS IgG resulted in reduced depolarization-dependent uptake of $^{45}Ca^{2+}$ into synaptosomes. There were no alterations in uptake between control and LEMS IgG with lower concentrations of serum. Higher concentrations of serum suppressed uptake to such an extent that any effects of the LEMS IgG on uptake may have been masked. Thus, the concentration of control serum should be 10% of the total incubation volume.

The reduction in uptake of $^{45}Ca^{2+}$ by LEMS IgG and serum was abolished by heat inactivating the serum. Heating serum will inactivate proteins or proteases present in the serum, such as complement, as C3, the pivotal component in both the classical and alternative pathway, is heat labile (Bokisch *et al.*, 1975; Stossel *et al.*, 1975).

Two of the most important components of complement are C3 and C5. The formation of the C3 convertase is the essential step of the complement reaction in generating activated C3 (C3b). C3b facilitates phagocytosis and generates C5 convertase (Hostteter *et al.*, 1984). It also functions as an important

intermediate in the activation of the alternative pathway (Gõetz, 1987). Cleavage of C5 by C5 convertases constitutes the final proteolytic reaction in both the classical and alternative pathway. Once present, C5b induces the assembly of the cytotoxic C5b-9 complex (MAC), which forms transmembrane channels that permit bidirectional flow of ions and ultimately macromolecules. This produces cellular injury and/or lysis (Bhakdi and Tranum-Jensen, 1978; Porter and Reid, 1978).

In the preceeding chapter, it was demonstrated that the decrease in $^{45}Ca^{2+}$ uptake by LEMS IgG was not a result of non-specific membrane damage. LDH release from synaptosomes incubated with LEMS IgG and control human serum was not increased over that of paired controls. Although membrane lysis did not occur, the present study sought to rule out the possibility that the MAC may cause discrete lesions that alter Ca^{2+} channel permeability (Bhakdi and Tranum-Jensen, 1978). Deficits in $^{45}Ca^{2+}$ uptake following incubation with LEMS IgG and C5-deficient serum indicate that the MAC does not play a role in the disruption of Ca^{2+} channel function in our system. This is consistent with results of Prior *et al.*, (1987) and Lambert and Lennon (1988) who demonstrated that the latter steps in the complement cascade (MAC) are not essential for the passive transfer of LEMS. Genetically C5-deficient mice are susceptible to the LEMS IgG as are mice with normal complement.

Incubation of LEMS IgG with C3-deficient serum prevented the reduction of synaptosomal $^{45}Ca^{2+}$ entry seen with control human serum. These findings are in contrast to those of Lambert and Lennon (1988) who report that the electro-

physiological characteristics of LEMS could be transferred passively to mice that had been depleted of C3 by cobra venom factor (CVF). Their results suggest that *in vivo* the effects of LEMS IgG may not be due to complement activation. This unaltered response in the absence of complement activity does not, however, mean that it is not involved when present. Many of the effects of IgG or alternative pathway activators on cells are greatly enhanced in the presence of complement (Rother, 1987). Furthermore, CVF interacts with Factor B of the APC to form a stable C3 convertase (Gõetz and Müller- Eberhard, 1971) thereby depleting C3 by continually activating it. In normal animals the C3 depletion induced by CVF is relatively short-lived (Klaus, 1987) and C3 depression must be maintained by repeated injections of CVF (Lennon and Lambert, 1988). Continual activation of C3 may result in binding of C3b to a non-specific acceptor, such as the plasma membrane (Law and Levine, 1977) or to the antigen-antibody complex itself (Capel *et al.*, 1978). Thus, there is still a possibility that activated C3 (C3b) may be important in mediating the LEMS pathogenesis *in vivo.* An alternative explanation, however, is that our *in vitro* model may not mimic precisely events occurring *in vivo*. As this is an artificial system, this possibility can not be excluded. Nonetheless, our results suggest a role for C3 in the action of LEMS IgG on synaptosomal Ca^{2+} channels and perhaps suggests that further experimentation is needed to clarify the participation of complement in the pathogenesis of LEMS.

The APC is a non-specific, natural defense system against pathogenic microorganisms (Joiner *et al.*, 1984; Hostteter *et al.*, 1984; Gõetz, 1987). In

addition, it is an amplification system for increasing C3b deposition and C3/C5 convertase activity after complement activation through the classical pathway (Hostetter *et al.*, 1984). Without Factor B, the APC can not function. The fact that a reduction in uptake was seen after 1 sec of K^+-induced depolarization following incubation of synaptosomes with 4 mg/ml LEMS IgG and Factor B-deficient serum suggests that at least at higher concentrations of LEMS IgG the APC is not contributing to the alterations in $^{45}Ca^{2+}$ uptake seen with the LEMS autoantibody and human serum. It may play a role at lower concentrations, however.

In summary, in synaptosomes, the presence of serum is important in mediating a functional change in Ca^{2+} channel activity by LEMS IgG. The optimal concentration of serum appears to be 10% (v/v) of the total incubation volume. This is a concentration of serum that alone does not reduce stimulated $^{45}Ca^{2+}$ uptake into synaptosomes, significantly. The MAC and APC do not appear to be involved. In contrast, the heat-labile, complement component, C3 is needed to disrupt $^{45}Ca^{2+}$ uptake into isolated nerve terminals following incubation of synaptosomes with LEMS IgG and control human serum. Formation of the MAC is not involved in the pathogenesis of LEMS *in vivo* (Prior *et al.*, 1987; Lennon and Lambert, 1988) or *in vitro* (present study). Enhanced phagocytosis of the antigen-antibody complex by macrophage infiltration cannot occur in our system. Perhaps, deposition of C3b ($\approx$ 180 kDa) adjacent to the autoantibody ($\approx$ 150 kDa) sterically hinders the gating process of Ca^{2+} channels.

CHAPTER SIX

PHARMACOLOGICAL DETERMINATION OF SYNAPTOSOMAL Ca^{2+} CHANNEL SUBTYPE(S) AFFECTED BY LAMBERT-EATON MYASTHENIC SYNDROME IMMUNOGLOBULIN

A. *Summary*

The data presented thus far support the hypothesis that a circulating autoantibody in the plasma of patients with LEMS interferes with nerve terminal Ca^{2+} channels. In the following study, the equilibrium binding of [^{3}H]-nitrendipine, [^{125}I]-ω-CgTx and [^{3}H]-verapamil was measured to determine whether binding sites reputed to be associated with pharmacologically identitified classes of Ca^{2+} channels are reduced or altered by acute exposure to LEMS IgG alone and/or LEMS IgG in the presence of serum. [^{3}H]-nitrendipine binds to synaptosomes with high affinity in both normal and elevated K^+ solutions (Shafer *et al.*, 1990). As no difference in the binding characteristics exists between the two solutions, all experiments were performed in non-depolarizing K^+ solutions. Scatchard analysis of binding of [^{3}H]-nitrendipine to synaptosomes in normal K^+ solution resulted in a ligand dissociation constant (K_d) value of 214.0 ± 84.0 pM and a maximum number of binding sites (B_{max}) value of 62.0 ± 12.0 fmol/mg protein. In the presence of 10% (v/v) control human serum, the values for K_d and B_{max} were 152.8 ± 48.0 pM and 62.5 ± 12.0 fmol/mg protein, respectively. Incubation of synaptosomes with control or LEMS IgG at 4 mg/ml prior to initiation of binding assays did not significantly alter binding of [^{3}H]-nitrendipine. Likewise, incubation with control or LEMS IgG in the presence of 10% (v/v) control human serum did not alter the binding characteristics of [^{3}H]-nitrendipine to synaptosomes. [^{125}I]-CgTx binds to a single high affinity binding site on synaptosomes, with half-saturation occurring in the mid-picomolar range. LEMS IgG at 4 mg/ml did not

alter the binding of [^{125}I]-CgTx as compared to synaptosomes incubated with an equivalent concentration of control IgG. The combination of LEMS IgG and control human serum failed to alter the binding characteristics as well. Finally, the binding characteristics of [^{3}H]-verapamil to synaptosomes were examined. LEMS IgG both in the presence and absence of control human serum, significantly decreased the maximum number of binding sites for verapamil by approximately 45%. The affinity of verapamil for the remaining receptors was not significantly altered, however. Thus, IgG from this patient with LEMS can interact with a Ca^{2+} channel binding site for verapamil but not for DHPs or CgTx.

B. ***Introduction***

The observation that multiple types of Ca^{2+} channels exists in nerve terminals (Penner and Dryer, 1986; Atchison and O'Leary, 1987; Miller, 1987) raises the question of the identity of the subtype of Ca^{2+} channels affected by LEMS IgG. Experimental evidence suggests that LEMS IgG inhibits Ca^{2+} entry into voltage-dependent Ca^{2+} channels from several sources, including motor nerve terminals (Lang *et al.*, 1987), SCC cells (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988*a*; Lang *et al.*, 1989*a*; 1989*b*), chromaffin cells (Kim and Neher, 1988), anterior pituitary cells (Login *et al.*, 1987), NG108-15 cells (Peers *et al.*, 1987), PC12 cells (Lang *et al.*, 1989*a*; 1989*b*) and synaptosomes (Chapters 4,5). Due to the inaccessibility of the putative target site of the LEMS IgG (motor nerve terminals), elucidation of the subtype(s) of Ca^{2+} channel affected by LEMS IgG has been

attempted in cell lines with the assumption that somal Ca^{2+} channels are similar to nerve terminal Ca^{2+} channels.

LEMS IgG was shown to reduce Ca^{2+} currents with the electrophysiological characteristics of L-type channels in NG108-15 and chromaffin cells. (Kim and Neher, 1988; Peers *et al.*, 1990). Data from studies using SCC cell lines (Roberts *et al.*, 1985; De Azipurua *et al.*, 1988*a*; 1988*b*) suggest an effect on both L- and N-type channels. Finally, LEMS autoantibodies have been shown to immunoprecipitate an [^{125}I]-CgTx labeled voltage-gated Ca^{2+} channel (N-type) from a human neuroblastoma cell line (Sher *et al.*, 1989).

The results from these studies imply that the L- and N- type Ca^{2+} channel are putative antigenic targets for the LEMS autoantibody. However, the types of Ca^{2+} channels that exist at the nerve terminal is unclear. In fact, experimental evidence suggests that Ca^{2+} channels at motor nerve terminals are neither N- nor L-, and are possibly of the P- (Anderson and Harvey, 1987; Sano *et al.*, 1987; Protti *et al.*, 1991; Uchitel *et al.*, 1992) or some as yet undetermined type.

Synaptosomes maintain the morphological and functional properties of intact nerve terminals and several pharmacological studies suggest that Ca^{2+} channels in the CNS resemble those in the periphery (Anderson and Harvey, 1987; Nachshen and Blaustein, 1979; Reynolds *et al.*, 1986; Suszkiw *et al.*, 1986;1989; Atchison, 1989; Protti *et al.*, 1991; Uchitel *et al.*, 1992). The DHP nitrendipine has been used in numerous binding and flux studies in synaptosomes to examine the properties of binding sites which are presumed to be associated with L-type Ca^{2+}

channels (Boles *et al.*, 1984; Greenberg *et al.*, 1984; Turner and Goldin, 1985; Wei and Chang, 1985; Carvalho *et al.*, 1986; Suszkiw *et al.*, 1986; Dunn, 1988). The phenylalkylamine, verapamil also appears to act as an L-type antagonist but has a separate binding site from that of the DHPs (Carvalho *et al.*, 1986; Miller, 1987; Glossmann and Striessnig, 1988). Finally, synaptosomes contain specific high-affinity receptors for the N-type channel antagonist, CgTx, as well (Abe *et al.*, 1986; Marqueze *et al.*, 1988; Wagner *et al.*, 1988; Scheer, 1990).

Thus, the goal of the present study was to determine whether binding sites reputed to be associated with Ca^{2+} channels are reduced or altered by acute exposure to LEMS IgG. The equilibrium binding of the Ca^{2+} channel antagonists [^{3}H]-nitrendipine, [^{125}I]-CgTx and [^{3}H]-verapamil to synaptosomal membranes following incubation with control and LEMS IgG, both in the presence and absence of serum was measured.

C. *Materials and Methods*

1. Materials

The radioisotopes [^{3}H]-nitrendipine (74 Ci/mmol), [^{3}H]-verapamil (66 Ci/mmol), and [^{125}I]-CgTx (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabelled CgTx was purchased from Penninsula Laboratories (Belmont, CA). Polyethylenimine, verapamil and nifedipine were purchased from Sigma Chemical Co. (St. Louis, MO). Disease-free control human plasma was purchased from the American Red Cross (Lansing, MI). Control human serum was donated by a healthy volunteer. Plasma from a patient with the

clinical and electromyographic features of LEMS was donated by Mark Glasberg, M.D., Henry Ford Hospital, Detroit, MI.

2. Immunoglobulin Isolation

Immunoglobulin from control and LEMS patients was isolated and quantitated as described in detail in Chapter Four. The LEMS IgG used in these studies was identical to that used in Chapter Four and Five as well.

3. Preparation of Synaptosomes

Synaptosomes were prepared according to a modification of the method of Gray and Whittaker (1962), as described in detail in Chapter Three. The final pellet was resuspended in physiologic saline buffer. Equal aliquots of synaptosomal suspension were incubated at 4°C under 100% O_2 for 60 min with either physiologic saline buffer, 10% (v/v) serum, 4 mg/ml control or LEMS IgG alone or IgG plus 10% (v/v) serum. The proper volume of physiologic saline buffer was added to maintain a constant incubation volume. Prior to the initiation of binding experiments, synaptosomes were allowed to equilibrate for 30 min at 37°C. Equilibrium binding assays were carried out at room temperature.

4. Solutions

Stock solutions of the radiolabels were made in non-depolarizing K^+ buffer. The incubations were terminated and the filters washed by the addition of cold quench solution. Contents of these solutions are described in Chapter Three.

5. Binding Experiments

Binding of [^{3}H]-nitrendipine was initiated by addition of 40 μl of synaptosomal suspension (200 -300 μg protein) to 400 μl of non-depolarizing K^+ buffer containing 10-1500 pmol of [^{3}H]-nitrendipine in ethanol (EtOH) for 60 min at 25°C in a room illuminated by a sodium lamp. The final concentration of EtOH was always less than 0.1%. Non-specific binding was measured in the presence of 5 μM unlabelled nifedipine. Incubation was terminated by addition of five ml of cold quench solution and rapid filtration through glass fiber filters (Whaman GF\B) that had been presoaked in quench solution containing 0.1% polyethylenimine. The filters were rinsed twice with five ml of cold quench solution. Scintillation cocktail was added 12 hr before the radioactivity trapped on the filters was estimated in a liquid scintillation counter having an approximate efficiency of 45% for ^{3}H. Protein content was determined by the method of Lowry *et al.*, (1951) using BSA as a standard. To determine total ligand concentration, a 40 μl aliquot was taken from the stock solution and the radioactivity was estimated. The average of triplicate values was used for Scatchard Analysis (Scatchard, 1949) to determine the equilibrium dissociation constant (K_d) for binding and the maximum density of binding sites (B_{max}).

Initiation of [^{125}I]-CgTx binding was essentially identical to that described above except that 40 μl of synaptosomal suspension was added to 400 μl of non-depolarizing K^+ buffer containing 30-480 pmol of [^{125}I]-CgTx in deionized H_20 and incubated for 60 min at 25°C. In addition, both the stock solutions and quench

buffer contained 0.5 mg/ml BSA to limit non-specific binding to of CgTx test tubes and filters. Non-specific binding was measured in the presence of 1 μM unlabelled CgTx. Radioactivity remaining on the filters was estimated using a gamma counter with an efficiency of 80% for ^{125}I. Since binding of CgTx is essentially irreversible, scatchard analysis of the data could not be performed. Thus, the average of duplicate values were used for estimation of B_{max} by saturation analysis.

[^{3}H]-verapamil binding was again essentially identical to the methods described for [^{3}H]-nitrendipine binding. Binding of [^{3}H]-verapamil was initiated by addition of 40 μl of synaptosomal suspension to 400 μl of non-depolarizing K^+ buffer containing 0.2-128 nmol of [^{3}H]-verapamil in EtOH. They were next incubated for 60 min at 25°C in a room illuminated by sodium lamp. The final concentration of EtOH was always less than 0.1%. Both the stock solutions and quench buffer contained 0.5 mg/ml BSA to limit non-specific binding of verapamil to test tubes and filters. Non-specific binding was measured in the presence of 25 mM unlabelled verapamil. Radioactivity trapped on the filters was estimated in a liquid scintillation counter having an approximate efficiency of 45% for ^{3}H. The average of duplicate values were used for Scatchard Analysis (Scatchard, 1949) to determine the equilibrium dissociation constant (K_d) for binding and the densitity of binding sites (B_{max}).

6. Statistical Analysis

Data from the equilibrium binding of [^{3}H]-nitrendipine and [^{3}H]-verapamil were analyzed by mixed design ANOVA followed by Student's *t*-test for paired

samples. Data from CgTx binding experiments were analyzed using a randomized complete block ANOVA. Differences were considered significant at $p < 0.05$.

D. *Results*

1. Nitrendipine Binding

A single high affinity binding site for [^{3}H]-nitrendipine (10-1500 pmol) was observed (Fig. 6.1). Non-specific binding accounted for approximately 25% of total binding. Scatchard analysis of the data (n = 3) yielded a K_d value of 214 ± 84 pM and an apparent B_{max} value of 62.0 ± 12.0 fmol/mg protein. Hill slope was approximately equal to one. These values are consistent with those reported by others for [^{3}H]-nitrendipine binding in synaptosomes (Boles *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986). Addition of control human serum (10% v/v) did not significantly alter the linearity of [^{3}H]-nitrendipine binding or the K_d and B_{max} values which were 153 ± 48 and 62.5 ± 12.0, respectively (Fig. 6.1).

Comparative effects of LEMS IgG and control IgG (4 mg/ml) on [^{3}H]-nitrendipine binding are depicted in Figure 6.2A. The calculated B_{max} and K_d values for binding of nitrendipine in the presence of control and LEMS IgG were not significantly different from one another, nor were they different from values obtained following incubation in buffer or serum alone (Table 6.1). Likewise, incubation of synaptosomes with LEMS IgG in the presence of 10% (v/v) control human serum did not alter either the K_d or B_{max} values for binidng of nitrendipine as compared to those observed using equivalent concentrations of control IgG and serum (Fig. 6.2B; Table 6.1).

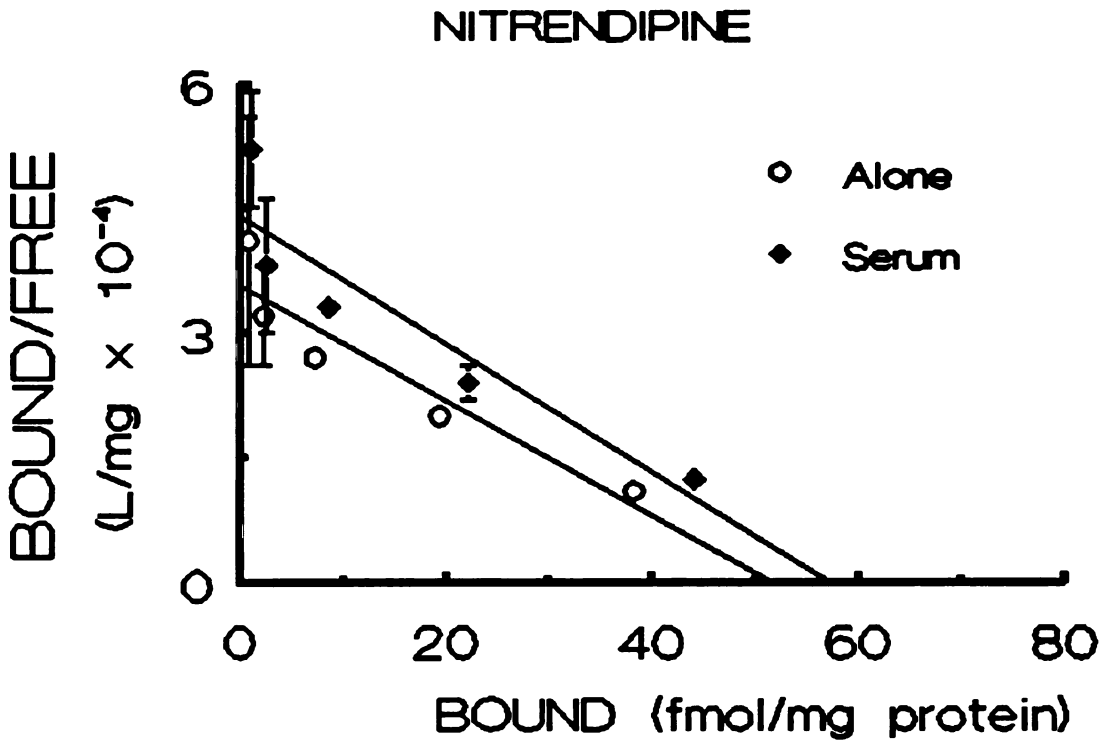


Figure 6.1. Effect of serum on nitendipine binding to synaptosomes. Specific binding of [^{3}H]-nitrendipine to synaptosomes in the presence and absence of control human serum (10%, v/v) in non-depolarizing K^+ buffer. Values are the mean ± SEM of three experiments. When SE bars are not shown the SE is smaller than the size of the symbol.

Table 6.1

[^{3}H]Nitrendipine Binding Characteristics

	B_{max}	K_d	n
Buffer	62.0 ± 12.0	214.1 ± 84.0	3
Serum alone	62.5 ± 12.0	152.8 ± 48.0	3
Control IgG	75.2 ± 10.2	236.0 ± 49.0	6
LEMS IgG	72.4 ± 18.5	289.6 ± 77.0	6
Control IgG + serum	75.1 ± 14.5	256.0 ± 76.4	6
LEMS IgG + serum	65.2 ± 9.9	190.6 ± 33.0	6

Equal aliquots of synaptosomal suspension were incubated at 4°C under 100% O_2 for 60 min with either physiologic saline buffer, 10% (v/v) serum, control or LEMS IgG alone (4 mg/ml) or IgG (4 mg/ml) plus 10% (v/v) serum. Prior to the initiation of binding experiments, synaptosomes were allowed to equilibrate for 30 min at 37°C. Equilibrium binding assays were carried out at room temperature. Synaptosomes were added to non-depolarizing K^+ buffer containing 10-1500 pmol of [^{3}H]-nitrendipine for 60 min at 25°C. Incubation was terminated by addition of five ml of cold quench solution followed by suction-filtration. The average of triplicate values was used for Scatchard Analysis to determine the equilibrium dissociation constant (K_d; pM) for binding and the maximum density of binding sites. (B_{max}; fmol/mg protein).

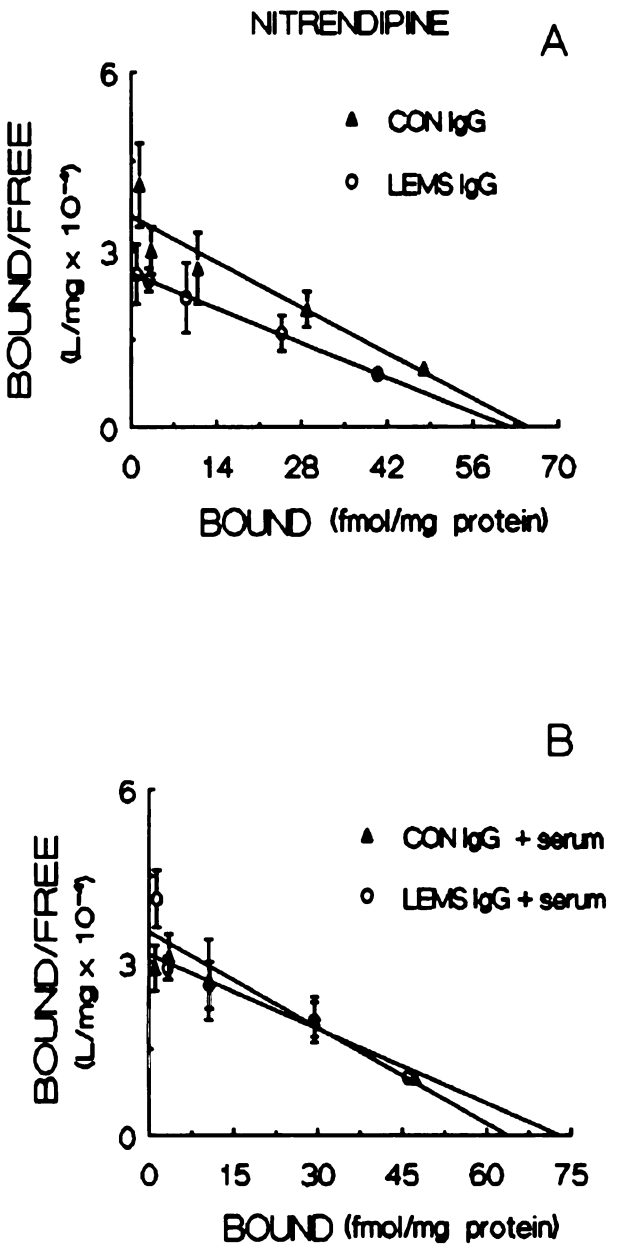


Figure 6.2. Comparative effects of Control and LEMS IgG in the absence (A) and presence (B) of control human serum on nitrendipine binding to synaptosomes. Values are the mean ± SEM of 6 individual experiments.

2. ω-Conotoxin Binding

The specific binding of [^{125}I]-CgTx to synaptosomes in the presence of control and LEMS IgG (4 mg/ml) is depicted in Figure 6.3A. Non-specific binding accounted for less than 10% of total binding. CgTx bound to synaptosomes with a half-saturation of between 0.1 to 0.2 nM. Since binding of CgTx is essentially irreversible, the maximum density of binding sites was estimated by extrapolation of the saturated component to the Y-axis. B_{max} values for CgTx binding in synaptosomes treated with control or LEMS IgG were 260.6 ± 6.7 and 259.2 ± 1.0. These values are consistent with those reported by others (Cruz and Olivera, 1986; Wagner *et al.*,1988; Marqueze *et al.*, 1988). Almost identical binding curves were obtained when synaptosomes were incubated with control or LEMS IgG (4 mg/ml) in the presence of 10% (v/v) control human serum prior to initiation of CgTx binding (Fig. 6.3B).

3. Verapamil Binding

Over a concentration range of 0.2-128 nmol of [^{3}H]-verapamil, a single high-affinity binding site was observed when synaptosomes were incubated with any IgG or IgG plus serum (Fig. 6.4A,B). Non-specific binding accounted for 40-50 % of total binding. Scatchard analysis of the data derived from incubation of synaptosomes with control IgG alone (4 mg/ml; n=3) yielded a K_d value of 19.9 ± 7.6 nM and an apparent B_{max} of 106.1 ± 12.2 fmol/mg of protein. Following incubation with LEMS IgG, the value for K_d was unchanged at 19.9 ± 8.7 nM, but

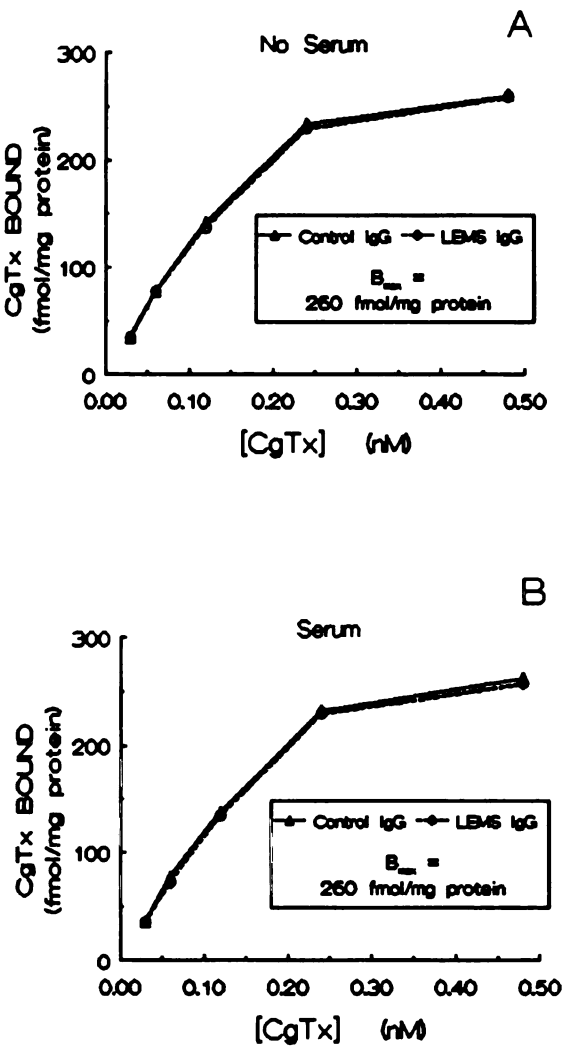


Figure 6.3. Comparative effects of control and LEMS IgG on CgTx binding to rat forebrain synaptosomes. Equal aliquots of synaptosomes were incubated with control or LEMS IgG (4 mg/ml) alone (A) or control and LEMS IgG (4 mg/ml) in the presence of 10% (v/v) human serum (B) 90 min prior to initiation of binding. Specific binding of [^{125}I]-CgTx was determined as described in methods. Values shown are the mean ± SEM of three experiments. When SE bars are not shown the SE is smaller than the size of the symbol.

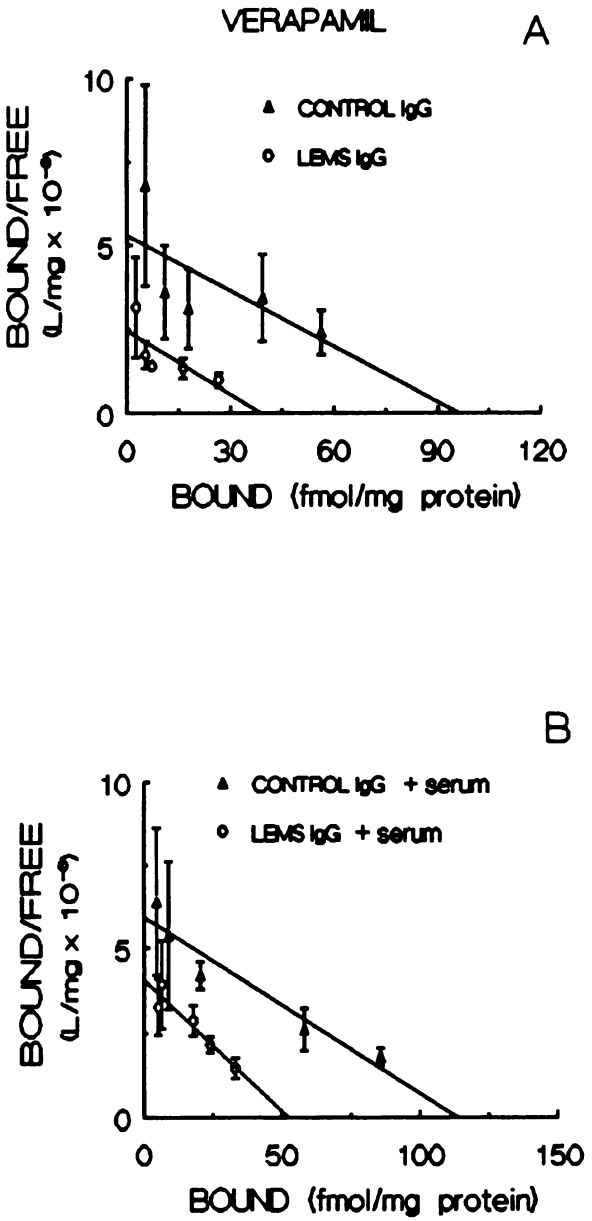


Figure 6.4. Comparative effects of control and LEMS IgG on verapamil binding to synaptosomes. Equal aliquots of synaptosomes were incubated with control or LEMS IgG (4 mg/ml) alone (A) or control and LEMS IgG (4 mg/ml) in the presence of 10% (v/v) human serum (B) 90 min prior to initiation of binding. Specific binding of [^{3}H]-verapamil was determined as described in methods. Values shown are the mean ± SEM of six experiments. When SE bars are not shown the SE is smaller than the size of the symbol.

the B_{max} was decreased significantly to 48.8 ± 14.0 fmol/mg protein (n = 4) (Fig. 6.4A). Similar results were obtained following incubation of synptosomes with control and LEMS IgG in the presence of control human serum (Fig. 6.4B). The K_d values for binding of [^{3}H]-verapamil in the presence of control and LEMS IgG plus serum were 17.1 ± 4.2 and 14.6 ± 9.1 nM respectively. The B_{max} value was reduced from 94.7 ± 15.0 to 37.3 ± 3.8 fmol/mg of protein (n = 4) by incubation with LEMS IgG and serum as compared to incubation with control IgG plus serum (Fig. 6.4B).

E. *Discussion*

The aim of the present study was to to determine whether pharmacologically distinct binding sites reputed to be associated with Ca^{2+} channels are reduced in number or altered in their affinity for antagonist by acute exposure to LEMS IgG. Specifically, we sought to determine if the equilibrium binding of the Ca^{2+} channel antagonists [^{3}H]-nitrendipine, [^{125}I]-ω-CgTx and [^{3}H]-verapamil to synaptosomal membranes was altered following incubation with control and LEMS IgG. These studies were performed both in the presence and absence of control human serum because functional alterations in uptake of $^{45}Ca^{2+}$ into synaptosomes by LEMS IgG are seen only in the presence of serum (Chapter Five). It could be that the activated C3 component of complement alters the binding of Ca^{2+} channel antagonists alone or in conjunction with the LEMS IgG.

Our results indicate that IgG from a patient with LEMS does not alter the equilibrium binding characteristics of [^{3}H]-nitrendipine nor [^{125}I]-CgTx either in the

presence or absence of control human serum. Conversely, LEMS IgG both in the presence and absence of control human serum significantly decreased the B_{max} for [^{3}H]-verapamil while having no effect on its K_d. These results indicate that LEMS IgG alone interacts with a binding site on synaptosomal membranes that is specific for verapamil.

Synaptosomes are likely to contain more than one subtype of Ca^{2+} channel but the types present have yet to be identified clearly. Synaptosomal Ca^{2+} influx is blocked by the phenylalkylamine verapamil ($IC_{50} \approx 20\ \mu M$) (Nachshen and Blaustein, 1979; Norris *et al.*,1983) suggesting that L-type channels may be involved in Ca^{2+} influx. However, DHPs such as nitrendipine, which are also L-type antagonists, do not have consistent effects on $^{45}Ca^{2+}$ influx into synaptosomal preparations. Various investigators report direct block of synaptosomal K^+-stimulated Ca^{2+} influx and neurotransmitter release by nifedipine, nimodipine and nitrendipine (Turner and Goldin, 1985; White and Bradford, 1986; Dunn, 1988). Still, others report no effect of DHPs on K^+-stimulated Ca^{2+} uptake into synaptosomes (Nachshen and Blaustein, 1979; Daniell *et al.*, 1983; Wei and Chang, 1985; Suszkiw *et al.*, 1986; Reynolds *et al.*, 1986; Vickroy *et al.*, 1992) even though a high affinity binding site for DHPs exists on synaptosomes (Boles *et al.*, 1984; Greenberg *et al.*, 1984; Turner and Goldin, 1985; Wei and Chang, 1985; Carvalho *et al.*, 1986; Suszkiw *et al.*, 1986; Dunn, 1988). In either case, the concentration of DHP required to block $^{45}Ca^{2+}$ influx into synaptosomes is much greater than the concentrations which bind specifically to synaptosomes.

Previously, LEMS IgG was shown to reduce Ca^{2+} currents with the electrophysiological and pharmacological characteristics of L-type channels in undifferentiated NG108-15 cells (Peers *et al.*, 1990). L-type channels contributing to Ca^{2+} currents in adrenal medullary chromaffin cells were reduced by LEMS IgG as well (Kim and Neher, 1988). In the present study, [^{3}H]-nitrendipine bound to a single class of high affinity sites in rat forebrain synaptosomes. The values for K_d and B_{max} correlate well with those observed by others (Boles *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986). The affinity of binding as well as the maximum number of binding sites were unaltered by prior incubation of synaptosomes with LEMS IgG in the presence or absence of serum. If L-type channels do exist in isolated nerve terminals, this would suggest that they are not altered at the DHP binding site by LEMS IgG nor an activated serum component. The possibility that the antibody binds to an alternate site on the L-type channel can not be excluded. These results do correlate with the fact that L-type channels are not normally involved in transmitter release at motor nerve terminals, however (Atchison, 1989).

Synaptosomes contain specific high-affinity receptors for the N-type channel antagonist, CgTx, as well (Abe *et al.*, 1986; Marqueze *et al.*, 1988; Wagner *et al.*, 1988; Scheer, 1990). Rapid $^{45}Ca^{2+}$ flux measurements into rat brain synaptosomes demonstrates partial antagonism ($\approx$20-40%) of voltage-dependent Ca^{2+} channels by CgTx (Reynolds *et al.*, 1986; Suszkiw *et al.*, 1987; Woodward *et al.*, 1988*b*; Suszkiw *et al.*, 1989) suggesting N-type channels may mediate a proportion of

Ca^{2+} uptake and neurotransmitter release from synaptosomes.

In the present study, CgTx bound to a single class of high affinity sites on rat forebrain synaptosomes as did nitrendipine. Like nitrendipine, CgTx binding was unaltered by incubation of synaptosomes with LEMS IgG in the presence or absence of serum. This suggests that N-type channels in isolated nerve terminals are not altered at the CgTx binding site by LEMS IgG nor an activated serum component either. This is in agreement with the work of Sher and colleaques (1989) who demonstrated that LEMS IgG was unable to directly inhibit the binding of [^{125}I]-CgTx to neuroblastoma cell membranes. LEMS IgG was able to immunoprecipitate voltage-dependent Ca^{2+} channels prepared from the same human neuroblastoma cell line which were prelabeled with radiolabeled CgTx, however (Sher *et al.*, 1989). Therefore, the antibody must recognize antigenic determinants on the N-type Ca^{2+} channel in the cell line which are separate from the site occupied by the toxin. This possibility in synaptosomes can not be excluded. However, it should be noted that, in either case, release of ACh from mammalian motor nerve terminals is unaltered by CgTx (Anderson and Harvey, 1987; Protti *et al.*, 1991).

Finally, synaptosomal Ca^{2+} influx is blocked by the phenylalkylamine verapamil (Nachshen and Blaustein, 1979; Norris *et al.*,1983). Likewise, single-channel recordings of synaptosomal Ca^{2+} channels in lipid bilayers confirm that verapamil is a potent blocker of synaptosomal Ca^{2+} channels (Nelson, 1985). A verapamil-sensitive Ca^{2+} current exists at mouse motor nerve terminals as well

(Penner and Dryer, 1986; Anderson and Harvey, 1987).

The B_{max} for verapamil binding to synaptosomal membranes was decreased significantly by prior incubation of synaptosomes with LEMS IgG both in the presence and absence of serum as compared to synaptosomes incubated with equivalent concentrations of control IgG with and without serum. The K_d was unchanged. Verapamil is presumed to act as an L-type antagonist but it is believed to have a separate binding site from that of the DHPs (Carvalho *et al.*, 1986; Miller, 1987; Glossmann and Striessnig, 1988). More recent evidence suggests that verapamil may not be as selective for L-type channels as was recently thought. In cerebellar granule cells (Carboni and Wojcik, 1988) and neocortical neurons (Mangano *et al.*, 1991) in primary culture, Ca^{2+} influx resulting from cell depolarization is only partially inhibited by nitrendipine. Verapamil, in contrast, completely blocked the depolarization-induced influx suggesting that verapamil either blocks all subtypes of voltage-sensitive Ca^{2+} channels or blocks the activity of a novel Ca^{2+} channel (Mangano *et al.*,1991). In any case, these results indicate that LEMS IgG interacts with a verapamil-binding site on rat brain synaptosomes. Further experiments will be required to determine whether this binding site represents an L-type channel only, all subtypes of channels, or a novel voltage-dependent Ca^{2+} channel.

Caution, however must be taken when interpreting these data since the correlation of DHP, CgTx or verapamil binding sites correspond to functional Ca^{2+} channels is unclear. For each compound, the concentration required to block

$^{45}Ca^{2+}$ influx into synaptosomes is in excess of that which specifically binds to synaptosomal membranes. Although the pharmacological binding potencies of Ca^{2+} channel antagonists do not necessarily correlate with their functional effects, binding assays nonetheless provide a measure of the ability of LEMS IgG to compete with known Ca^{2+} channel antagonists for their binding sites on nerve terminal membranes. The results from this study demonstrate that IgG from a patient with LEMS does not alter the equilibrium binding characteristics of [^{3}H]-nitrendipine nor [^{125}I]-CgTx but does decrease significantly the B_{max} of [^{3}H]-verapamil binding. Interaction with the verapamil binding site takes place both in the presence and absence of serum suggesting that the IgG molecule itself interacts at this site. Interaction of LEMS IgG at this site may be necessary but is not sufficient to alter synaptosomal Ca^{2+} channel function as reductions of $^{45}Ca^{2+}$ uptake are evident only in the presence of both the IgG and the serum (Chapter 5).

F. *Note*

ω-Agatoxin and FTX, are potent blockers of K^{+}-induced $^{45}Ca^{2+}$ uptake into rat brain synaptosomes suggesting that a P-type channel may also mediate neurotransmitter release from these terminals (Mintz *et al.*, 1992; Uchitel *et al.*, 1992). As Agatoxin and FTX are not available commercially and attempts to procure it elsewhere were unsuccessful, it was not possible in this dissertation to examine whether LEMS IgG interacted with a binding site specific for either one of the toxins.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

Summary and Conclusions

The overall aim of this dissertation was to test the hypothesis that a circulating autoantibody in the plasma of patients with the autoimmune, neuromuscular disorder Lambert-Eaton Myasthenic Syndrome interferes with nerve terminal Ca^{2+} channels associated with neurotransmitter release. The reduction in nerve-evoked release of transmitter at the NMJ of patients with LEMS is thought to be caused by a circulating autoantibody to Ca^{2+} channels of presynaptic motor nerve terminals. The small size and close anatomical association of motor nerve terminals with muscle fibers restrict direct measurements of Ca^{2+} channel function in intact NMJ preparations. In fact, data from such experiments represent indirect measurements of Ca^{2+} channel activity as the postsynaptic response measured results from many processes ongoing in the presynaptic nerve terminal. Therefore, studies in this dissertation were performed on isolated nerve terminals derived from rat brain. Although LEMS patients do not normally exhibit CNS symptomology, it was our contention that if the LEMS autoantibody could gain access to the calcium channels of the CNS by incubation *in vitro*, it might be possible to induce a passive transfer of the nerve terminal dysfunction to central neurons.

Specfically, the ability of serum, plasma and IgG from patients with LEMS to interact with synaptosomal Ca^{2+} channels was examined. In most of the studies, radiotracer flux measurements using $^{45}Ca^{2+}$ were used to determine Ca^{2+} channel activity. In addition, studies were performed to assess the ion channel specificity

of the LEMS immunoglobulin as well as to determine the mechanism by which this antibody may block or alter the function of synaptosomal Ca^{2+} channels. The final studies in this dissertation attempted to characterize pharmacologically the subtype(s) of Ca^{2+} channel in synaptosomes that is affected by LEMS IgG.

Preliminary experiments characterized the ability of serum and plasma from several patients with LEMS to block Ca^{2+} influx into isolated nerve terminals. Incubation of rat cortical synaptosomes with LEMS serum or plasma reduced depolarization-induced uptake of $^{45}Ca^{2+}$ as compared to uptake in synaptosomes that had been treated with control serum or plasma. These deficits were not due to a decrease in synaptosomal viability as lactate dehydrogenase release from synaptosomes was not increased over that released by control sera/plasma (Chapter Three). Lactate dehydrogenase is a cytosolic enzyme that can be released into the extrasynaptosomal fluid if the membrane is permeabilized. As LDH is rather large, discrete changes in the synaptosomal membrane would not be detected by this assay.

As an IgG autoantibody is presumed to mediate the pathogenesis of LEMS, the effects of isolated IgG derived from the plasma of a single patient with LEMS on synaptosomal Ca^{2+} channels, in particular, and synaptosomal ion channels, in general, were assessed. Although a stronger conclusion could be drawn after studying multiple patient sera, we feel that the examination of one patient allows for a more detailed, in depth, investigation. Furthermore, there is precedence in the literature for detailed analysis of one or two patients.

Incubation of synaptosomes with IgG from a patient with LEMS in the presence of control human serum resulted in a reduction in one sec depolarization-dependent uptake of $^{45}Ca^{2+}$. This phase of influx is mediated by voltage-dependent Ca^{2+} channels (Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1983) and has been consistently correlated with neurotransmitter release (Drapeau and Blaustein, 1983; Floor, 1983; Suszkiw and O'Leary, 1983; Nachshen, 1985; Leslie *et al.*, 1985). The antibody appeared specific for Ca^{2+} channels as it was unable to disrupt $^{22}Na^{+}$ uptake into or $^{86}Rb^{+}$ efflux from synaptosomal Na^{+} or K^{+} channels, respectively. These deficits, again, were not due to gross alterations of synaptosomal membrane integrity, as assessed by LDH release, or to alterations in synaptosomal membrane potential, as measured by potentiometric, carbocyanine dye fluorescence, but appeared to result from a direct block of or alteration in Ca^{2+} channel function (Chapter Four).

In synaptosomes, the presence of serum is important in mediating a functional change in Ca^{2+} channel activity by LEMS IgG. In the absence of human serum, LEMS IgG did not reduce uptake of $^{45}Ca^{2+}$ into synaptosomes. Heat-inactivating the serum abolished this effect as well. This suggested a role for complement in the pathogenesis of synaptosomal Ca^{2+} channel dysfunction by LEMS IgG. Neither the membrane attack complex nor the alternative pathway of complement contributed to disruption of Ca^{2+} channel function by LEMS IgG in synaptosomes. In contrast, the C3 component of the complement pathway appears to be important in mediating Ca^{2+} channel dysfunction in synaptosomes

following incubation with serum and LEMS IgG (Chapter 5).

The data presented support the hypothesis that a circulating autoantibody in the plasma of patients with LEMS interferes with nerve terminal Ca^{2+} channels. This raised the question of the identity of the subtype(s) of Ca^{2+} channels affected by LEMS IgG. To date, there are three well characterized Ca^{2+} channel types in DRG neurons: N, L,and T (Nowycky *et al.*, 1985*a*; Tsien, 1988); and one in mammalian Purkinje cells: P (Llinas *et al.*, 1989). The N- and L-type channels have been proposed to regulate neurotransmitter release from a variety of central, peripheral and clonal cell lines while the P-type channel has been implicated in release of transmitter at the squid giant synapse, mammalian neuromuscular junction and in synaptosomes. The results from several studies using primary cell or clonal cell cultures suggest that the L- and N-type Ca^{2+} channel are putative antigenic targets for the LEMS autoantibody (Roberts *et al.*, 1985; Login *et al.*, 1987; Kim and Neher, 1988; De Azipurua *et al.*, 1988*a*,*b*; Sher *et al.*, 1989; Peers *et al.* 1990). However, the type(s) of Ca^{2+} channel affected by LEMS IgG in the motor nerve terminal is unclear. The goal of the final study in this dissertation was to determine whether pharmacological binding sites reputed to be associated with Ca^{2+} channels in a nerve terminal preparation were reduced in number or altered in affinity following acute exposure to LEMS IgG. IgG from a patient with LEMS does not alter the equilibrium binding characteristics of the L-type antagonist, [^{3}H]-nitrendipine nor the N-type antagonist, [^{125}I]-CgTx but does decrease significantly the maximum number of binding sites of [^{3}H]-verapamil binding. LEMS IgG

reduced verapamil binding both in the presence and absence of serum suggesting the IgG molecule itself interacts at this site. Interaction of LEMS IgG at this site may be necessary for alterations of Ca^{2+} channel activity but is not sufficient as reductions in $^{45}Ca^{2+}$ uptake are evident only in the presence of both IgG and serum.

The studies presented in this dissertation were the first to examine specifically the ability of a circulating humoral factor in serum from patients with LEMS to interact with nerve terminal Ca^{2+} channels. A thorough characterization of the autoantibodies effects at the nerve terminal has strengthened the hypothesis that Ca^{2+} channels are the antigenic target of LEMS IgG. In addition, results described provide new insight into the mechanism of Ca^{2+} channel dysfunction by LEMS IgG showing that complement may indeed be important. Furthermore, the type(s) of nerve terminal Ca^{2+} channel(s) affected by LEMS IgG have been explored.

The ability of sera/plasma and IgG derived from LEMS patients to block Ca^{2+} influx into a hetereogeneous preparation may represent a common action of the LEMS autoantibody at all nerve terminals. This may be an unexpected benefit of these studies as it suggests that Ca^{2+} channels of nerve terminals secreting different transmitters may share common epitopes recognized by the LEMS autoantibody and perhaps share the same type(s) of Ca^{2+} channel reponsible for neurotransmitter release. Antigenic similarities between peripheral and central nerve terminal Ca^{2+} channels must exist as an autoantibody whose primary target is the

somatic motor nerve terminal Ca^{2+} channel can interact and alter activity of central nerve terminal Ca^{2+} channels as well.

The preferential action of the LEMS autoantibody for nerve terminal Ca^{2+} channels as opposed to Na^{+} or K^{+} channels suggests that there is no common determinant or epitope on different voltage-gated channel proteins to which the antibody can bind. However, the possibility that some patients with LEMS may synthesize antibodies with different specificities can not be excluded. These findings are important as the existence of autoantibodies which are specific for Ca^{2+} channels involved in the neurosecretory process may serve as molecular tools for studying the steps involved in quantal transmitter release both in the peripheral and central nervous system.

The complement component C3 appears to be necessary for mediating the functional alteration in Ca^{2+} channel activity following incubation of synaptosomes with LEMS IgG and control human serum. This finding is in contrast to that of Lambert and Lennon (1988) who reported that the electrophysiological characteristics of LEMS could be transferred passively to mice that had been depleted of C3 by cobra venom factor (CVF). It is also inconsistent with the work of Lang and colleagues (1987) who found that quantal content of the EPPs was reduced in a murine nerve-muscle preparation following chronic injection of LEMS $F(ab)'_2$ fragments. $F(ab)'_2$ fragments lack the F_c portion but retain the binding capacity of the full immunoglobulin molecule. The F_c portion of immunoglobulin is important in activation of the complement cascade, however.

There can be several possible explanations for this discrepancy. First, our *in vitro* model may not mimic precisely events occurring *in vivo*. As this is an artificial system, this possibility can not be excluded. Next, the possibility that activated C3 (C3b) may be important in mediating the LEMS pathogenesis *in vivo* may exist. C3 depletion induced by CVF in normal animals is relatively short-lived (Klaus, 1987) and C3 depression must be maintained by repeated injections of CVF (Lennon and Lambert, 1988). Continual activation of C3 may result in binding of C3b to a non-specific acceptor, such as the plasma membrane (Law and Levine, 1977) or to the antigen-antibody complex itself (Capel *et al.*, 1978). Finally, the characteristic electrophysiological abnormalities produced by IgG and $F(ab)'_2$ fragments or the reduction in $^{45}Ca^{2+}$ uptake into synaptosomes following incubation with IgG and serum may be patient specific. In each study, the IgG or the $F(ab)'_2$ fragments of IgG from a single patient with LEMS was examined. It may be that like the triggering factor for LEMS, the mechanism of Ca^{2+} channel dysfunction differs between patients. Complement, like small cell carcinoma of the lung, may be important in mediating the pathogensis of LEMS in some patients but not in others.

The density of binding sites for [^{3}H]-verapamil but not [^{3}H]-nitrendipine nor [^{125}I]-ω-conotoxin was reduced by approximately 45% following incubation with LEMS IgG alone or LEMS IgG with serum. The affinity of verapamil for the remaining receptors was not significantly altered, however. The reduction of verapamil binding corresponds nicely with the reduction of depolarization-induced

$^{45}Ca^{2+}$ uptake observed following incubation of synaptosomes with LEMS IgG and serum (Chapter 4,5).

Verapamil is presumed to act as an L-type antagonist with a separate binding site from that of the DHPs (Carvalho *et al.*, 1986; Miller, 1987; Glossmann and Striessnig, 1988). Although verapamil functions as a potent selective inhibitor of Ca^{2+} channels in cardiac, smooth and skeletal muscle, it is capable of blocking Na^{+} channels as well as Ca^{2+} channels in neuronal preparations (Nachshen and Blaustein, 1979; Norris, 1983; Carvalho *et al.*, 1986). However, the concentration of verapamil needed to block active Na^{+} channels (50 to 100 μM) is greater than that needed to block Ca^{2+} channels selectively (1 to 20 μM) (Nachshen and Blaustein, 1979; Norris *et al.*, 1983; Nelson, 1985). Furthermore, the concentration range (0.2-128 nM) used in chapter 6 for binding of [^{3}H]-verapamil is consistent with that of Carvalho *et al.*,(1986) demonstrating specific, saturable, high affinity binding to Ca^{2+} channels in synaptosomal membranes.

Recent evidence suggests that verapamil may not be as selective for just L-type Ca^{2+} channels as was recently thought (Carboni and Wojcik, 1988; Mangano *et al.*, 1991). In cerebellar granule cells (Carboni and Wojcik, 1988) and neocortical neurons (Mangano *et al.*, 1991) in primary culture, Ca^{2+} influx resulting from cell depolarization is only partially inhibited by nitrendipine but completely blocked by verapamil suggesting that verapamil may block all subtypes of voltage-sensitive Ca^{2+} channels or may block the activity of a novel Ca^{2+} channel. Further experiments will be required to determine whether this binding site represents an

L-type channel only, all subtypes of channels, or a novel voltage-dependent Ca^{2+} channel.

It is important to characterize all the effects of LEMS IgG and try to synthesize a sequence of events that contribute to their pathogenesis. The results described in this dissertation along with the data which preceded this work indicate that LEMS autoantibodies are potentially myasthenogenic if they can fix complement (Chapter 5), cross-link neighboring Ca^{2+} channels (Lang *et al.*,1987; Nagel *et al.*, 1988), or interfere with Ca^{2+} channels opening or Ca^{2+} fluxes (Kim and Neher, 1988; Hewett and Atchison, 1991; Chapter 4,5). Reduction of the density of functional channels in the presynaptic membrane of nerve terminals appears to be the ultimate cause of neuromuscular transmission failure in LEMS (Fukunaga *et al.*, 1982; 1983; Nagel *et al.*, 1988).

Further progress in clarifying the molecular mechanisms of LEMS will come by elucidating the autoimmune mechanism at the molecular level. This will involve the identification of the voltage-dependent Ca^{2+} channel subunit(s) that are the targets for the autoantibody and the mapping of B and T cell epitopes with recombinant and synthetic peptides as soon as the human cDNA's are available. Detailed knowledge of the amino acid sequences that form these epitopes may eventually allow for the development of specific immunotherapy.

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