EFFECTS OF THE NEUROTOXICANT METHYLMERCURY ON NEURONAL Ca^{2+} CHANNELS: EFFECTS ON Ca^{2+} CHANNELS INVOLVED IN TRANSMITTER RELEASE

Volume I

By

Timothy Joseph Shafer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology
and Institute for Environmental Toxicology

1991

ABSTRACT

EFFECTS OF THE NEUROTOXICANT METHYLMERCURY
ON NEURONAL Ca^{2+} CHANNELS:
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The ability of the environmental neurotoxicant methylmercury (MeHg) to disrupt neuronal Ca^{2+} channel function was examined. In rat brain synaptosomes, MeHg (25-125 μM) blocked depolarization-induced $^{45}Ca^{2+}$ influx. Increasing the concentration of extracellular Ca^{2+} ($[Ca^{2+}]_e$) only slightly antagonized block by MeHg of the Ca^{2+} channel-mediated component of ^{45}Ca influx, whereas block of the Na^+/Ca^{2+} exchange-mediated component of influx was relieved. MeHg also disrupted Ca^{2+} channel function by altering ionic selectivity and inactivation kinetics of synaptosomal Ca^{2+} channels. Effects of MeHg did not depend on the state of synaptosomal Ca^{2+} channels but were exacerbated by increasing membrane depolarization. MeHg also inhibited [^{3}H]-nitrendipine binding to synaptosomal preparations. MeHg blocked depolarization induced $^{45}Ca^{2+}$ influx into differentiated and undifferentiated rat pheochromocytoma (PC12) cells, and inhibited binding of the Ca^{2+} channel ligand ω-conotoxin (CgTx) to its receptors in this cell line. Studies of effects of MeHg on Ca channel function in PC12 cells using the whole-cell patch voltage clamp technique demonstrated that 5-20 μM MeHg reduced rapidly Ba^{2+} current mediated by N- and L-type Ca^{2+} channels in PC12 cells. Increasing $[Ba^{2+}]_e$ antagonized block by MeHg between 10 and 20 mM Ba^{2+} but not between 20 and 30 mM Ba^{2+}. MeHg

disrupted Ca^{2+} channel function in a manner which was voltage- but not state-dependent. Ionic selectivity and inactivation of whole cell current were altered by MeHg. Effects of MeHg on nerve terminal Ca^{2+} channels in intact nerve muscle preparations were examined by measuring effects of MeHg on Ba^{2+}-dependent voltage changes in the perineurial sheath of mouse *triangularis sterni* preparations. 50-100 μM MeHg blocked the Ba^{2+}-dependent potential as well as the Na^{+}-dependent component. These results suggest that MeHg: 1) blocks Ca^{2+} channels in synaptosomal preparations and intact motor nerve terminals, 2) disrupts function of N- and L-type Ca channels in PC12 cells, 3) disrupts Ca^{2+} channel ionic selectivity and inactivation, and 4) disrupts Ca channel function in a manner which is voltage-, but not state-dependent in synaptosomes and PC12 cells. These actions may contribute to the ability of MeHg to disrupt synaptic transmission as well as other neurotoxic effects of MeHg.

For Kathryn, Rosie and Loren

ACKNOWLEDGEMENTS

I would like to thank my parents, family and friends for their love, support and encouragement. Also, I would like to thank my wife Jenny for her love, patience and help. These people have contributed to this dissertation in ways that cannot be measured.

I would like to thank my advisor, Dr. William Atchison, for his genuine interest in my graduate training. He has provided significant ideas, suggestions and criticisms which shaped this dissertation and has been an excellent teacher of science, laboratory techniques and writing skills. In addition, I would like to express my sincere thanks to my guidance committee for their input, ideas and suggestions, as well as their willingness to share their time to serve on the committee.

The time and effort expended by student workers, technicians and other graduate students to help with experiments is appreciated. The excellent technical assistance from Ms. Lynne Adgate and Aizhen Yao and Drs. Paul Levesque and John Spitsbergen is especially appreciated. Finally, I would like to thank Ms. Diane Hummell and Mrs. Gretchen Humphries for their excellent clerical assistance.

PREFACE

Some of the data contained in this dissertation has been published previously. Chapter Three appeared as Shafer and Atchison (1989), Chapter Four as Shafer *et al.* (1990) and Chapter Five as Shafer and Atchison (1991).

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LIST OF ABBREVIATIONS

ACh - acetylcholine

ANOVA - analysis of variance

ATP - adenosine 5'-triphosphate (disodium salt)

$[Ba^{2+}]_e$ - extracellular Ba^{2+} concentration

B_{max} - density of binding sites for a ligand

CAT - choline acetyltransferase

cAMP - cyclic adenosine monophosphate

$[Ca^{2+}]_e$ - sum of the extracellular concentration of all calcium isotopes

$[Ca^{2+}]_i$ - intracellular Ca^{2+} concentration

CgTx - ω-conotoxin GVIA

DA - dopamine

DEX - dexamethasone

DHP - dihydropyridine

DRG - dorsal root ganglion

EGF - epidermal growth factor

EGTA - ethyleneglycol bis-(ß-aminoethyl ether)-N,N,N',N',-tetraacetic acid

EPP - endplate potential

EtOH - ethanol

FGF - fibroblast growth factor

FTX - synthethic funnel-web spider toxin

GTP - guanosine triphosphate

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

I_A - whole cell current through the inactivating K^+ channel

I_{Ba} - whole cell current mediated by barium

I_{Ca} - whole cell current mediated by calcium

I_K - whole cell current mediated by the delayed rectifier K^+ channel

I_{Na} - whole cell current mediated by sodium

IC_{50} - concentration of chemical that causes 50% inhibition of the maximal response of the system

K_D - equilibrium dissociation constant for ligand binding

lsd - least significant difference

MEPP - miniature endplate potential

MeHg - methylmercury

ms - milliseconds

$[Na^+]_e$ - extracellular concentration of Na^+

NGF - nerve growth factor

NMJ - neuromuscular junction

pA - picoamperes

PC12 cells - pheochromocytoma cells

PKA - protein kinase A

PKC - protein kinase C

PI - phosphoinositide

SEM - standard error of the mean

TEACl - tetraethylammonium chloride

TTX - tetrodotoxin

CHAPTER ONE

INTRODUCTION

A) GENERAL INTRODUCTION

Methylmercury (MeHg) is a neurotoxic heavy metal found as a contaminant throughout the environment, but especially in aquatic ecosystems. The organic component of this molecule imparts to MeHg the ability to bioconcentrate in aquatic organisms and biomagnify within the food chain. Consumption of contaminated seafood has resulted in numerous cases of MeHg-intoxication in human populations. In addition, agricultural use of MeHg has resulted in human intoxication from accidental consumption of MeHg-treated grain. The neurotoxic potential of MeHg was dramatically illustrated by chronic and acute episodes of human exposure in Japan (Takeuchi *et al.*, 1962; 1968) and Iraq (Bakir *et al.*, 1973), respectively. The characteristic clinical symptoms of MeHg-intoxication observed included cerebellar ataxia, speech and visual impairments, and, in the Iraqi episode, weakness of the extremities (Chang, 1980). Central and peripheral lesions of the nervous system have been observed in cases of MeHg intoxication (Chang and Hartmann, 1972a,b), but the cellular mechanisms responsible for these lesions are unknown.

In individuals from the Iraqi episode, neostigmine alleviated clinically observable symptoms of motor weakness produced by MeHg (Rustam *et al.*, 1975), indicating that MeHg possibly disrupts cholinergic transmission at the motor end-plate. This prompted further *in vitro* study of the effects of MeHg on neuromuscular transmission. In isolated nerve-muscle preparations, MeHg exerts two prominent presynaptic actions: 1) block of nerve-evoked neurotransmitter release, and 2)

increased frequency, followed by complete block of spontaneous neurotransmitter release (Barrett *et al.*, 1974; Juang, 1976; Atchison and Narahashi, 1982).

Release of neurotransmitter following invasion of the action potential into the nerve terminal is dependent on influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels in the membrane (Katz and Miledi, 1967a,b). Several divalent heavy metal cations which produce effects on evoked and spontaneous transmitter release similar to those of MeHg block the movement of Ca^{2+} through these channels. Traxinger and Atchison (1987b) demonstrated that, under certain conditions, effects of MeHg on nerve-evoked transmitter release are relieved by increasing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$). MeHg also blocks depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes. Effects of MeHg on $^{45}Ca^{2+}$ influx are also partially antagonized by increasing $[Ca^{2+}]_e$ (Atchison *et al.*, 1986). These results indicate that the ability of MeHg to disrupt synaptic transmission at the neuromuscular junction may, in part, be due to a disruption of Ca^{2+}-dependent events at the motor nerve terminal. This dissertation reports results of experiments designed to examine the following hypothesis: disruption of voltage-sensitive Ca^{2+} channel function by MeHg contributes to block of synaptic transmission observed in neuromuscular preparations. The emphasis of studies undertaken was to characterize effects of MeHg on Ca^{2+} channels, determine types of Ca^{2+} channels affected by MeHg, and examine the ability of MeHg to affect Ca^{2+} channels in intact motor nerve terminals.

Entry of Ca^{2+} into nerve terminals at the neuromuscular junction is difficult to measure directly. Thus, effects of MeHg on channel-mediated $^{45}Ca^{2+}$ influx into

synaptosomes (nerve terminals isolated from the central nervous system) were examined in initial experiments. In addition, non-channel mediated entry of $^{45}Ca^{2+}$ was also examined as a measure of the specificity of MeHg for Ca^{2+} channel function. MeHg readily blocks the channel-mediated components of influx in a manner which is not completely antagonized by increasing $[Ca^{2+}]_e$. MeHg also affects non-channel-mediated components of influx, including Na^+/Ca^{2+} exchange and depolarization-independent entry of Ca^{2+}. For components of Ca^{2+} influx not mediated by Ca^{2+} channels, the ability of increasing $[Ca^{2+}]_e$ to antagonize the actions of MeHg and/or the concentrations at which MeHg effectively blocks entry are different than for effects on voltage-sensitive Ca^{2+} channels by MeHg (Shafer and Atchison, 1989). Furthermore, the ability of $Ca^{2+}{}_e$ to antagonize the block of channel-mediated $^{45}Ca^{2+}$ influx by MeHg is decreased when compared to block by divalent cations.

When compared to the actions of inorganic Ca^{2+} channel blockers, among the unique effects of MeHg on Ca^{2+} channel function in synaptosomes are alteration of the apparent rate of inactivation of synaptosomal Ca^{2+} channels as well as the ionic permeability of Ca^{2+}, Sr^{2+} and Ba^{2+} through the Ca^{2+} channel. In addition, the effects of MeHg do not depend directly on the state of the channel, but block of $^{45}Ca^{2+}$ influx is increased in magnitude as a function of the magnitude of the depolarizing stimulus (Shafer *et al.*, 1990).

Because effects of MeHg are expressed in the central as well as the peripheral nervous system, the use of nerve terminal preparations derived from mammalian

central neurons provides meaningful and relevant data about effects of MeHg. Continued studies in central nervous system preparations in the future will be necessary to understand completely the mechanisms of MeHg's neurotoxic effects. Because synaptosomal preparations are heterogeneous with respect to the neurotransmitter content of individual terminals within the preparation, the ability of MeHg to block Ca^{2+} uptake into synaptosomes may represent a common action of MeHg at all types of terminals, and because Ca^{2+} entry is required for neurotransmitter release, the ability of MeHg to disrupt this process may be a component underlying its neurotoxicity. However, while these experiments demonstrate that MeHg is capable of producing unique alterations of Ca^{2+} channel function in a population of isolated nerve terminals, they provide only preliminary evidence to support the stated hypothesis. The purpose of studies performed in the latter portion of this dissertation was to collect data which would provide stronger evidence for or against the hypothesis.

Transmitter release from central and peripheral neurons is likely to be mediated at least in part by N- and/or L-type Ca^{2+} channels (Fox *et al.*, 1987a,b) or as yet uncharacterized Ca^{2+} channel types which inactivate slowly (Smith and Augustine, 1988; Suszkiw *et al.*, 1989). The hypothesis predicts that MeHg should be capable of blocking one or both of these types of channels if block of Ca^{2+} channels by MeHg contributes to the disruption of synaptic transmission. Therefore, the ability of MeHg to interact with N- and/or L-type Ca^{2+} channels was determined using whole-cell patch voltage-clamp techniques to examine effects of MeHg on Ca^{2+} (or

Ba^{2+}) current in rat pheochromocytoma (PC12) cells. PC12 cells express both N- and L-type Ca^{2+} channels when cultured in the presence of nerve growth factor. The use of patch voltage-clamp techniques allows unambiguous identification of Ca^{2+} channel types as well as the ability to measure channel function with temporat resolution that approaches physiological rates of channel function. However, this method of examining Ca^{2+} channel function requires use of supraphysiological concentrations of Ca^{2+} or other permeant ions such as Ba^{2+} or Sr^{2+}. If MeHg is able to block one or both of the aforementioned types of Ca^{2+} channel, this would be strong evidence in favor of the hypothesis.

The hypothesis was also examined by measuring directly Ca^{2+} channel function at the terminal regions of motor neurons. Whole cell patch-voltage clamp recording, while allowing for unambiguous determination of Ca^{2+} channel types, nonetheless measures function of predominantly somatic rather than terminal currents. By precisely placing electrodes in the perineurial sheath near the motor nerve terminal, one can record voltage changes within the perineurial sheath in response to movement of Ba^{2+} through Ca^{2+} channels in the nerve terminal (Penner and Dreyer, 1986). Measurement of effects of MeHg on Ca^{2+}-dependent voltage waveforms from the perineurial sheath will provide the most direct assessment of the ability of MeHg to alter Ca^{2+} channel function in intact motor nerve terminals.

MeHg is a highly reactive compound, especially with sulfhydryl groups. As such, MeHg binds to a large variety of proteins and disrupts a large variety of cellular processes, including channel function, mitochondrial function (Levesque and

Atchison, 1987; 1988; 1991), microtubule structure (Abe *et al.*, 1975; Sager *et al.*, 1983) and protein synthesis (Yoshino *et al.*, 1966). Furthermore, exposures to MeHg which produce significant pathological changes are also likely to alter numerous cellular functions. Thus, it is important to characterize all events which might contribute to compromised cellular function in the presence of MeHg rather than attempt to define a single critical event which results in altered cellular function (Reuhl, 1987). The plasma membrane, along with its resident channels, receptors and transporter proteins is the initial site of interaction for all foreign and endogenous compounds with the cell (*See* Narahashi, 1980) and plays a vital role in cellular homeostasis. In the nervous system, the proper functioning of Na^+ and K^+ channels is crucial for propagation of action potentials, and Ca^{2+} channel function is critical to the process of neurotransmitter release. By characterizing the ability of MeHg to disrupt Ca^{2+} channel function, the effects at this initial site of interaction with the cell will be better understood. The significance of this information may exceed possible effects on neurotransmitter release, as Ca^{2+} channels are involved in cellular Ca^{2+} homeostasis; alterations of Ca^{2+} homeostasis may therefore contribute to disruption of cellular function by MeHg.

B) BACKGROUND

1) Methylmercury in humans and the environment.

Chronic and acute episodes of human intoxication with MeHg have occurred previously. In the mid- to late-1950's, ingestion of MeHg-contaminated shellfish from

Minamata Bay in Japan led to 1500 cases of intoxication and 46 deaths (Takeuchi *et al.*, 1962, 1968). The cause of contamination was due to discharge of organic and inorganic mercurial waste into the bay from a chemical plant producing acetaldehyde from acetylene. Mercury metal used as a catalyst reacted with the acetylene, ultimately forming MeHg as a contaminant. In Iraq, the use of MeHg-treated seed grain for bread (despite government warnings of adverse health effects) resulted in 450 deaths due to acute MeHg poisoning in 1972 (Bakir, *et al.*, 1973). Thus, environmental contamination with MeHg, as well as any significant exposure to MeHg, represent legitimate human health concerns. MeHg still remains a considerable human health hazard, and certian populations, particularly those which consume large omounts of fish, remain at risk (Hansen, 1990).

There are two main sources of environmental contamination by MeHg: MeHg itself, and elemental Hg which is converted to MeHg. Because of its antifungal properties, MeHg has been employed by the agricultural, lumber, paper and leather industries, primarily as a preservative. Consequently, it is found as a contaminant in seed grain dressings, agricultural runoff, and waste-water discharges from these industries. Elemental Hg has been used by the electrical apparatus, instrumentation, paint and chloralkali industries, and is released into the environment *via* atmospheric venting or discharge along with waste-water. Mercury is also released into the environment during the combustion of fossil fuels. In the aquatic environment, microorganisms can methylate inorganic mercury (Wood *et al.*, 1968; Ridley *et al.*, 1977), thus increasing the pool of MeHg in the environment.

The conversion of Hg to MeHg in the environment has important toxicological implications. Prior to knowledge of this conversion, inorganic Hg discharged into the environment was considered to be relatively harmless to humans as it is poorly absorbed by the gastrointestinal tract. By contrast, the more lipophilic MeHg tends to bioaccumulate and biomagnify in the food chain, and up to 95% is absorbed by the gastrointestinal tract.

MeHg is transported in the plasma and red blood cells and distributes evenly to most tissues, but concentrates in the blood and central nervous system. In rats chronically exposed to MeHg, the spinal dorsal root ganglia accumulated the highest levels of MeHg, while the cerebral cortex and cerebellum also contained high levels of MeHg (Somjen *et al.*, 1973). The blood-brain barrier is easily crossed and also damaged by MeHg (Steinwall and Klatzo, 1966; Steinwall and Olsson, 1969; Chang and Hartmann 1972c). Pathological examination of victims of MeHg toxicity in Minamata indicated that MeHg lesions the calcarine (visual) cortex, as well as cerebellar granule cells and the dorsal root ganglia (Chang, 1977; 1979). Histochemical analysis has shown that MeHg is closely associated with the mitochondria, golgi, endoplasmic reticulum, and the nuclear envelope. In nerves, MeHg is found primarily in myelin sheaths and mitochondria, and causes axonal degeneration (Chang, 1977). The high affinity of MeHg for membranous structures in the neuron may contribute to the toxicity of this compound by disrupting important cellular functions which take place at these sites.

MeHg is slowly excreted; its half-life in humans is approximately 70 days. MeHg slowly decomposes to inorganic Hg, is completely reabsorbed from the bile, and has a low rate of urinary excretion compared to elemental Hg (Magos, 1975). Thus, frequent consumption of MeHg-contaminated food or water can result in significant accumulation of MeHg in the body. Consumption of contaminated seafood has resulted in the accumulation of toxic doses of MeHg in Japanese (Takeuchi *et al.*, 1962; 1968) and Swedish (Berglund *et al.*, 1971) populations. Such episodes have resulted in concern regarding the safety of fish from the Great Lakes. However, mercury levels in fish in the Great Lakes have declined since 1976, and the average Hg concentration in fish in each of the five Great Lakes is below the action level established by the Food and Drug Administration (1.0 mg/kg) and the Michigan Department of Public Health (0.5 mg/kg) (D'Itri, 1985).

2) Effects of MeHg on Synaptic Transmission.

The observation that neostigmine alleviated symptoms of motor weakness spurred research into the mechanism underlying this action of MeHg. Neuromuscular preparations such as the rat phrenic nerve-hemidiaphram preparation have served as useful model systems for study of synaptic transmission between nerve and muscle. The neuromuscular junction (NMJ) has been well characterized with respect to its structure, function and pharmacological response to various drugs and toxins. In addition, the synthesis, storage and Ca^{2+}-dependent release of acetylcholine (ACh) at the NMJ are similar in many respects to the synthesis, storage and release of other

neurotransmitters in central and peripheral neurons. As MeHg not only affects neurons which release ACh, but also those neurons which release catecholamines, serotonin, GABA, glutamate and glycine (Borowitz, 1974; Nakazato, *et al.*, 1979; Bondy, *et al.*, 1979; Tuomisto and Komulainen, 1983), information obtained using this preparation may also provide preliminary indications as to how MeHg affects other neurons as well.

Following acute bath application to isolated nerve-muscle preparations, MeHg causes a rapid block of nerve-evoked transmitter release and an increase in the frequency, followed by a complete block, of spontaneous neurotransmitter release (Barrett *et al.*, 1974; Juang, 1976; Atchison and Narahashi, 1982; Miyamato,1983; Atchison *et al.*, 1984; Atchison, 1986; Levesque and Atchison 1987; 1988). At the neuromuscular junction, these effects are observed electrophysiologically as alterations of the amplitude of end plate potentials measured in the muscle cell in response to nerve-evoked (EPP) or spontaneous (MEPP) neurotransmitter release. Block of the EPP by 100 μM MeHg occurs rapidly and with no apparent gradual reduction; after 8-20 min the EPP is completely abolished (Atchison and Narahashi, 1982; Atchison *et al.*, 1984; Traxinger and Atchison, 1987b). By contrast, changes in MEPP frequency occur after a latent period, which can be shortened without increasing the peak MEPP frequency observed by increasing the concentration of MeHg (Atchison and Narahashi, 1982) or by K^+-induced depolarization (Atchison, 1986). The ultimate block of transmitter release produced by MeHg is not due to depletion of ACh stores; treatment with La^{3+} after complete block of MEPPs by

MeHg results in their restoration (Atchison, 1986; Levesque and Atchison, 1988), indicating that the nerve terminal has not been depleted of ACh-containing vesicles.

Although post-synaptic actions of MeHg have not yet been carefully examined, several observations indicate that the block of EPP and increase in MEPP frequency caused by MeHg are the result of actions of MeHg on the presynaptic terminal. The experimental evidence supporting a presynaptic action of MeHg include the following observations: 1) the response of the endplate to iontophoretically applied ACh is unchanged even after 1 hr of exposure to 100 μM MeHg (Atchison and Narahashi, 1982). At this time, neither EPPs nor MEPPs are observed. 2) MEPP amplitude is not affected at the time of complete EPP block by MeHg (Atchison and Narahashi, 1982). These results indicate that the postsynaptic cell still responds to ACh at a time when synaptic transmission is impaired by MeHg. In addition to this experimental evidence, changes in MEPP frequency are thought to be due to presynaptic and not postsynaptic events.

Not only do the actions of MeHg appear to be presynaptic, but the differences in the time course of MeHg's effects on EPPs and MEPPs suggest different mechanisms of action by MeHg on each of these forms of release. Indeed, the mechanisms underlying the EPP and MEPP themselves are different. The MEPP is a depolarization of the end-plate membrane resulting from release of one quantum of neurotransmitter and subsequent interaction with receptors located on the postsynaptic membrane (Fatt and Katz, 1952). Although MEPPs are affected by extracellular Ca^{2+}, spontaneous release can occur in Ca^{2+}-free solutions. The

frequency of MEPP occurence reflects the intraterminal [Ca^{2+}] (Hubbard *et al.*, 1968). Thus, it has been proposed that the actions of MeHg on MEPP frequency are due to a disruption of intraterminal Ca^{2+} buffering organelles, such as the smooth endoplasmic reticulum and mitochondria, leading to an increase in $[Ca^{2+}]_i$ (Atchison *et al.*, 1984; Atchison 1986; Levesque and Atchison, 1987; 1988; 1991). The EPP, however, is the postsynaptic response resulting from synchronous release of many quanta of neurotransmitter following the depolarization of the nerve terminal by an action potential and subsequent entry of extracellular Ca^{2+} (Katz and Miledi, 1967a,b; Llinas *et al.*, 1981). Under physiological conditions, EPPs require $Ca^{2+}{}_e$. The rapid and complete block of EPPs by MeHg indicates that MeHg may block conduction of the action potential, electrotonic spread of depolarization into the terminal and/or entry of Ca^{2+} into the nerve terminal.

Initially, MeHg-induced block of evoked release at the NMJ is not reversible simply by methods designed to overcome a competitive block of divalent cation influx by MeHg. Block of the EPP by MeHg is not reversed by perfusing the preparation with MeHg-free solutions containing normal or elevated [Ca^{2+}] or with MeHg-free solutions containing agents (4-aminopyridine) which prolong Ca^{2+} entry into the nerve terminal. However, increasing the intensity or duration of the stimulus in MeHg-free solutions does partially and occasionally reverse MeHg-induced block of evoked release (Traxinger and Atchison, 1987b) (Figure 1.1). This indicates that MeHg may affect impulse conduction at either the axonal membrane or the unmyelinated portion of the nerve terminal. Previous studies have indicated that

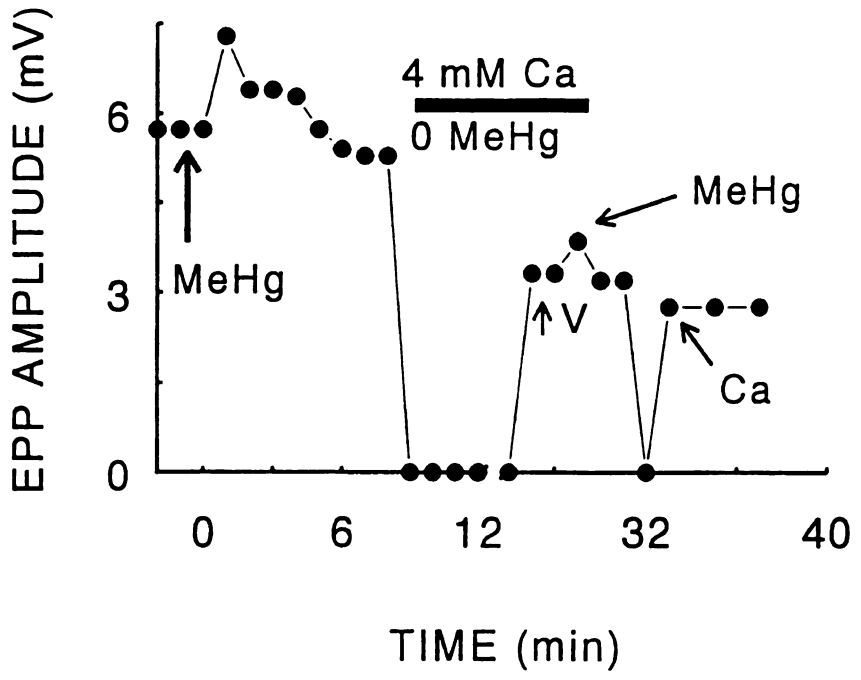

Figure 1.1 Effects of stimulus intensity and Ca^{2+}_e on EPP amplitude in the presence of MeHg. EPPs were evoked in rat hemidiaphram by stimulation of the phrenic nerve at 0.25 Hz throughout the experiment. MeHg was applied continuously for 9 min by which time the EPP was blocked. Immediately following block, the preparation was perfused with a MeHg-free Ca^{2+} ($[Ca^{2+}]$ = 4 mM) solution for 15 min. During this time, EPPs remained blocked. While maintaining the preparation in 4 mM Ca^{2+} solution, the stimulus intensity was increased (↑ V); this brought about an immediate return of EPPs. Subsequent re-exposure of the preparation to MeHg in normal (2 mM) Ca^{2+} solutions caused EPPs to be abolished within 3 min. Wash of the preparation at this time with MeHg-free, 4 mM Ca^{2+} solution caused a prompt recovery of the EPP. All values are from a single representitive preparation.

Modified from: Traxinger and Atchison, *Toxicol. Appl. Pharmacol.* 90:23-33 (1987b).

MeHg may interact with Na^+ channels or sulfhydryl groups in the membrane which are important in action potential propagation (Shrivistav, *et al.*, 1976; Quandt *et al.*, 1982). An increase in the duration or intensity of stimulation would be expected to overcome a MeHg-induced decrease in excitability in these regions (Konishi, 1985). Under conditions of increased stimulus intensity, re-exposure of the preparation to MeHg resulted in a re-occurrence of block of EPPs. This subsequent block of EPPs could be reversed by increasing $[Ca^{2+}]_e$ (Traxinger and Atchison, 1987b). Thus, block of EPPs by MeHg may in part be due to block by MeHg of Ca^{2+} entry associated with neurotransmitter release. This hypothesis is strengthened by the observation that MeHg blocks depolarization-induced $^{45}Ca^{2+}$ influx into rat forebrain synaptosomes, and that the block of influx by MeHg over 10 s is antagonized, although not completely overcome by increasing $[Ca^{2+}]_e$ (Atchison *et al.*, 1986).

In addition to experimental evidence suggesting that MeHg may block Ca^{2+} entry associated with neurotransmitter release, one may suspect, based on its similarities in physico-chemical properties (i.e., relatively similar ionic radius, positive charge, ability to gain or lose electrons) (Miller, 1984) with other heavy and/or transition metals, that MeHg could exert this action. Other heavy metals such as Ni^{2+} (Kita and Van der Kloot, 1973), Cd^{2+} (Forshaw, 1977; Cooper and Manalis 1983) and Pb^{2+} (Kostial and Vouk, 1957; Manalis and Cooper, 1973; Silbergeld *et al.*, 1974a,b; Atchison and Narahashi, 1984; Cooper and Manalis, 1983; Büsselberg *et al.*,1990), block evoked transmitter release in a fashion similar to MeHg. However, in contrast to the effects of MeHg, block of synaptic transmission at the NMJ by Pb^{2+}

(Kober and Cooper, 1976; Cooper and Manalis, 1983; Atchison and Narahashi, 1984; Pickett and Bornstein, 1978; 1984), Cd^{2+} (Nilson and Volle, 1976; Cooper and Manalis, 1984a,b), or Co^{2+} (Weakly, 1973) is reversed by simply increasing $[Ca^{2+}]_e$ or washing with normal, metal-free solutions. In synaptosomes, block of $^{45}Ca^{2+}$ influx by divalent cations such as Pb^{2+}, Ni^{2+}, Cd^{2+} and Hg^{2+} (Nachshen, 1984; Atchison *et al.*, 1986), but not MeHg (Atchison *et al.*, 1986; Shafer and Atchison, 1989), is completely reversed by increasing $[Ca^{2+}]_e$ concentration. As MeHg has enhanced lipophilicity and a monovalent charge, differences in charge and lipophilicity may underlie the differences in the action of MeHg and inorganic, divalent cations on nerve terminal Ca^{2+} fluxes.

3) Synaptic Transmission

Successful transmission of neuronal signals, whether between nerve and muscle, post-synaptic nerve or other cell type, requires a complex sequence of events. Disruption of any one event, such as block of Na^+ channels by tetrodotoxin (Narahashi *et al.*, 1964) or inhibition of acetylcholinesterase by organophosphate insecticides, can lead to deleterious consequences. Many neurotoxic compounds (including heavy metals) disrupt processes at the synapse. Thus, it is conceivable that MeHg may also act at this site.

Transmission at peripheral and central chemical synapses involves the conversion of an electrical signal into a chemical signal in the presynaptic cell, and then, depending on the postsynaptic cell, into an electrical or biochemical (i.e., 2nd

messenger) signal. Although the complete sequence of events resulting in neurotransmitter release is not yet known, several processes which take place prior to release have been identified. Action potentials propagated along the nerve axon by movement of Na^+ and K^+ into and out of the axon through their respective channels (Hodgkin and Huxley, 1952) cause depolarization of the non-myelinated terminal membrane by electrotonic spread into the terminal region (Brigant and Mallart, 1982; Mallart, 1985). This depolarization causes voltage-sensitive Ca^{2+} channels in the nerve terminal membrane to open, allowing extracellular Ca^{2+} to move down its electrochemical gradient into the nerve terminal (Katz and Miledi, 1967a,b; Llinas *et al.*, 1981). It is thought that these channels, whose activation is crucial for evoked release, are clustered in areas in which vesicles containing neurotransmitter are arranged in close apposition to the cell membrane (Pumplin *et al.*, 1981; Robitaille *et al.*, 1990; Cohen *et al.*, 1991). Influx of Ca^{2+} in these areas, or "active zones", gives rise to locally high $[Ca^{2+}]_i$, resulting in increased interaction of Ca^{2+} ions with Ca^{2+}-binding proteins. This interaction between Ca^{2+} and binding proteins is thought to trigger directly or indirectly the fusion of synaptic vesicles with the cell membrane, resulting in the release of chemical neurotransmitters into the synaptic cleft (Augustine *et al.*, 1987). The protein or proteins to which Ca^{2+} binds have yet to be identified, although synapsin I, synexin or other members of the calelectrin family and p38-synaptophysin have been suggested as the macromolecule(s) responsible for coupling Ca^{2+} entry to neurotransmitter release (Smith and Augustine, 1988). Injection of dephosphorylated synapsin I into the

preterminal region of squid giant synapse inhibited neurotransmitter release (Llinas *et al.*, 1985) and reduced spontaneous release (Lin *et al.*, 1990a). Furthermore, depolarization of synaptosomes caused translocation of synapsin I from the particulate (presumably cytoskeletal) fraction to the soluble (cytoplasmic) fraction in a Ca^{2+} and phosphorylation-dependent manner (Sihra *et al.*, 1989). It has been suggested that this protein may regulate the availability of synaptic vesicles for release (Llinas *et al.*, 1985). However, the exact role of this molecule in neurotransmitter release has yet to be determined.

The released transmitter diffuses across the synaptic cleft and binds to receptors located on the membrane of the post-synaptic cell, resulting in an electrical or chemical response in the post-synaptic cell. Continuous activation of the receptor is prevented by re-uptake of the transmitter into the pre-synaptic cell, as in adrenergic synapses, or by enzymatic destruction of the transmitter, as in cholinergic synapses (Figure 1.2).

4) Types of Ca^{2+} channel

Entry of extracellular Ca^{2+} into the nerve terminal is critical for release of neurotransmitter; Na^{+} and K^{+} do not support release, nor will significant release occur in Ca^{2+}-free medium (Katz and Miledi, 1967a,b). Therefore, an understanding of the nature of the Ca^{2+} channels that may regulate Ca^{2+} entry associated with neurotransmitter release is important to understand the process of synaptic transmission. However, it is not currently possible to characterize "active zone" Ca^{2+}

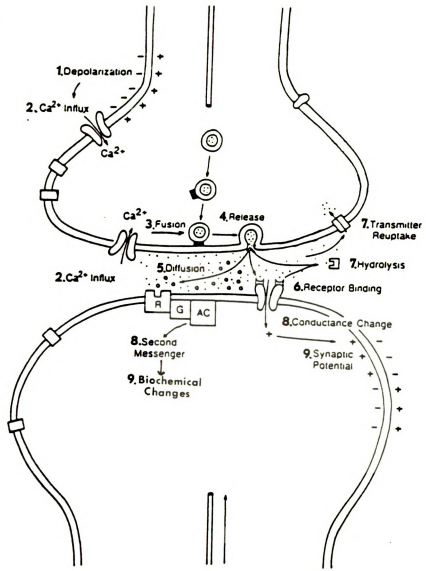

Figure 1.2. Sequence of events during synaptic transmission. 1) Depolarization of the terminal region by electrotonic spread of the action potential into the terminal region from pre-terminal nodes of Ranvier. 2) Activation of voltage-sensitive Ca^{2+} channels and influx of Ca^{2+} into the nerve terminal. 3) Ca^{2+}-triggered fusion of synaptic vesicles with the terminal membrane. 4) Release of neurotransmitter into the synaptic cleft and 5) diffusion to the post-synaptic cell where 6) binding to receptors occurs. Following binding, the transmitter is removed from the cleft by 7) hydrolysis and/or carrier-mediated re-uptake into the presynaptic terminal. Binding of the neurotransmitter to receptor activated channel complexes results in 8) conductance changes and 9) synaptic potentials, whereas binding to other receptor types results in changes in 8) second messingers and 9) cellular biochemistry.

Modified from: Shepherd, G. H. *In:* Synaptic organization of the brain. (Shepherd, G. H., ed.). p. 5, (1990).

channels using intracellular or patch electrodes, as this region accounts for only a small portion of the nerve terminal. Therefore, the best method presently available to determine which type or types of Ca^{2+} channel may be involved in transmitter release is to characterize the types of somatic Ca^{2+} channel,and then infer which types may be involved in release based on the properties and pharmacological sensitivities of Ca^{2+} channels and the release process. However, a lack of pharmacological effect on neurotransmitter release may result from inadequate access of drugs or toxins to the channel rather than channel insensitivity. Thus, caution must be observed when making inferences as to channel type(s) critical to neurotransmitter release bases solely on pharmacological sensitivity.

Two types of Ca^{2+} channel have been consistently demonstrated in neurons (Llinas and Yarom, 1981; Carbone and Lux, 1984). One type begins to activate (opens) at membrane potentials between -60 and -40 mV, has a very small unitary conductance, completely inactivates during a sustained voltage pulse and remains inactivated if the membrane potential is held positive to -60 mV. Depending on the nomenclature system used, these channels are termed "Low Voltage Activated" (LVA), "Type I", or "T-type" channels. The second type of Ca^{2+} channel is activated at membrane voltages of -20 to 0 mV, has a large unitary conductance, and generally does not inactivate during sustained voltage pulses of 100-500 ms. These channels are denoted as "High Voltage Activated" (HVA), "Type II", or "L-type" Ca^{2+} channels.

A third subtype has been described in avian (Nowycky *et al.*, 1985a; Fox *et al.*, 1987a,b) and mammalian (Kostyuk *et al.*, 1988) embryonic dorsal root ganglion

(DRG) cells and in nerve growth factor-differentiated rat pheochromocytoma (PC12) cells (Plummer *et al.*, 1989) as well as many other types of neuronal cells. It is also a high voltage activated channel, but the unitary conductance of this channel is smaller than the "L-type" channel and inactivation of this channel occurs during a sustained voltage pulse. This channel has been termed "N-type". However, the existence of this channel as a separate entity from the L channel has been questioned on the basis of analysis of tail current recordings from chick DRG neurons (Swandulla and Armstrong, 1987) and growth cone currents from PC12 cells (Streit and Lux, 1990). The evidence supporting three separate Ca^{2+} channel entities has been reviewed recently (Swandulla *et al.*, 1991).

Recently, an additional Ca^{2+} channel type has been described in Purkinje cell neurons of guinea pig cerebellum and in the presynaptic terminal of squid giant synapse (Llinas *et al.*, 1989). The biophysical characteristics of this channel differ from those of T-, N- and L-type channels. In addition, this channel is not sensitive to DHPs or ω-conotoxin GVIA, but is sensitive to a polyamine-like toxin from venom of funnel web spiders (Llinas *et al.*, 1989). Using a synthetic funnel-web spider toxin (FTX), proteins have been isolated from mammalian cerebellar tissue which formed FTX-sensitive channels when incorporated into bilayers. Furthermore, the molecular mass of this protein markedly differed from that of other characterized Ca^{2+} channel proteins (Cherksey *et al.*, 1990) This channel is referred to as the P channel since it was first described in Purkinje cells. Ca^{2+} current from rat brain mRNA expressed by *Xenopus* oocytes has a single exponential time course of inactivation of

approximately 650 ms and is not sensitive to DHPs nor ω-conotoxin GVIA (Leonard *et al.*, 1987), but has recently been reported to be partially sensitive to FTX (Lin *et al.*, 1990b). These results suggest that the channel type expressed from rat mRNA may be similar to the P-channel. The biophysical and pharmacological profiles of the different Ca^{2+} channel types are summarized in Table 1.

Pharmacological differences also exist between the Ca^{2+} channel subtypes and have been instrumental in distinguishing among the putative subclasses. The dihydropyridine (DHP)-class of organic Ca^{2+} channel agonists and antagonists interact preferentially with L-type channels, whereas N- and T-type channels are unaffected by nanomolar to low micromolar concentrations of these drugs. The peptide, ω-conotoxin GVIA, (CgTx) acts on a specific population of sensitive N-type channels in mammalian neurons. However, in amphibian and avian preparations, CgTx also blocks L-type channels, as defined by voltage sensitivity and current kinetics. In some cases, block of L channels by CgTx may be reversible or incomplete. Inorganic divalent cations block all three types of Ca^{2+} channels. However, the relative potencies of block differ: N and L channels are blocked more readily by Cd^{2+} than Ni^{2+}, whereas T channels are blocked more readily by Ni^{2+} than Cd^{2+} (Tsien *et al.*, 1988).

It should be noted that slight differences in electrophysiological properties and/or pharmacological sensitivities, such as the voltage at which current by a channel type increases, or the time constant of inactivation of whole-cell current exist for given Ca^{2+} channel types from one cell type to the next. In addition, since Ca^{2+}

Table 1.1

Biophysical and Pharmacological Characteristics of Ca^{2+} channel Types

	T	N	L	P
Activation Range (mV)	-70	-10	-10	-30 to -20
Inactivation Kinetics	fast τ = 20-50 ms	moderate τ = 95 ms	slow τ > .55 s	slow τ = ?
Divalent Cation sensitivity	$Ni^{2+} > Cd^{2+}$	$Cd^{2+} > Ni^{2+}$	$Cd^{2+} > Ni^{2+}$	Cd^{2+}, <100 μM
DHPs	-	-	+	-
ωCgTx	-	+	+*	-
FTX	-	-	-	+
Single channel conductance (pS) (110 mM Ba^{2+})	8	13	25	10-12 (80 mM Ba^{2+})

Data for chick DRG N,L and T channels modified from Tsien *et al.*, *TINS* 10:431-438, 1988. P-channel data compiled from Llinas *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1689-1693, 1989 and Lin *et al.*, *Proc. Natl. Acad. Sci. USA* 87:4538-4542, 1990b.

* Mammalian L-type channels are not sensitive to CgTx (Plumber *et al.*, *Neuron* 2:1453-1463, 1989).

channel function often depends on the presence of intracellular components (Bean, 1985), channel activty often changes or is lost during whole-cell or cell-free patch recording. This has hampered charactization of Ca^{2+} channel function and types. Thus, the characteristics attributed to Ca^{2+} channel types are more descriptive than absolute, as Ca^{2+} channel characteristics do not always fit neatly into one of the above categories or nomenclature systems. However, it is possible that there is considerable heterogeneity in Ca^{2+} channel function among different cell types or that additional channel types exist. This is supported by the recent isolation from rat brain of 4 different types of cDNA that code for putative Ca^{2+} channels; two of which are highly homologous (75% identical residues) with the α_1 subunit of the DHP-sensitive Ca^{2+} channel from skeletal muscle, whereas the other two are much less homologous (50% identical residues) but nonetheless related in sequence (Snutch *et al.*, 1990). Characterization of the primary structure and expression of these cDNAs suggest that several different Ca^{2+} channel gene families exist which are related in amino acid sequence, but may have subtle differences in pharmacological sensitivity (Starr *et al.*, 1990; Dubel and Snutch, 1990).

Despite considerable research characterizing neuronal Ca^{2+} channels, the channel type(s) involved in neurotransmitter release are still not defined clearly. This is largely due to the technical limitations of current techniques. Intracellular and patch clamp recording require the use of microelectrodes whose diameter is almost as large as the nerve terminal itself. Thus, biophysical characterization of channel types at the active zone is not possible. Exceptions to this problem are the terminals

of the neurohypophyseal neurons, which are large enough to allow use of patch-clamp methodology. It has been reported that both L and N type channels are present in these terminals, and that both may contribute to Ca^{2+} entry associated with transmitter release. Under normal conditions of release, L channels are more likely to be involved in release than N channels, due to rapid inactivation of the latter during trains of action potentials (Lemos and Nowycky, 1989). More recently, however, it has been reported that both Ca^{2+} channel types in this preparation may be involved in an initial burst of transmitters release, whereas L-type Ca^{2+} channels are important for sustained release of peptide transmitters (Lim *et al.*, 1990; Nowycky, 1991). Although they provide the only mammalian system in which Ca^{2+} currents can be examined in nerve terminals, these neurons are highly specialized structures which secrete the neurohormones oxytocin and vasopressin. Release of peptide neurotransmitters from these terminals may or may not be truly representative of release of small, classical transmitters from other central and peripheral neurons.

Indirect information on the types of Ca^{2+} channels which mediate Ca^{2+} influx associated with neurotransmitter release can be obtained by examining the pharmacological sensitivity of neurotransmitter release. At the rat neuromuscular junction, DHP-sensitive Ca^{2+} channels can have at best a modulatory role in neurotransmitter release (Atchison and O'Leary, 1987), but do not contribute to nerve-evoked release under normal conditions (Atchison, 1989). DHP-sensitive or L-type channels have been implicated in release of substance P from DRG cells (Rane

et al., 1987; Holz *et al.*, 1988) and ^{3}H-norepinephrine release from undifferentiated PC12 cells (Kongsamut and Miller, 1986). DHP-insensitive or N-type channels have been implicated as the important channel involved in neurotransmitter release in rat sympathetic neurons (Hirning *et al.*, 1988), peripheral neurons (Perney *et al.*, 1986), nerve growth factor differentiated PC12 cells (Kongsamut and Miller, 1986), various areas of the central nervous system (Dooley *et al.*, 1987a).

These data would suggest that N- and/or L-type Ca^{2+} channels are responsible for Ca^{2+} influx associated with neurotransmitter release at many terminals. However, substantial data exists which suggests that another as yet uncharacterized Ca^{2+} channel subtype may also mediate Ca^{2+} entry associated with neurotransmitter release. For example, the Ca^{2+} current associated with neurotransmitter release from the presynaptic terminal of the squid giant synapse activates at relatively hyperpolarized potentials, inactivates slowly, and is insensitive to DHPs and CgTx (Charlton and Augustine, 1990). This suggests the possibility that P-type channels may also be involved in transmitter release. In mammalian preparations, CgTx and DHPs do not affect release of ACh from motor nerve terminals (Anderson and Harvey, 1987) or aspartate release from hippocampal slices (Mangano *et al.*, 1991). Transmitter release from rat sympathetic ganglion cells is not impaired by DHPs or CgTx (Seabrook and Adams, 1989). Species and/or tissue differences in the type Ca^{2+} channel involved in neurotransmitter release may also exist in mammals, as *in vitro* CgTx produces a greater effect on transmitter release in smooth muscle preparations from guinea pigs than from rats (Maggi *et al.*, 1988). Wessler *et al.*,

(1990) demonstrated in rats that ^{3}H-ACh release from myenteric plexus neurons and the phrenic nerve is not affected by verapamil or diltiazem, whereas CgTx reduces release from the neocortex (IC_{50} = 13 nM) and myenteric plexus (IC_{50} = 0.7 nM), but not the phrenic nerve ($IC_{50} \geq$ 100 nM). These results suggest that channels which are neither N- nor L-type are involved in neurotransmitter release from some mammalian nerve terminals. Therefore, at many terminals it appears as though N- and/or L-type channels may mediate Ca^{2+} entry associated with neurotransmitter release, although additional Ca^{2+} channel subtypes may also be involved in neurotransmitter release. There is no evidence to date which indicates that the T-type Ca^{2+} channel is involved in release in any system, but this does not preclude an unknown role of this channel.

5) Environmental contaminants, natural toxins and pharmaceutical agents which affect Ca^{2+} channels.

Ca^{2+} channel function in neurons is affected by a wide variety of chemical agents including metals, peptides and organic compounds. Inorganic heavy metal cations are effective Ca^{2+} channel blockers. Lead (Audesirk, 1987; Audesirk and Audesirk, 1989) and cadmium, although the latter is not overtly neurotoxic, are environmental contaminants which block Ca^{2+} channels at concentrations between 1 and 10 micromolar. The transition metals nickel, mercury, copper and zinc are also capable of blocking Ca^{2+} channels at low micromolar concentrations (Nachshen, 1984). In isolated nerve terminals, block of $^{45}Ca^{2+}$ influx by divalent cations such as

Pb^{2+}, Ni^{2+}, Cd^{2+} and Hg^{2+} (Nachshen, 1984; Atchison *et al.*, 1986) is overcome by increasing $[Ca^{2+}]_e$. Thus, it is thought that these metals bind to a site in the pore of the Ca^{2+} channel with high affinity, impeding the flow of Ca^{2+} through the channel (Hagiwara and Takahashi, 1967; Hagiwara *et al.*, 1974; Lansman *et al.*, 1986; Hess *et al.*, 1986).

In addition to inorganic metals, the organic metals methylmercury (Atchison *et al.*, 1986; Shafer and Atchison, 1989) and ethylmercury (Hewett and Atchison, 1990) are capable of blocking $^{45}Ca^{2+}$ influx into nerve terminals. Both charge and lipophilicity may be important in determining the characteristics of Ca^{2+} channel disruption by mercurials, as only positively charged mercurials block Ca^{2+} channels, and only organic mercurials blocked $^{45}Ca^{2+}$ influx into synaptosomes in a voltage-dependent manner (Hewett and Atchison, 1990).

Several natural toxins which interact specifically with Ca^{2+} channels have been isolated from venom preparations. A family of polypeptide toxins with Ca^{2+} channel activity has been isolated from venom of marine snails of the *Conus* family (Olivera *et al.*, 1987). ω-Conotoxin GVIA, isolated from the venom of *Conus geographus* (Olivera *et al.*, 1985), has been thoroughly characterized with respect to its action on Ca^{2+} channels. This compound is a potent inhibitor of non-mammalian Ca^{2+} channels, binding to and blocking N- and L-type Ca^{2+} channels irreversibly in sub-picomolar concentrations (Cruz and Olivera, 1986). In mammalian preparations, the action of ω-conotoxin GVIA is less clear. It has been suggested that only the N-type Ca^{2+} channel is sensitive to CgTx in several preparations (Tsien *et al.*, 1988; Plummer

et al., 1989). This peptide blocks neurotransmitter release (Keith *et al.*, 1989) and synaptic transmission (Kamiya *et al.*, 1988; Dutar *et al.*, 1989) in the hippocampus. However, CgTx does not affect ACh release at the neuromuscular junction (Anderson and Harvey, 1987), and only partially reduces $^{45}Ca^{2+}$ influx into mammalian synaptosomes (Suszkiw *et al.*, 1989). In addition, expression of rat brain mRNA in oocytes results in Ca^{2+} currents which are insensitive to CgTx (Leonard *et al.*, 1987). As discussed in the previous section, these results suggest a unique Ca^{2+} channel type in these preparations.

Maitotoxin, which is produced by the marine dinoflagellate *Gambierdiscus toxicus*, increases Ca^{2+} channel function in cultured neuronal cells (Takahashi *et al.*, 1982; 1983). Spider venoms also contain peptides and polyamine-like compounds which block Ca^{2+} channels. The ω-aga-toxins are peptides that have been isolated from the venom of funnel web spider (*Agelenopsis aperta*) and are potent blockers of Ca^{2+} channels (Adams *et al.*, 1990). These toxins can be subdivided into three classes based on the ability to inhibit CgTx binding in chick synaptosomes; ω-AgaI toxins do not affect binding, whereas ω-AgaII and ω-AgaIII (Venema and Adams, 1990) toxins inhibit CgTx binding. ω-AgaIIIA blocks N- and L-type channels in rat DRG cells (Minty *et al.*, 1990). However, the pharmacological characterization of these compounds has not been completed to date. A polyamine-like toxin which is a potent Ca^{2+} channel blocker also has been purified from the venom of funnel-web spiders (Llinas *et al.*, 1989). The structure of this toxin has not yet been determined, although several compounds with similar activities have been synthesized (Cherksey

et al., 1989). Pending further characterization of spider toxins, these compounds may be useful tools in neurobiology. Finally, the endogenous peptide endothelin has been demonstrated to bind to and depolarize dorsal horn neurons (Yoshizawa *et al.*, 1989). Endothelin is a vasoactive peptide which is presumed to increase function of L-type Ca^{2+} channels in muscle cells. That it has actions in the spinal cord may indicate that there are also endogenous regulators of Ca^{2+} channel activity in the nervous system as well as the cardiovascular system.

Several therapeutic compounds also exert Ca^{2+} channel block as either a predominant mechanism of therapeutic action, or at least one of several potential mechanisms of therapeutic and/or toxic action. Prominent among these compounds are several agents used for treatment of cardiovascular disorders such as angina pectoris, hypertension and arrythymias. These so called "Ca^{2+} channel blockers" also bind readily to CNS preparations (Gould *et al.*, 1985). Therapeutic Ca^{2+} channel antagonists fall into three chemical classifications: the dihydropyridines (DHP) such as nifedipine, the phenylalkylamines such as verapamil and the benzothiazopines, such as diltiazem. DHPs with Ca^{2+} channel agonist properties have also been synthesized. These are represented by (+)-Bay K 8644 and (+)-S-202-791. The DHPs, and presumably the phenylalkylamines and benzothiazopines, interact preferentially with L-type Ca^{2+} channels as opposed to other Ca^{2+} channel types (Fox *et al.*, 1987a,b). Other therapeutic agents whose action is postulated to involve Ca^{2+} channel block include anticonvulsants such as phenytoin (Twombly *et al.*, 1988), the barbiturates (Blaustein and Ector, 1975; Werz and Mcdonald, 1985; Gross and

Mcdonald, 1988; Gunderson *et al.*, 1988), as well as the benzodiazepines (Leslie, 1987) and certain general anesthetics such as halothane (Takenoshita and Steinbach, 1991). The neuroleptic chlorpromazine blocks Ca^{2+} influx into synaptosomes (Hoss and Formaniak, 1984) and Ca^{2+} currents in neuroblastoma cells (Ogata and Narahashi, 1990). Finally, the aminoglycoside antibiotics may also interact with neuronal Ca^{2+} channels and this interaction may contribute to the toxicity of these therapeutic agents. In synaptosomes, neomycin and polymyxin block synaptosomal Ca^{2+} channels in a manner which is reversed by increasing extracellular [Ca^{2+}] (Atchison *et al.*, 1988) and aminoglycoside antibiotics compete with binding of radiolabelled ω-conotoxin GVIA (Knaus *et al.*, 1987).

Low concentrations of ethanol (EtOH) decrease $^{45}Ca^{2+}$ influx into synaptosomes (Friedman *et al.*, 1980; Leslie *et al.*, 1983; Skattebol and Rabin, 1987) and block Ca^{2+} currents in neuroblastoma cells (Twombly *et al.*, 1990). Chronic exposure to low millimolar concentrations of EtOH results in an increase in depolarization-induced $^{45}Ca^{2+}$ influx into PC12 cells. This increase in Ca^{2+} influx is presumably channel mediated, as it is blocked by DHPs and inorganic divalent cations (Greenberg *et al.*, 1987a). In addition, chronic exposure to ethanol alters the binding of Ca^{2+} channel ligands to PC12 cells (Marks *et al.*, 1989).

CHAPTER TWO

SYNAPTOSOMES AND PHEOCHROMOCYTOMA CELLS AS MODEL SYSTEMS FOR STUDY OF Ca^{2+} CHANNELS

A) SYNAPTOSOMES AND SYNAPTOSOMAL Ca^{2+} CHANNELS

Because of the limitations imposed by the small size of most nerve terminals, direct electrophysiological study of channel function is severely restricted. Synaptosomal suspensions are preparations containing large numbers of pinched-off nerve terminals which allow for biochemical study of channel function and neurotransmitter release *in vitro*. Synaptosomes are derived by homogenization of central nervous system tissue followed by differential centrifugation to separate synaptosomal material from other CNS components. Synaptosomes are spherical particles 1 - 2 μm in diameter and contain intracellular organelles including mitochondria, endoplasmic reticulum and synaptic vesicles (Gray and Whittaker, 1962). Many metabolic pathways and synthetic pathways remain intact in synaptosomes, including glycolysis, Kreb's cycle and neurotransmitter synthesis (Suszkiw and O'Leary, 1982; Eder-Colli *et al.*, 1989). Synaptosomes when incubated in glucose-containing medium remain viable for biochemical studies of metabolic function, channel function and neruotransmitter release for several hours after isolation (Bradford, 1975). The activity of Na^+/K^+ ATPase is very high in synaptosomal preparations, allowing for maintenance of a membrane potential between -30 and -80 mV (Blaustein and Goldring, 1975; Suszkiw *et al.*, 1986). Synaptosomes also contain voltage-sensitive Na^+, K^+ and Ca^{2+} channels (Nelson *et al.*, 1983; Nelson, 1985; 1986) and release neurotransmitter in a Ca^{2+}-dependent manner following depolarization (Blaustein, 1975; Floor, 1983; Leslie, *et al.*, 1985). This preparation has been widely employed in studies of channel function (Nachshen

and Blaustein, 1979; 1980; 1982), neurotransmitter synthesis and release, receptor binding (see Chapter four), and regulation of metabolic processes (Blaustein *et al.*, 1978) at the nerve terminal. Results of experiments using this preparation have contributed significantly to knowledge of processes that occur at this portion of the neuron.

Depolarization-dependent Ca^{2+} influx into synaptosomes occurs in two distinct phases (Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1983; Nachshen, 1985): a "fast" phase, which inactivates after 1-2 s of depolarization and a "slow" phase which remains active for 20-90 s after depolarization (Figure 2.1). The slow phase of influx may be mediated by a Ca^{2+} channel and a reversed Na^+/Ca^{2+} exchange mechanism; the latter may predominate, as removal of Na^+_e largely reduces "slow" influx (Coutinho *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986).

The fast phase of influx, which is associated with neurotransmitter release (Drapeau and Blaustein, 1983; Floor, 1983; Suszkiw and O'Leary, 1983; Leslie *et al.*, 1985), is presumably mediated by voltage-dependent Ca^{2+} channels and is not dependent on Na^+_e. In many ways, $^{45}Ca^{2+}$ influx into synaptosomes correlates with Ca^{2+} channel function in other systems. Depolarization of synaptosomes by K^+ results in influx of $^{45}Ca^{2+}$ in a manner which is related to the $[K^+]_e$. Presuming that $[K^+]_e/[K^+]_i$ (Blaustein and Goldring, 1975) determines the membrane potential, approximately 20-40 mV depolarization is required to initiate $^{45}Ca^{2+}$ influx (Nachshen and Blaustein, 1980; Suszkiw *et al.*, 1986; 1989; Adam-Vizi and Ligeti, 1986). Predepolarization of the synaptosomal suspension in the presence or absence

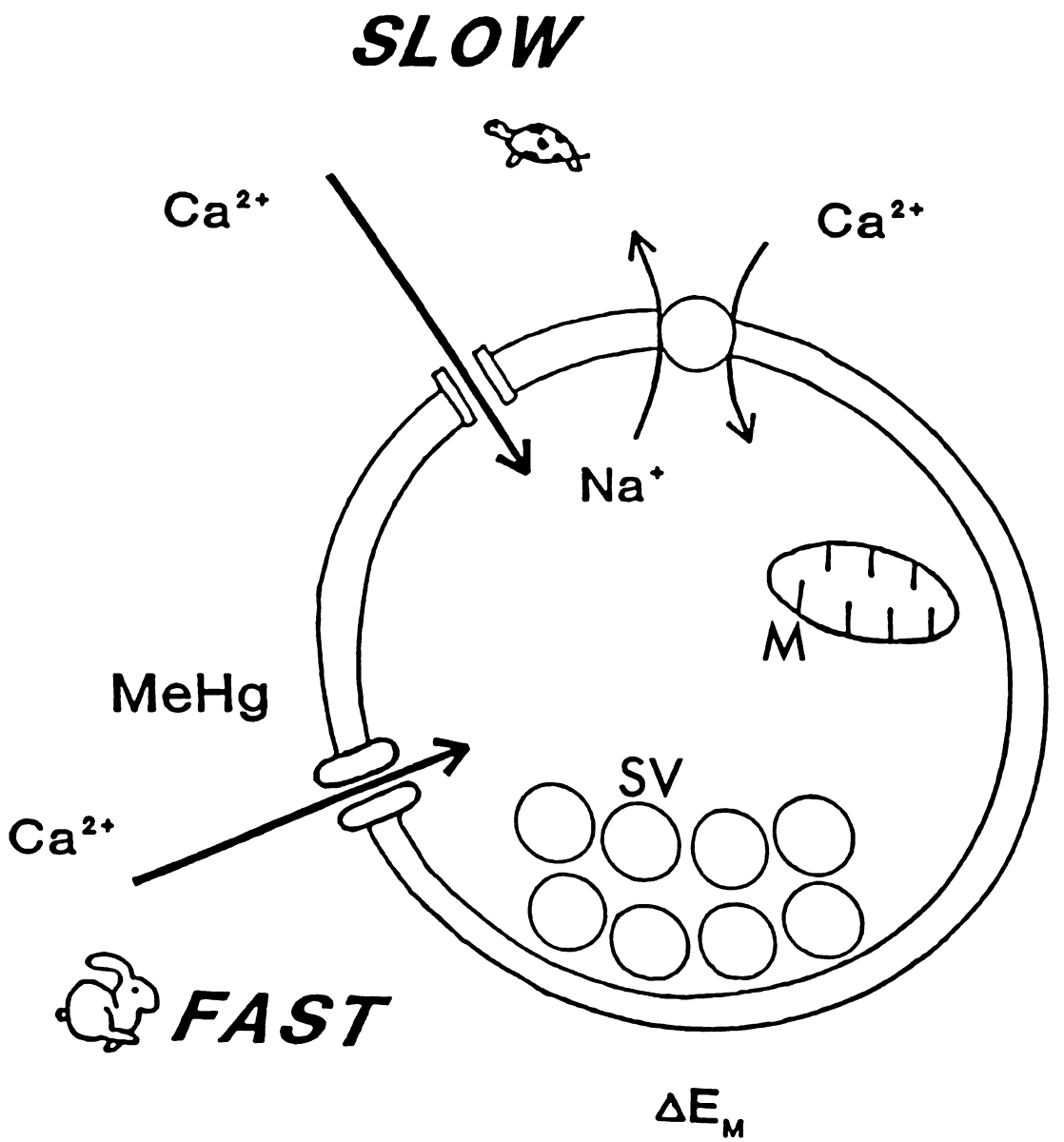

Figure 2.1. Components of Ca^{2+} influx into synaptosomes. Schematic diagram of a synaptosome containing mitochondria (M) and synaptic vesicles (SV). Depolarization of the membrane (ΔE_M) results in fast, channel-mediated Ca^{2+} influx (FAST) and slow influx (SLOW). The slow component is mediated by reversed Na^+/Ca^{2+} exchange and slowly- or non-inactivating Ca^{2+} channels.

of Ca^{2+} eliminated the fast component (Nachshen and Blaustein, 1980). Ca^{2+} channels in synaptosomes also undergo both voltage- and Ca^{2+}-dependent inactivation (Suszkiw *et al.*, 1986; 1989). In addition to Ca^{2+}, Sr^{2+} and Ba^{2+} are also permeant through these Ca^{2+} channels; the relative influx of Ca^{2+}:Sr^{2+}:Ba^{2+} for the fast component of synaptosomal influx is 6:3:2 (Nachshen and Blaustein, 1982). Incorporation of synaptosomal Ca^{2+} channels into lipid bilayers indicated that the single channel conductance was $gBa^{2+} > gCa^{2+} = gSr^{2+}$ (Nelson, 1986). This is similar to cardiac Ca^{2+} channels, for which whole cell current indicated that the relative premeability was $Ca^{2+} > Sr^{2+} > Ba^{2+}$, but single channel conductance measurements indicated that $gBa^{2+} > gCa^{2+}$ (Hess *et al.*, 1986).

Like Ca^{2+} channels in other systems, fast $^{45}Ca^{2+}$ influx into synaptosomes is sensitive to block by inorganic divalent and trivalent cations. The most potent blockers of influx are La^{3+}, Pb^{2+} and Cd^{2+}, which have K_i values of less than 10 μM. Other metals including Co^{2+}, Ni^{2+}, Hg^{2+} and Zn^{2+} also block the fast component of infux, although higher concentrations (K_i = 10 - 100 μM) are required (Nachshen, 1985). The organic Ca^{2+} channel blocker verapamil also decreases $^{45}Ca^{2+}$ influx into synaptosomes (Nachshen, 1985). Single-channel recordings of synaptosomal Ca^{2+} channels in lipid bilayers also confirm that La^{3+}, Cd^{2+} and verapamil are potent blockers of synaptosomal Ca^{2+} channels (Nelson, 1985). Block of Ca^{2+} channels by verapamil suggests the possibility that L-type channels may be involved in Ca^{2+} influx. However, this is a subject of considerable controversy, as DHPs do not have consistent effects on $^{45}Ca^{2+}$ influx in synaptosomal preparations (see below).

Fast influx has been associated with release of Substance P (Floor, 1983), acetylcholine (Suszkiw and O'Leary, 1983) and dopamine (Drapeau and Blaustein, 1983, Leslie *et al.*, 1985) from synaptosomes. Thus, the fast phase couples Ca^{2+} entry and subsequent neurotransmitter release from nerve terminals.

The slow component of $^{45}Ca^{2+}$ influx into synaptosomes differs in many respects from the fast component. It does not inactivate for up to 90 s during depolarization and is much less sensitive to La^{3+} than the fast phase (Nachshen and Blaustein, 1980). Furthermore, the relative flux of Ca^{2+}:Sr^{2+}:Ba^{2+} for the slow component is 6:3:1 (Nachshen and Blaustein, 1982), indicating that this component has different properties of selectivity than the fast component. Finally, this component is extremely sensitive to $[Na^+]_e$ (Blaustein and Oborn, 1975). Inasmuch as preparation and incubation of synaptosomes in Na^+-free solutions drastically reduces influx via this component, it has been proposed that a reversed Na^+/Ca^{2+} exchange contributes significantly to this phase of influx (Blaustein and Oborn, 1975; Åkerman and Nicholls, 1981; Coutinho *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986). This does not preclude involvement of Ca^{2+} channels in this component, as small fluxes are measured in the absence of Na^+ (Suszkiw *et al.*, 1986; Shafer and Atchison, 1989) and DHPs have been reported to decrease this component of influx in Na^+-containing solutions (Martinez-Serrano *et al.*, 1989).

Initial experiments in this dissertation examine effects of MeHg on synaptosomal $^{45}Ca^{2+}$ influx Thus, information regarding Ca^{2+} channel types in this system is important. Synaptosomes are likely to contain more than one type of Ca^{2+}

channel, but the types of Ca^{2+} channel present have not been identified. DHP antagonists bind to synaptosomal preparations with high affinity (Rampe, 1984, Turner and Goldin, 1984; Suszkiw *et al.*, 1986; Dooley *et al.*, 1987b; Massieu and Tapia, 1988). However, synaptosomal $^{45}Ca^{2+}$ influx and/or neurotransmitter release have been variously reported to be sensitive (Turner and Goldin, 1985; Dunn, 1988; Martinez-Serrano, 1989) or insensitive (Nachshen and Blaustein, 1979; Daniell *et al.*, 1983; Reynolds *et al.*, 1986; Rivier *et al.*, 1987; Suszkiw *et al.*, 1986; 1989; Massieu and Tapia, 1988) to block by DHP antagonists. Additionally, in mammalian synaptosomes, $^{45}Ca^{2+}$ influx is only partially sensitive to block by ω-conotoxin GVIA (Suszkiw *et al.*, 1989). Furthermore, the activation, inactivation (Suszkiw *et al.*, 1989) and pharmacological sensitivity (Suszkiw *et al.*, 1989; Lundy *et al.*, 1991) of $^{45}Ca^{2+}$ influx into synaptosomes suggest that these preparations contain a high voltage activatid channel which is neither N- nor L-type. In some respects, Ca^{2+} influx into synaptosomes has characteristics similar to current mediated by P-channels. However, further characterization is required to verify the type or types of Ca^{2+} channels present in synaptosomal preparations.

B) Ca^{2+} CHANNEL FUNCTION IN PC12 CELLS

PC12 cells contain many voltage-sensitive channel types important to neuronal function. These include tetrodotoxin- (TTX) sensitive and -insensitive Na^+ channels (Rudy *et al.*, 1987), voltage-sensitive K^+ channels (Pun and Behbehani, 1989), and Ca^{2+} channels. PC12 cells have been utilized extensively to study the functions,

expression and regulation of Ca^{2+} channels. Furthermore, this cell line has been employed for numerous studies of neuronal function. As such, many biochemical and phsyiological processes of this cell line are well understood. Neurotransmitter release and receptor function in PC12 cells are reviewed in the Appendix.

By contrast to synaptosomes, the type of Ca^{2+} channels present in PC12 cells are much better defined. It is generally agreed that NGF-differentiated PC12 cells contain both DHP-sensitive L- (Figure 2.2, top panel) and ω-conotoxin GVIA-sensitive N- (Figure 2.2, bottom panel) type Ca^{2+} channels (Plummer *et al.*, 1989; Janigro *et al.*, 1989; Usowicz *et al.*, 1990; Shafer and Atchison, 1991). The presence of T-type Ca^{2+} channels in differentiated PC12 cells also has been reported (Garber *et al.*, 1989), although pharmacological characterization is required to confirm the presence of T-type channels in PC12 cells.

The existence of DHP-sensitive (L-type) channels in both undifferentiated and NGF-differentiated cells was established by both ion flux assays (Stallcup, 1979; Toll, 1982; Takahashi and Ogura, 1983; Takahashi *et al.*, 1985) and electrophysiological studies (Streit and Lux, 1987; Plummer *et al.*, 1989; Janigro *et al.*, 1989; Usowicz et al, 1990; Shafer and Atchison, 1991). The presence of DHP-insensitive (N-type) channels in PC12 cells has been more difficult to establish. Release of ^{3}H-NE from PC12 cells grown in NGF was less sensitive to DHPs than was release from undifferentiated PC12 cells (Takahashi *et al.*, 1985; Kongsamut and Miller, 1986), although $^{45}Ca^{2+}$ influx remained largely DHP-sensitive (Kongsamut and Miller, 1986). Depolarization of undifferentiated PC12 cells by K^{+} resulted in Ca^{2+}-dependent

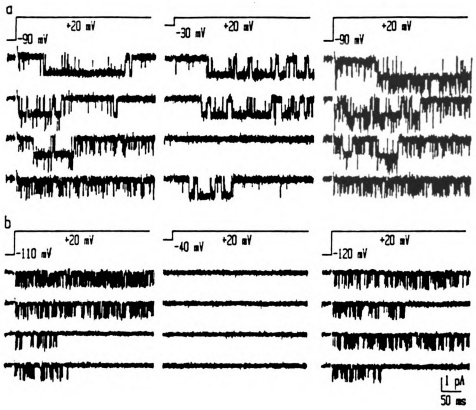

Figure 2.2. N and L type Ca^{2+} channels in PC12 cells. DHP-sensitive and -insensitive single Ca^{2+} channel current in differentiated PC12 cells recorded using the cell-attached patch voltage-clamp technique. (a) current records from a patch containing one "L-type" and one "N-type" channel. Four consecutive sweeps are shown for each set of depolarizations from two different holding potentials, as indicated in the voltage protocol above the sweeps. (b) Current traces from a patch containing a single "N-type" channel. In each case, the DHP agonist (+)-(S)-202-791 is present at 1 μM, and Ba^{2+} (110 mM) is the charge carrier.

From Plummer *et al.* *Neuron* 2:1453-1463 (1989).

release of transmitter which is sensitive to DHP Ca^{2+} channel agonists and antagonists (Figure 2.3). Differentiation with NGF resulted in decreased sensitivity of neurotransmitter release to DHP drugs (Figure 2.3B,C). Indeed, in most $^{45}Ca^{2+}$ flux studies in PC12 cells, no DHP-insensitive components were reported with the exception of a small (<10%) component in one study (Shafer *et al.*, 1990). Although this latter study and studies of neurotransmitter release indicated expression of DHP-insensitive or N-type Ca^{2+} channels in differentiated PC12 cells, they did not provide conclusive evidence that N-type channels were indeed being expressed. By contrast to radiolabel flux experiments, expression of DHP-insensitive or N-type channels has been demonstrated in NGF-differentiated PC12 cells using both whole-cell (Plummer *et al.*, 1989; Usowicz et al, 1990; Shafer and Atchison, 1991) and cell-attached (Plummer *et al.*, 1989) patch voltage-clamp techniques. In undifferentiated PC12 cells, small N-type current components have been reported (Plummer *et al.*, 1989; Janigro *et al.*, 1989).

In addition to flux and electrophysiological measurements on Ca^{2+} channel function, numerous studies of Ca^{2+} channel ligand binding have been performed to identify and characterize pharmacologically the Ca^{2+} channel subtypes expressed in PC12 cells. The most commonly used ligands are the DHP (+)-PN 200-110 and the peptide ω-conotoxin GVIA. Differentiation of PC12 cells increases the number of conotoxin binding sites by 2-3 fold and increases the number of DHP binding sites by less than 2 fold (Usowicz et al, 1990). In PC12 cells, conotoxin is specific for N-type channels, as there is no interaction between DHP binding and conotoxin binding

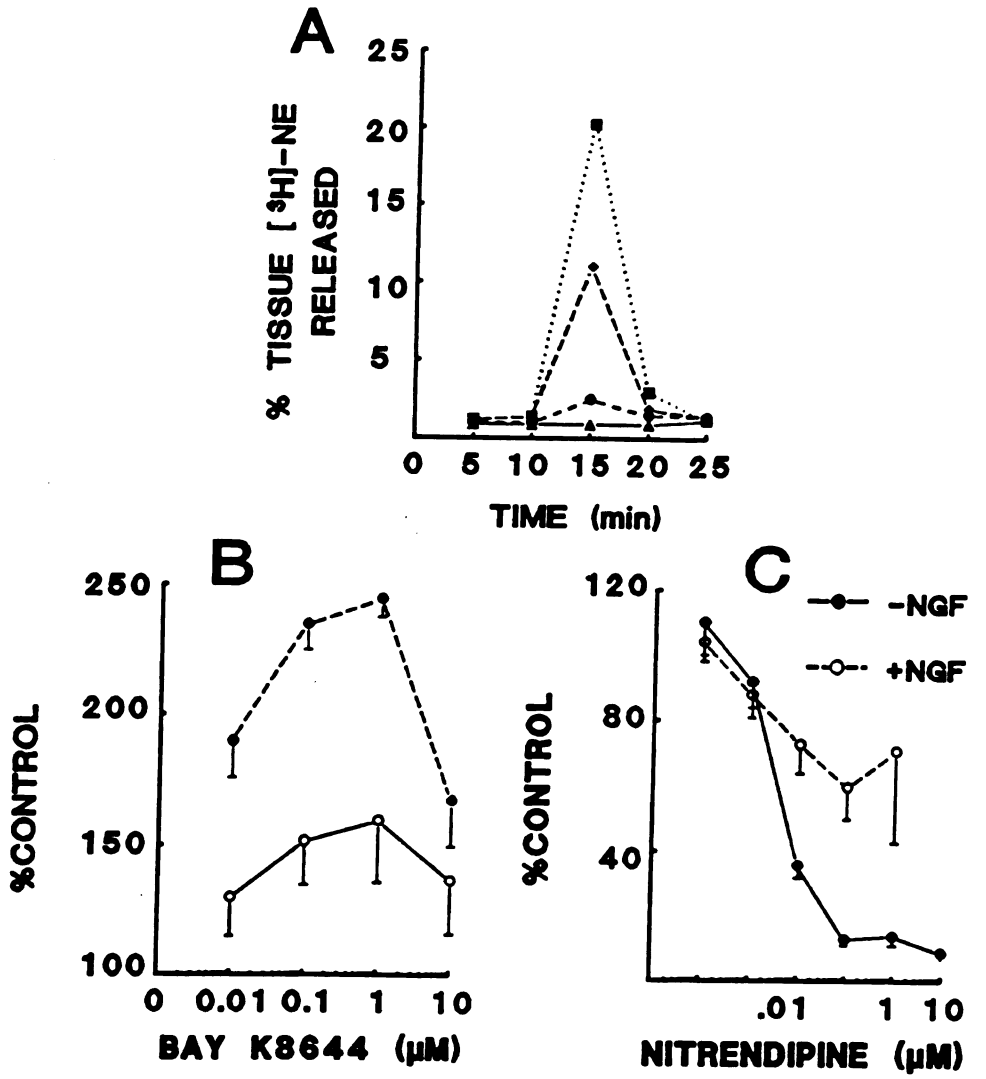

Figure 2.3. Norepinephrine release from PC12 cells. Release of [^{3}H]-norepinephrine from PC12 cells. A.) Release of [^{3}H]-norepinephrine from PC12 cells expressed as a percentage of tissue stores present at the beginning of the particular fraction. The first two 5 min. fractions were release in non-depolarizing K^+ solutions, with or without incubation of drugs. The third fraction was release induced by depolarization with 70 mM K^+ solution. The final two fractions are release in non-depolarizing solutions. (diamond) 70 mM K^+ alone, (square) 70 mM K^+ plus 10^{-6} M Bay K 8644, (circle) 70 mM K^+ plus 10^{-6} M nitrendipine, (triangle) 70 mM K^+ Ca^{2+}-free plus 0.2 mM EGTA. B. and C.) Dose-response relationship for effects of Bay K 8644 (B) and nitrendipine (C) on release of NE from NGF-differentiated (open circle) and undifferentiated (solid circle) PC12 cells. Results are expressed as a percentage of control-stimulated release in depolarizing solutions. Values are the mean± SEM of 6-12 experiments.

Modified from Kongsamut and Miller, *Proc. Natl. Acad. Sci. (U.S.A.)* 83:2243-2247 (1986).

(Usowicz *et al.*, 1990), and conotoxin blocks only the inactivating component of N-type current (Plummer *et al.*, 1989). In an NGF-insensitive PC12 mutant line, no changes in Ba^{2+} current or conotoxin binding were observed in response to NGF (Usowicz *et al.*, 1990). In addition, high concentrations of ouabain (0.1 mM), which did induce morphological differentiation of the mutant line, did not induce changes in Ca^{2+} channel expression (Usowicz *et al.*, 1990). These results suggest that NGF-differentiation induces increased expression of N-type Ca^{2+} channels in PC12 cells, and that morphological differentiation and changes in Ca^{2+} channel expression are not necessarily co-requisite events.

As indicated by binding studies, the proportion of N- to L- type channels changes with differentiation by NGF. PC12 cells grown in absence of NGF express predominantly L-type Ca^{2+} channels, whereas those treated with NGF exhibit a substantial increase in the expression of N-type Ca^{2+} channels without a concomitant decrease in expression of L-type channels (Streit and Lux, 1987; Plummer *et al.*, 1989; Usowicz et al, 1990). Ca^{2+} channels are regionally distributed in differentiating PC12 cells; growth cones contain a higher density of Ca^{2+} current than neurites and Ca^{2+} current shows more inactivation in growth cones than in the soma (Streit and Lux, 1989). Streit and Lux conclude that the increase in the inactivating component of current (which resembles currents mediated by N-type channels) with NGF treatment may result from changes in the distribution, but not types (eg. N and L) of Ca^{2+} channels in PC12 cells (Streit and Lux, 1990). However, this conclusion is based on

whole cell recordings and does not consider pharmacological sensitivity to conotoxin of Ca^{2+} currents in PC12 cells nor single channel current kinetics.

The apparent differential expression of Ca^{2+} channel subtypes in PC12 cells is important for two reasons. First, N- and L-type Ca^{2+} channels have been suggested to modulate or possibly even to mediate Ca^{2+} entry associated with neurotransmitter release from several neuronal preparations (Atchison and O'Leary 1987; Rane *et al.*, 1987; Holz *et al.*, 1988; Atchison, 1989; Lemos and Nowycky, 1989). Second, one can control to some extent, by use or omission of NGF, the proportion and distribution of N- vs L-type channels in PC12 cell cultures. This change induced by NGF may help to define whether N- and L-type channels are indeed separate entities or whether the biophysical and pharmacological differences arise from different "functional states" of one channel type.

There is some evidence that L-type Ca^{2+} channels in PC12 cells are regulated by second messengers, including protein kinase C (PKC) and G-proteins. Activators of PKC reduce DHP-sensitive $^{45}Ca^{2+}$ influx into PC12 cells (Harris *et al.*, 1986; Di Virgilio *et al.*, 1986), while inhibitors of PKC have little effect on influx (Greenberg *et al.*, 1987b). Calmidazolium and trifluoroperazine, inhibitors of calmodulin, decrease influx of $^{45}Ca^{2+}$ into PC12 cells, indicating that calmodulin or calmodulin-dependent enzymes may also regulate Ca^{2+} channels (Greenberg *et al.*, 1987b). G-proteins may also be involved in Ca^{2+} channel regulation. Stable GTP analogs increase the displacement PN 200-110 binding by the DHP agonist Bay K 8644. Pertussis toxin inhibits this action of Bay K 8644, as well as Bay K 8644-induced

potentiations of depolarization-dependent $^{45}Ca^{2+}$ influx into PC12 cells (Bergamaschi *et al.*, 1990). Chronic K^+-induced depolarization or treatment with ionomycin, a Ca^{2+} ionophore, reversibly decreased depolarization-induced $^{45}Ca^{2+}$ influx and the number of [^{3}H]-nitrendipine (a DHP antagonist) binding sites (DeLorme *et al.*, 1988). Elevations in $[Ca^{2+}]_i$ may be involved in Ca^{2+} channel down regulation, although $[Ca^{2+}]_i$ returned to normal levels while Ca^{2+} channel function remained depressed (DeLorme and M^{c}Gee, 1988). One second messenger which does not appear to affect Ca^{2+} channel function in PC12 cells is cAMP (Rabe *et al.*, 1982; Nishizawa *et al.*, 1990). Further characterization of the regulation of Ca^{2+} channels by second messengers is needed to understand more fully the role of Ca^{2+} channels in neurotransmitter release and other cellular functions in PC12 cells.

In general, PC12 cells have not been used extensively as a tool in neurotoxicological studies, although the interactions of several neurotoxic agents with Ca^{2+} channels have been examined. PC12 cells have been used to characterize interactions of the neurotoxicant methylmercury (MeHg) with Ca^{2+} channels. MeHg blocks $^{45}Ca^{2+}$ influx into undifferentiated and NGF-differentiated PC12 cells. In addition, MeHg alters the binding of conotoxin to intact PC12 cells (Shafer *et al.*, 1990). In more recent studies, acute effects of MeHg on current carried by Ba^{2+} (I_{Ba}) in NGF-differentiated PC12 cells have been examined. MeHg blocks both N- and L-type Ca^{2+} current in PC12 cells in a manner which is voltage- but not state-dependent (see chapter 5). The effects of MeHg on Ca^{2+} channels are not reversed by washing with MeHg-free solutions, but are antagonized by increasing extracellular

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[Ba^{2+}] (Shafer and Atchison, 1991). These most recent results suggest that the effects of MeHg on I_{Ba} in PC12 cells mirror the actions of MeHg on synaptosomal $^{45}Ca^{2+}$ influx (Atchison *et al.*, 1986; Shafer and Atchison, 1989; Shafer *et al.*, 1990). EtOH also alters Ca^{2+} channel function in PC12 cells. Chronic exposure to low millimolar concentrations of EtOH results in an increase in depolarization-induced $^{45}Ca^{2+}$ influx into PC12 cells. This increase in Ca^{2+} influx is presumably channel mediated, as it is blocked by DHPs and inorganic divalent cations (Greenberg *et al.*, 1987a). Furthermore, chronic exposure to EtOH increased the number of (+)-PN 200-110 binding sites in PC12 cell membranes (Figure 2.4). This action was not reduced by co-treatment with Bay K 8644, indicating that increased channel number was not the result of decreased Ca^{2+} influx into PC12 cells (Marks *et al.*, 1989). Further study of the effects of EtOH on Ca^{2+} channel numbers in PC12 cells may provide insight into the mechanisms of regulation of expression of Ca^{2+} channels in PC12 cells. Chronic exposure (3 days) to immunoglobulin G isolated from serum of patients with Lambert-Eaton Myasthenic Syndrome has also been demonstrated to decrease $^{45}Ca^{2+}$ influx into PC12 cells (Lang, *et al.*, 1989). The effects of volatile anesthetics (Kress *et al.*, 1987), maitotoxin (Takahashi *et al.*, 1982; 1983; Choi *et al.*, 1990) and α-latrotoxin (Vicentini and Meldolesi, 1984; Meldolesi *et al.*, 1986) on Ca^{2+} channel function have also been examined using PC12 cells. Thus, PC12 cells are clearly useful models for the study of interactions between neurotoxic compounds and Ca^{2+} channels.

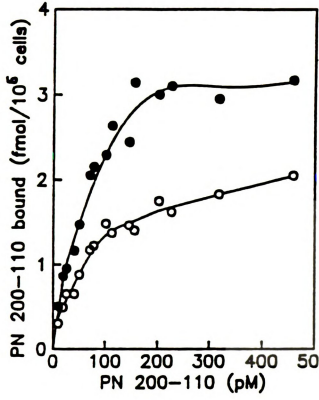

Figure 2.4. Effects of EtOH on Ca^{2+} channel density in PC12 cells. Saturable binding of the DHP Antagonist (+)-[^{3}H]PN 200-110 to intact PC12 cells grown for 6 days in the absence (open circles) or presence (closed circles) of 200 mM ethanol. Data shown are the average of triplicate values from a single representative experiment.

From: Marks *et al.* *J. Neurochem.* 53:168-172 (1989).

CHAPTER THREE

BLOCK OF $^{45}Ca^{2+}$ INFLUX INTO SYNAPTOSOMES BY METHYLMERCURY: Ca^{2+} AND Na^{+} DEPENDENCE

ABSTRACT

Block of Ca^{2+} influx into isolated nerve terminals by the neurotoxicant methylmercury (MeHg) was studied for its dependence on extracellular Ca^{2+} and Na^{+}. Depolarization-independent entry of $^{45}Ca^{2+}$ was determined in rat forebrain synaptosomes incubated in 5 mM K^{+} solution. $^{45}Ca^{2+}$ influx was similarily measured following 1 ("fast" phase) or 10 s ("total") of elevated K^{+} - (41.25 mM) induced depolarization, or during 10 s of elevated K^{+}-induced depolarization after synaptosomes had been predepolarized for 10 s in Ca^{2+}- and MeHg-free solutions ("slow" phase). In 5 mM K^{+} solutions, MeHg concentrations of 125 μM and greater significantly reduced synaptosomal $^{45}Ca^{2+}$ accumulation measured during 1 or 10 s of incubation. In K^{+}-depolarized synaptosomes, the estimated IC_{50} for block of "total", "fast" and "slow" $^{45}Ca^{2+}$ influx was 75 μM: 250 μM MeHg reduced influx by approximately 90%. The ability of $[Ca^{2+}]_e$ to antagonize block was tested by increasing $[Ca^{2+}]_e$ from 0.01 to 1.15 mM in the presence and absence of MeHg. When compared to control, 50 μM MeHg reduced "total" influx of $^{45}Ca^{2+}$ by $\geq$ 70% and reduced "fast" influx by 20-60% at all $[Ca^{2+}]_e$ tested. At $[Ca^{2+}]_e$ concentrations of 0.01-0.15 mM, MeHg (50 μM) reduced "slow" influx by 75-90%, but did not affect slow influx at higher $[Ca^{2+}]_e$ ($\geq$ 0.30 mM). When the dependence of block of $^{45}Ca^{2+}$ influx on $[Na^{+}]_e$ was tested, equivalent levels of inhibition were caused by MeHg (25 μM) for fast influx by synaptosomes in Na^{+}-containing and Na^{+}-free solutions. Conversely, 25 μM MeHg reduced slow influx by 26.1$\pm$4.5 and 96.0$\pm$4.0% in Na^{+}-containing and Na^{+}-free solutions, respectively. In Na^{+}-free solutions, block of the

slow phase of $^{45}Ca^{2+}$ influx was not overcome by increasing $[Ca^{2+}]_e$. Results of this study indicate that 1) MeHg affects Ca^{2+} influx into synaptosomes in the absence of depolarization; 2) block of the fast phase of $^{45}Ca^{2+}$ influx by MeHg is only weakly antagonized by Ca^{2+}_e; and 3) block of fast $^{45}Ca^{2+}$ influx was independent of $[Na^+]_e$; 4) block and reversal of block of the slow phase of $^{45}Ca^{2+}$ influx by MeHg are highly Na^+-dependent.

INTRODUCTION

The hypothesis states that effects of MeHg on voltage-sensitive Ca^{2+} channels which are involved in neurotransmitter release at the motor nerve terminal may contribute to disruption of neurotransmitter release produced by MeHg. This would be best tested by simultaneously measuring Ca^{2+} entry and neurotransmitter release in intact motor nerve terminals. However, several limitations discussed in Chapter One preclude direct measurement of function of Ca^{2+} channels in intact motor nerve terminals. Although Ca^{2+} channel function in intact nerve terminals is difficult to examine using conventional electrophysiological techniques, biochemical measurement of Ca^{2+} channel function can be accomplished easily by measuring $^{45}Ca^{2+}$ flux into isolated nerve terminals, or synaptosomes. Thus, experiments to examine the ability and nature of MeHg interaction with nerve terminal Ca^{2+} channels were performed using synaptosomal preparations.

Heavy metals are known to disrupt Ca^{2+} influx into nerve terminals leading to decreased neurotransmitter release. Divalent heavy metal cations such as Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+} block the influx of $^{45}Ca^{2+}$ into K^+-depolarized synaptosomes in a reversible manner (Nachshen, 1984; Suszkiw *et al.*, 1985; Atchison *et al.*, 1986). Micromolar concentrations of monovalent MeHg also block $^{45}Ca^{2+}$ influx into K^+-depolarized synaptosomes (Atchison *et al.*, 1986). In contrast to inorganic divalent cations, MeHg blocked $^{45}Ca^{2+}$ influx into synaptosomes during 10 s of depolarization in a manner which was not completely antagonized by increasing $[Ca^{2+}]_e$ (Atchison *et al.*, 1986). However, the ability of increasing $[Ca^{2+}]_e$ to

antagonize block by MeHg of fast and slow components of $^{45}Ca^{2+}$ influx was not examined. Studies at the neuromuscular junction demonstrated that after increasing stimulus duration and/or intensity, the subsequent block of nerve-evoked endplate potentials (EPPs) by MeHg could be reversed by increasing $[Ca^{2+}]_e$ (Traxinger and Atchison, 1987b). Since increasing $[Ca^{2+}]_e$ can, under the conditions described above, reverse the effects of MeHg at the neuromuscular junction, and antagonize block of synaptosomal $^{45}Ca^{2+}$ influx during 10 s of K^+-induced depolarization (total influx), experiments were designed to examine the ability of increasing $[Ca^{2+}]_e$ to antagonize block by MeHg of the fast and slow phases of Ca^{2+} influx into synaptosomes.

When examining block by MeHg of the fast and slow phases of $^{45}Ca^{2+}$ influx into synaptosomes, consideration must be given to the mechanisms mediating Ca^{2+} influx during each of the two phases. As mentioned previously, the fast phase is mediated by voltage-dependent Ca^{2+} channels, while the slow phase is mediated largely by a reversed Na^+/Ca^{2+} exchange mechanism. Because the two phases differ in their dependence on $[Na^+]_e$, it is possible that the block of $^{45}Ca^{2+}$ influx by MeHg during the fast and slow phases may also exhibit a differential dependence on $[Na^+]_e$. Thus, experiments were designed to test the dependence of block of each phase by MeHg on $[Na^+]_e$.

MeHg is also capable of forming mercaptides with sulfhydryl groups (Klaassen, 1985) and reacting with amino, carbonyl and hydroxyl groups (Bidstrup, 1964), all of which can be constituents of membranes or membrane proteins. Because of its reactivity, MeHg can affect a variety of membrane-associated functions. MeHg alters

the resting membrane potential and blocks the action potential in the squid giant axon (Huneeus-Cox *et al.*, 1966), and increases its threshold for excitation (Shrivastav *et al.*, 1976). In addition, MeHg alters Na^+- and K^+-channel function (Quandt *et al.*, 1982; Shrager, 1977) and permeability (Passow and Rothstein, 1960). At rest, permeability of the membrane to Ca^{2+} may be mediated in part by random openings of Ca^{2+} channels, active transport proteins or non-specific permeability. Therefore, effects of MeHg on depolarization-independent entry of $^{45}Ca^{2+}$ into synaptosomes were examined as a measure of the specificity of MeHg for Ca^{2+} channel-mediated transport.

MATERIALS AND METHODS

Materials. Methylmercuric acetate was obtained from Pfaltz and Bauer, Inc. (Stamford, CT) and was dissolved in 4% (v/v) glacial acetic acid to make a 2 mM stock solution. Aliquots of this solution were diluted in the appropriate buffers and adjusted to pH 7.4 at room temperature (25 °C) to yield the final concentrations of MeHg indicated in the text of chapters 3 and 4. N-methyl-glucamine, ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from United States Biochemical Corporation (Cleveland, OH). $^{45}CaCl_2$, $^{133}BaCl_2$ and $^{85}SrCl_2$ (specific activities 15-47, 11 and 5.8 mCi/mg, respectively) were purchased from New England Nuclear Co. (Boston, MA). For incubation of synaptosomes, normal physiological saline contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; d-glucose, 10; and HEPES, 10. For $^{45}Ca^{2+}$ influx experiments, elevated K^+ solution contained (mM): NaCl, 72.5; KCl, 77.5; $MgCl_2$, 1; $CaCl_2$, 0.04; d-glucose, 10; and HEPES, 10. Normal K^+ (5 mM) solution contained the same constituents and amounts as did elevated K^+ solution except that $[K^+]$ was lowered to 5 mM by replacement of KCl with 72.5 mM choline chloride. Ca^{2+}-free solutions contained no added $CaCl_2$, but may have contained residual amounts of Ca^{2+} (1-2 μM). In Na^+-free experiments, NaCl was replaced in all solutions by choline chloride on an equimolar basis. Quenching solution contained (mM): KCl, 5; $MgCl_2$, 2; EGTA, 1; d-glucose, 10; HEPES, 10; and N-methylglucamine, 145. N-methylglucamine, which replaces Na^+

in the buffer solution, does not permeate the voltage-dependent Na^+ channel (Hille, 1971). All solutions were adjusted to pH 7.4 at room temperature (25 °C).

Synaptosomal preparation. Synaptosomes were prepared from forebrains of male Sprague-Dawley rats (Harlan, 175-200 gm) using a modification of the method of Gray and Whittaker, (1962) as described by Atchison *et al.* (1986, 1988). Forebrains were homogenized in 0.32 M sucrose and centrifuged for 10 min at 1000xg. The pellet was discarded and the supernatant was centrifuged at 17,500xg for 20 min. This pellet was resuspended in fresh 0.32 M sucrose and layered onto a discontinuous sucrose gradient of 0.8 and 1.2 M sucrose. After centrifuging for 2 h at 69,000xg, the synaptosome-enriched fraction was removed from the interface between the 0.8 and 1.2 M sucrose layers and diluted slowly 1:1 (v/v) with physiological saline. This step facilitates return of the synaptosomal preparation to physiological osmolarity. The synaptosomes were repelleted by centrifugation at 10,000xg for 10 min and then resuspended to an approximate concentration of 20-40 mg/ml in physiological saline (Lowry *et al.*, 1951). This step returns the synpatosomal preparation to physiological osmolarity and removes sucrose from the solution. As the synaptosomal preparation was performed at 0-4 °C, synaptosomal suspensions were incubated in a 37 °C water bath for 20 min prior to their use.

$^{45}Ca^{2+}$ influx measurements. $^{45}Ca^{2+}$ influx was measured as described by Nachshen and Blaustein (1980; 1982) by adding 50 μl of synaptosomal suspension (approximately 20 mg protein/ml) to 50 μl of either elevated (77.5 mM) or normal (5 mM) [K^+] solution containing 0.06 mM $^{45}Ca^{2+}$ and MeHg, when appropriate.

Actual volume of synaptosomes in the suspension is presumed to be negligable, as protein concentrations are low. Therefore, the final K^+ concentration of the depolarizing solution was 41.25 mM. After 1 s (fast phase) or 10 s (total influx) of incubation, 2 ml of ice-cold quench solution was added to stop $^{45}Ca^{2+}$ influx. A metronome was used to facilitate measurements at 1 s. Predepolarized (slow phase) influx was measured by adding 25 μl of synaptosomal suspension (approximately 40 mg protein/ml) to 25 μl of Ca^{2+}- and MeHg-free elevated $[K^+]$ solution for 10 s, followed by the addition of 50 μl of elevated $[K^+]$ solution containing 0.06 mM of $^{45}Ca^{2+}$ and MeHg (when appropriate). After an additional 10 s, the reaction was quenched as above. A stopwatch was used to measure 10 s intervals. The final extracellular concentration of Ca^{2+} plus $^{45}Ca^{2+}$ ($[Ca^{2+}]_e$) was always 50 μM, unless otherwise noted. In experiments in which $[Ca^{2+}]_e$ was increased, the molar ratio of $^{45}Ca^{2+}/Ca^{2+}$ was maintained at a constant value.

Immediately following addition of quenching solution, samples were filtered through Millipore filters (0.45 μm) and washed with two 5 ml aliquots of ice-cold quenching solution. Filters were placed into scintillation vials containing 1.5 ml of Triton X-100/ HCl solubilizer and 10 ml of scintillation cocktail was added after 10 min. Radiotracer content was determined in a Searle Mark III scintillation counter with a 70% efficiency for $^{45}Ca^{2+}$, or in a Searle model 1197 gamma counter with efficiencies of 47 and 53% for $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$, respectively. Depolarization-induced $^{45}Ca^{2+}$ influx was determined by subtracting the average of three replicate values obtained in 5 mM K^+ solution from the average of three replicate values

obtained in 41.25 mM K^+ solutions and converting the net value to nmol $^{45}Ca^{2+}/\mu g$ of synaptosomal protein (Lowry *et al.*, 1951).

Statistical analysis. All dose-response data were analyzed using a randomized block analysis of variance (ANOVA) and Dunnett's one-tailed t-test (Steele and Torrie, 1980). Data from experiments in which $[Ca^{2+}]_e$ was increased were analyzed using a split-plot ANOVA and, if significant differences were found, comparisons were made using the least significant difference test. Comparisons between data obtained in Na^+-containing and Na^+-free solutions were made using a paired Student's t-test. For all comparisons, statistical significance was defined by $p \leq 0.05$.

RESULTS

Effects of MeHg on depolarization-independent entry of $^{45}Ca^{2+}$ into synaptosomes. Because of the ability of MeHg to alter a variety of membrane-associated functions, its ability to disrupt those mechanisms which help to regulate cytosolic Ca^{2+} in synaptosomes in the absence of depolarization was investigated. Synaptosomes were incubated in normal (5 mM) K^+ solutions in the presence of MeHg and $^{45}Ca^{2+}$ for 1 or 10 s, and then assessed for $^{45}Ca^{2+}$ content. Compared to untreated controls, MeHg had no significant effect on depolarization-independent $^{45}Ca^{2+}$ entry into synaptosomes at concentrations up to 50 μM, but significantly reduced $^{45}Ca^{2+}$ entry at concentrations of 125 and 250 μM (Figure 3.1). Thus, during exposures of 1 and 10 s, $^{45}Ca^{2+}$ entry into synaptosomes is suppressed, but only by high concentrations of MeHg. As noted in the Methods section, this component of influx is subtracted from depolarization-induced influx. Thus, this effect did not contribute to block of depolarization-induced influx by MeHg.

Effects of MeHg on depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes. Depolarization of synaptosomes with elevated [K^+] solutions resulted in two components of influx (Figure 3.2). A rapid component which inactivated after approximately 2 s and a slower component which saturated after approximately 20 s. Furthermore, influx was linearly related to the concentration of protein in the synaptosomal preparation (Figure 3.3).

The effects of increasing concentrations of MeHg on total $^{45}Ca^{2+}$ influx into synaptosomes are shown in Figure 3.4A. In contrast to the effects of MeHg described

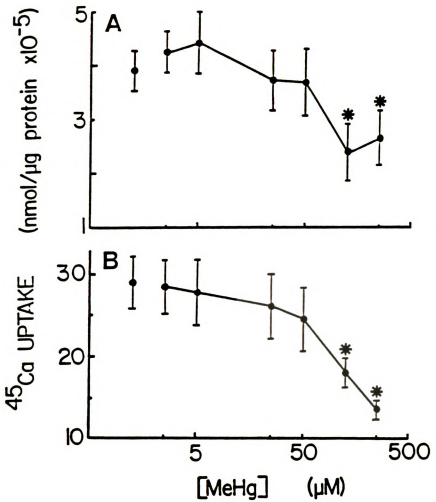

Figure 3.1. MeHg blocks resting $^{45}Ca^{2+}$ entry into synaptosomes. Effects of MeHg on depolarization-independent $^{45}Ca^{2+}$ influx into synaptosomes during 1 s (A) or 10 s (B). Synaptosomes were incubated in 5 mM K^+ solutions for 1 or 10 s in the presence of $^{45}Ca^{2+}$ and various concentrations of MeHg. Values are the mean ± SEM of three or five separate experiments, respectively. Data points not connected indicate MeHg-free control values and the asterisks (*) indicate values which are significantly less than control ($p \leq 0.05$).


$^{45}Ca^{2+}$ INFLUX

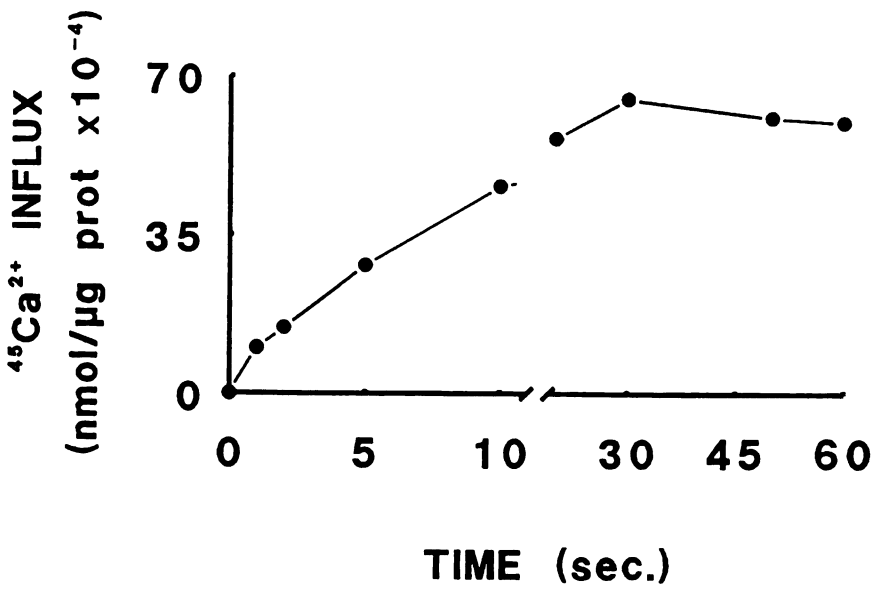

Figure 3.2. Influx of $^{45}Ca^{2+}$ into synaptosomes vs duration of depolarization. $^{45}Ca^{2+}$ influx into synaptosomes induced by depolarization in elevated [K^+] solutions as a function of time. Two phases are illustrated: a fast component which lasts approximately 2 s, and a slower component which lasts up to 60 s. The values shown are the mean ± SEM of three experiments, where the values for any one experiment are the average of three replicates.

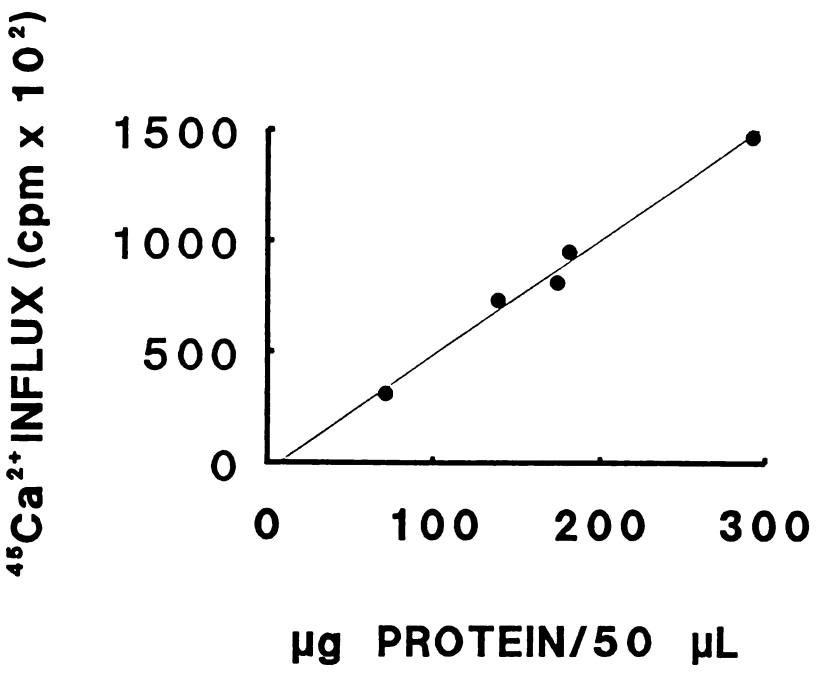

Figure 3.3. Influx of $^{45}Ca^{2+}$ into synaptosomes as a function of protein concentration. Values from a single representative experiment comparing $^{45}Ca^{2+}$ influx with protein content of the synaptosomal suspension. The line is the linear least-squares regression of influx on protein content. Values for influx are the average of three replicates.

for depolarization-independent influx of Ca^{2+}, depolarization-induced influx was more prominently suppressed by MeHg. At concentrations above 5 μM, MeHg caused a concentration-dependent suppression of $^{45}Ca^{2+}$ influx which reached approximately 100% inhibition with 250 μM MeHg. Concentrations of MeHg $\geq$ 25 μM significantly depressed $^{45}Ca^{2+}$ influx when compared to untreated controls. An estimated IC_{50} value for MeHg effects on total influx is 75 μM. These results are similar to those of Atchison *et al.* (1986).

When effects of MeHg on the two distinct phases of depolarization-induced $^{45}Ca^{2+}$ influx were examined, tracer influx was again reduced in a concentration-dependent manner by MeHg. Increasing concentrations of MeHg caused a dose-dependent suppression of $^{45}Ca^{2+}$ influx during the fast phase (Figure 3.4B) which reached a maximal effect of approximately 90% with 250 μM MeHg. Nearly identical effects of MeHg were observed on the slow phase of $^{45}Ca^{2+}$ influx (Figure 3.4C). The estimated IC_{50} value for MeHg is also approximately 75 μM for both the fast and slow phases of $^{45}Ca^{2+}$ influx.

Antagonism of block of $^{45}Ca^{2+}$ influx in response to MeHg by increasing $[Ca^{2+}]_e$. The effect of increasing $[Ca^{2+}]_e$ from 0.01 to 1.15 mM on block of total $^{45}Ca^{2+}$ influx by MeHg is shown in Figure 3.5A. This concentration of MeHg approximated the estimated IC_{50} value. Block of $^{45}Ca^{2+}$ influx by 50 μM MeHg was decreased from approximately 98% to 75% by increasing $[Ca^{2+}]_e$ from 0.01 to 1.15 mM. However, at each $[Ca^{2+}]_e$ tested, there was significantly less influx of $^{45}Ca^{2+}$ in the presence of 50 μM MeHg than for the corresponding untreated control.

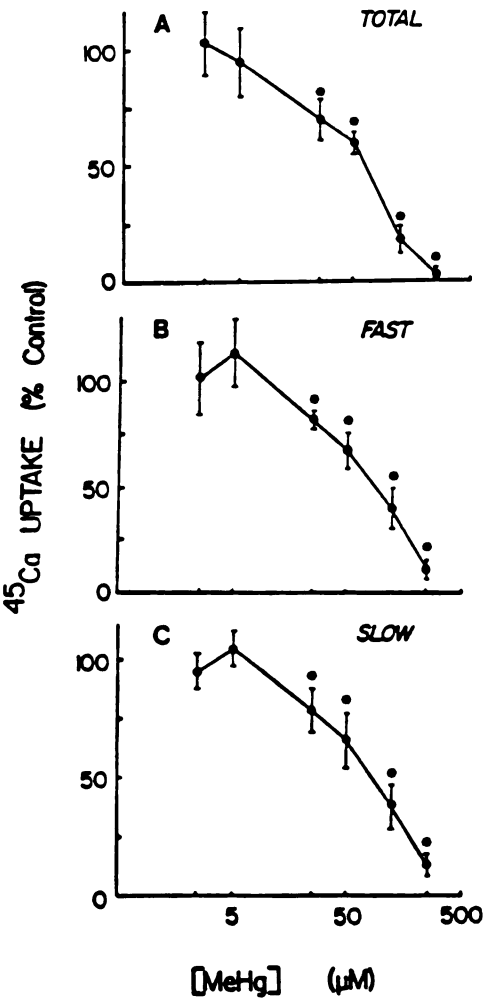

Figure 3.4. MeHg blocks depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes. Effects of MeHg on influx of $^{45}Ca^{2+}$ into rat forebrain synaptosomes during 10 (TOTAL, A) and 1 (FAST, B) s of depolarization with 41.25 mM KCl, or during 10 s of depolarization which was preceded by a 10 s predepolarization in the absence of Ca^{2+} and MeHg to inactivate the fast phase (SLOW, C). Synaptosomes in physiological saline solution (5 mM K^+) were added to a reaction mixture which contained 77.5 mM KCl, 40 μM $CaCl_2$, 0.06 mM $^{45}CaCl_2$, and MeHg. Final concentrations of MeHg ranged from 2.5 to 250 μM. Influx in 5 mM K^+ solutions was subtracted from that in 41.25 mM K^+ as depolarization-independent background. All values are the mean ± SEM of four to six different experiments. The values for any individual experiment are the average of three replicates. When SE bars are not shown, the SE is smaller than the size of the symbol. The asterisks (*) indicate results that are significantly less than control values ($p \leq 0.05$).

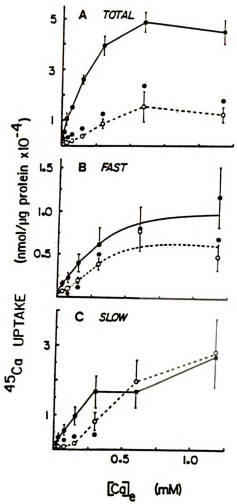

Figure 3.5. Block of Ca^{2+} channels by MeHg is not reversed by increasing $[Ca^{2+}]_e$. Dependence on $[Ca^{2+}]_e$ of $^{45}Ca^{2+}$ influx into synaptosomes in the absence (solid circles) and presence (open circles) of 50 μM MeHg during A) 10 s, or B) 1 s of K^+-induced depolarization, or C) 10 s of depolarization following a 10 s predepolarization in the absence of $[Ca^{2+}]_e$. $^{45}Ca^{2+}$ influx during TOTAL, FAST and SLOW phases was measured as described in Figure 3.4. $[Ca^{2+}]_e$ ranged from 0.01 to 1.2 mM, and the mole to mole ratio of $^{45}Ca^{2+}$ to Ca^{2+} was held constant at 0.22. Values are the mean± SEM of five to seven different experiments. The values for any individual experiment are the average of three replicates. When SE bars are not shown, the SE is smaller than the size of the symbol. The asterisks (*) indicate results that are significantly less than control values ($p \leq 0.05$).

Figure 3.5B depicts the effect of increasing $[Ca^{2+}]_e$ on block of the fast phase of $^{45}Ca^{2+}$ influx by 50 μM MeHg. Once again, in the presence of MeHg, increasing $[Ca^{2+}]_e$ did not overcome the block of depolarization-dependent $^{45}Ca^{2+}$ influx.MeHg reduced $^{45}Ca^{2+}$ influx by approximately 50% at all $[Ca^{2+}]_e$ tested, except 0.01 and 0.62 mM. With the exception of the value for 0.62 mM $[Ca^{2+}]_e$, $^{45}Ca^{2+}$ influx at each $[Ca^{2+}]_e$ tested was significantly lower in the presence of MeHg, when compared to the respective control values.

In contrast to the results observed for both total and fast influx, block of the slow phase of $^{45}Ca^{2+}$ influx was reversed completely by increasing $[Ca^{2+}]_e$ (Figure 3.5C). At $[Ca^{2+}]_e$ below 0.3 mM, 50 μM MeHg caused significantly less influx of $^{45}Ca^{2+}$ when compared to control values, reducing $^{45}Ca^{2+}$ influx by 50 to 90%. However, above 0.3 mM $[Ca^{2+}]_e$, $^{45}Ca^{2+}$ influx returned to control levels despite the presence of MeHg. It should be noted that the sum of the fast and slow components does not equal the amount of total $^{45}Ca^{2+}$ influx. Two factors account for this. The first is that the fast phase is largely inactivated by the 10 s predepolarization step when slow influx is measured. However, the fast phase does contribute an unknown amount to the 10 s of $^{45}Ca^{2+}$ influx measured for the total component. The second factor is that total influx is measured over the first 10 s of depolarization, whereas the slow component of influx is measured over seconds 10-20 of depolarization following a 10 s predepolarization. Small differences in the slow component of influx may therefore exist in the two intervals during which total and slow influx are measured.

Dependence of MeHg-induced block of $^{45}Ca^{2+}$ influx on $[Na^+]_e$. The dependence of block of $^{45}Ca^{2+}$ influx on $[Na^+]_e$ in each phase of influx was examined by using synaptosomes incubated in solutions in which NaCl had been replaced by choline chloride on an equimolar basis. MeHg (50 μM) caused nearly complete suppression of $^{45}Ca^{2+}$ influx into synaptosomes incubated in Na^+-containing and Na^+-free solutions (data not shown). Figure 3.6A depicts effects of 25 μM MeHg on the fast phase of $^{45}Ca^{2+}$ influx into synaptosomes incubated in Na^+-containing and Na^+-free solutions. Synaptosomal $^{45}Ca^{2+}$ influx was significantly reduced to approximately 50% of the MeHg-free control value in both Na^+-containing and Na^+-free solutions.

Figure 3.6B depicts effects of 25 μM MeHg on the slow phase of $^{45}Ca^{2+}$ influx in Na^+-containing and Na^+-free solutions. In the absence of MeHg (open bars), influx of $^{45}Ca^{2+}$ into synaptosomes incubated in Na^+-free solutions is reduced greatly compared to that in Na^+-containing solution. Since slow phase $^{45}Ca^{2+}$ influx into synaptosomes may be due largely to reversal of electrogenic Na^+/Ca^{2+} exchange (Coutinho *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986), removal of Na^+ from the incubation medium would deplete the synaptosomes of Na^+, eliminating or greatly reducing Ca^{2+} uptake via Na^+/Ca^{2+} exchange. In the presence of 25 μM MeHg, $^{45}Ca^{2+}$ influx into Na^+-containing and Na^+-free synaptosomes was reduced to values significantly less than their respective MeHg-free controls.

The comparative blocking effect of 25 μM MeHg in both Na^+-containing and Na^+-free solutions in shown for both fast and slow phases of $^{45}Ca^{2+}$ influx in Figure 3.7. In the slow phase, 25 μM MeHg inhibited 26.1±4.5 and 96.0±4.0% of control

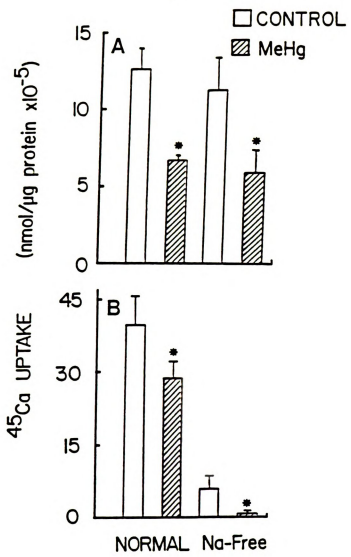

Figure 3.6. Fast and slow components of influx have differential dependence on Na_e. Dependence of block of $^{45}Ca^{2+}$ influx into synaptosomes by MeHg on $[Na^+]_e$ during A) 1 s (FAST) of K^+-induced depolarization or B) 10 s of K^+-induced depolarization following a 10 s predepolarization in the absence of Ca^{2+} and MeHg (SLOW). Synaptosomes were incubated in normal Na^+ (145 mM) or Na^+-free (NaCl replaced by choline chloride) solutions in the absence (open bars) or presence (striped bars) of 25 μM MeHg. Values shown are the mean ± SEM of five individual experiments. Values for any individual experiment are the average of three replicates. The asterisks (*) indicate results that are significantly less than control values ($p \leq 0.05$).

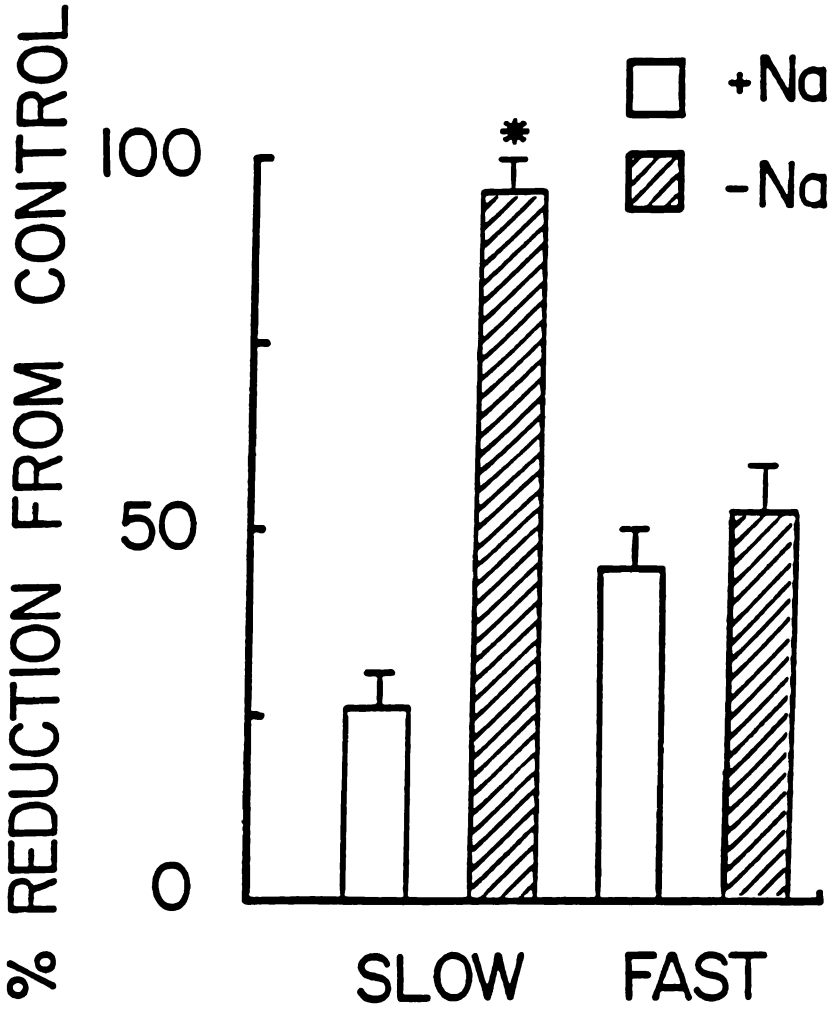

Figure 3.7. In the absence of Na_e, block of slow influx by MeHg is much greater in magnitude. Reduction of $^{45}Ca^{2+}$ influx from control values by 25 μM MeHg during the slow and fast phases of influx in normal (open bar) and Na^+-free (striped bars) solutions. All other conditions are the same as those in Figure 3.4. The asterisk (*) indicates a significantly greater percent reduction by 25 μM MeHg in Na^+-free solution than in normal Na^+-containing solution ($p \leq 0.05$).

tracer influx in synaptosomes incubated in Na^+-containing and Na^+-free solutions, respectively. Thus, block of the slow phase of $^{45}Ca^{2+}$ influx was significantly more pronounced when Na^+_e was deleted from the solutions. For the fast phase, 25 μM MeHg inhibited control tracer influx by 44.6±5.1% in Na^+-containing and by 51.8±6.3% in Na^+-free solutions, respectively. This result is consistent with the Na^+-independence of the fast phase of $^{45}Ca^{2+}$ influx.

Complete antagonism of block by MeHg of slow $^{45}Ca^{2+}$ influx by increasing $[Ca^{2+}]_e$ may be due to competitive inhibition of the various components of influx associated with this phase, or to an irreversible block of one component by MeHg which is counteracted by increasing $[Ca^{2+}]_e$ due to a lack of effect of MeHg on some other component(s) of influx. For example, MeHg may not affect the exchange-mediated component of the slow phase of influx, but may reduce or block the other component(s) of this phase of influx. This type of action by MeHg would enable an operating exchange component to induce a functional antagonism of MeHg-induced block of the other component(s) of influx. Thus, the true efficacy of the blocking action of MeHg on the other component(s) of slow influx can only be observed when the exchange-mediated component is made inoperative, such as by the removal of Na^+ from the medium. As $[Ca^{2+}]_e$ is increased, the amount of Ca^{2+} uptake by the reversed Na^+/Ca^{2+} exchanger is greater due to the increased driving force. If $[Ca^{2+}]_e$ is increased to a high enough level, the resulting uptake may completely mask the block by MeHg of other component(s) of influx. If MeHg blocks slow influx by this mechanism, block of $^{45}Ca^{2+}$ influx should not be reversed when Na^+ is removed from

the medium. This hypothesis was tested and the results are illustrated in Figure 3.8. At $[Ca^{2+}]_e$ = 0.15 mM, $^{45}Ca^{2+}$ influx was $2.4 \pm 0.7 \times 10^{-5}$ and $0.6 \pm 0.6 \times 10^{-5}$ nmol $^{45}Ca^{2+}/\mu g$ protein in the absence and presence of 25 μM MeHg, respectively. When $[Ca^{2+}]_e$ was increased to 1.15 mM, the values for $^{45}Ca^{2+}$ influx in the absence and presence of 25 μM MeHg were $16.1 \pm 4.9 \times 10^{-5}$ and $0.5 \pm 0.5 \times 10^{-5}$ nmol $^{45}Ca^{2+}/\mu g$ protein, respectively. Thus, MeHg-induced block of $^{45}Ca^{2+}$ influx was not antagonized by increasing $[Ca^{2+}]_e$ in the absence of Na^{+}_e.

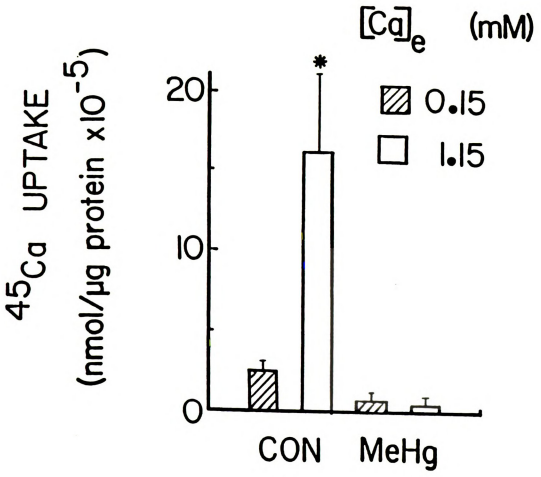

Figure 3.8. In the absence of Na_e, block of slow influx by MeHg is not reversed by increasing $[Ca^{2+}]_e$. Effects of increasing $[Ca^{2+}]_e$ on block of slow $^{45}Ca^{2+}$ influx by MeHg (25 μM) in Na^+-free solutions. $^{45}Ca^{2+}$ influx was measured in solutions which contained 0.15 or 1.15 mM Ca^{2+}. All other conditions are as in Figure 3.4. Values shown are the mean± SEM of five individual experiments. Values for any individual experiment are the average of three replicates. The asterisk (*) indicates results that are significantly increased by increasing $[Ca^{2+}]_e$ from 0.15 to 1.15 mM ($p \leq 0.05$).

DISCUSSION

This study confirms previous results showing a dose-dependent suppression of depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes by MeHg, and partial antagonism of MeHg-induced block of total influx by increasing $[Ca^{2+}]_e$ (Atchison *et al.*, 1986). In addition, the present experiments characterize further the mechanisms of blocking action of MeHg on Ca^{2+} influx into nerve terminals. More specifically, these results suggest that: 1) high concentrations of MeHg depress depolarization-independent $^{45}Ca^{2+}$ entry into synaptosomes; 2) block of the fast and slow phases of $^{45}Ca^{2+}$ influx by MeHg differ with respect to their sensitivity to antagonism by increasing $[Ca^{2+}]_e$; 3) the magnitude of block of the slow phase by MeHg is increased in the absence of Na^+_e; and 4) block of the slow phase is not antagonized by increasing $[Ca^{2+}]_e$ when synaptosomes are incubated in Na^+-free solutions.

Incubation of synaptosomes in non-depolarizing solution containing MeHg resulted in decreased $^{45}Ca^{2+}$ entry after 1 and 10 s when compared to MeHg-free controls. These results indicate that high concentrations of MeHg reduce the permeability of synaptosomes for Ca^{2+}. MeHg increases K^+ permeability in yeast (Passow and Rothstein, 1960) and increases leakage conductance in squid axon (Shrivastav *et al.*, 1976). Interestingly, MeHg has been shown to increase Ca^{2+} permeability in synaptosomes (Komulainen and Bondy, 1987), but only after long periods of incubation. The decrease in Ca^{2+} permeability produced by MeHg in the present experiments may be due specifically to block of Ca^{2+} entry via random openings of channels, a decrease in the frequency of random openings resulting from

a K^+-induced hyperpolarization, or an effect on membrane Ca^{2+} transport systems. Given the ability of MeHg to block depolarization-dependent Ca^{2+} influx via Ca^{2+} channels, it is possible that MeHg may also block Ca^{2+} entry via random openings of channels in non-depolarized membranes. Alternatively, MeHg alters Na^+ and K^+ gating mechanisms in neuroblastoma cells (Quandt *et al.*, 1982) and may therefore alter Ca^{2+} channel function indirectly. If MeHg were to increase K^+ permeability in synaptosomes as it does in yeast (Passow and Rothstein, 1960), then hyperpolarization of the membrane would result in a decreased frequency of random openings of Ca^{2+} channels, thus decreasing the permeability of synaptosomes for Ca^{2+}. The lack of alteration by MeHg of decay of miniature endplate potential (MEPP) frequency (Traxinger and Atchison, 1987a) and the ability of increasing $[Ca^{2+}]_e$ to reverse block of the slow phase of depolarization-induced influx indicate that membrane Ca^{2+} transport systems, such as Na^+/Ca^{2+} exchange may be less susceptible to disruption by MeHg. Finally, MeHg causes release of Ca^{2+} from intraterminal stores, such as the mitochondria (Levesque and Atchison, 1987; 1988; 1990). Increases in intrasynaptosomal $[Ca^{2+}]$ may decrease random opening of Ca^{2+} channels resulting from Ca^{2+}-dependent channel inactivation (Eckert and Chad, 1984). Thus, MeHg may reduce Ca^{2+} permeability by decreasing Ca^{2+} entry via random openings of channels, or indirectly by decreasing the frequency of random openings of Ca^{2+} channels.

In contrast to its effects on depolarization-independent $^{45}Ca^{2+}$ entry into synaptosomes, these results indicate that MeHg is a potent and effective inhibitor of

depolarization-induced $^{45}Ca^{2+}$ influx. Atchison *et al.*, (1986) have shown previously that MeHg reduces influx of $^{45}Ca^{2+}$ into synaptosomes and that increasing $[Ca^{2+}]_e$ only partially antagonizes block of total influx of $^{45}Ca^{2+}$ by MeHg. Inasmuch as total influx consists of at least two distinct components, the effect of increasing $[Ca^{2+}]_e$ on MeHg-induced block of each phase was examined to determine the mechanism of interaction of MeHg with Ca^{2+} channels, as well as exchange processes.

Block of the fast phase of influx by MeHg was not significantly antagonized when $[Ca^{2+}]_e$ was increased. Thus, MeHg appears to block the voltage-dependent Ca^{2+} channels which mediate fast influx in a non-competitive, irreversible manner. Fast influx has been associated with release of Substance P (Floor, 1983), acetylcholine (Suszkiw and O'Leary, 1983) and dopamine (Drapeau and Blaustein, 1983, Leslie *et al.*, 1985) from synaptosomes. Thus, the fast phase couples Ca^{2+} entry and subsequent neurotransmitter release from nerve terminals. The ability of increasing $[Ca^{2+}]_e$ to antagonize block of fast influx by MeHg is consistent with the ability of increased $[Ca^{2+}]_e$ to cause partial restoration of EPPs at the neuromuscular junction following block by MeHg (Traxinger and Atchison, 1987b). In isolated nerve terminals, then, MeHg appears to block Ca^{2+} channels which are associated with depolarization-dependent Ca^{2+} entry and subsequent neurotransmitter release. Complete restoration of EPPs following block by MeHg is also dependent on increased duration and/or intensity of stimulus, indicating that MeHg may exert additional effects on membrane excitability at the nonmyelinated portion of the neuron or along the axonal membrane (Traxinger and Atchison, 1987b). It is possible

that MeHg acts at both sites simultaneously or that block of Ca^{2+} channels follows diminished membrane excitability by MeHg.

Increasing $[Ca^{2+}]_e$ resulted in reversal of block of the slow phase of influx by MeHg. This result is by comparison much different from those observed with total or fast influx. However, given the mechanism by which slow Ca^{2+} influx occurs in synaptosomes, this result is neither contradictory nor unexpected. Because the slow phase of influx may be mediated by both a channel component and a reversed Na^+/Ca^{2+} exchange, an explanation of the actions of MeHg on this phase of influx cannot be derived simply on the basis of the results of this experiment, as MeHg may affect either non-inactivated Ca^{2+} channels, or Na^+/Ca^{2+} exchange, or both.

Na^+/Ca^{2+} exchange in synaptosomes is extremely sensitive to the internal and external concentrations of Na^+ (Blaustein and Oborn, 1975; Åkerman and Nicholls, 1981; Nachshen *et al.*, 1986) and is thought to be reversed in synaptosomes due to their tendency to become Na^+-loaded (Coutinho *et al.*, 1984). When the Na^+-dependence of the blocking action of MeHg was examined, MeHg block of fast $^{45}Ca^{2+}$ influx was unaffected by the absence of extracellular Na^+. This is consistent with the Na^+-independent, channel-mediated mechanism of fast influx. The large decrease in slow influx observed in MeHg-free synaptosomes in the absence of Na^+ is also consistent with previous results indicating that the slow phase is mediated largely by reversed Na^+/Ca^{2+} exchange, which can be abolished by the removal of Na^+_e (Coutinho *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986).

When Na^+/Ca^{2+} exchange is inactivated by removal of Na^+, inhibition of the remaining component of slow influx by MeHg is increased greatly. Therefore, MeHg may exert a preferential effect on the channel-mediated component of this phase, which could be offset by an unaffected Na^+/Ca^{2+} exchange in the presence of Na^+. At the neuromuscular junction, MeHg did not alter the rates of decay of frequency of miniature endplate potentials (MEPPs) after repetitive stimulation with Sr^{2+} and Ba^{2+} (Traxinger and Atchison, 1987a). MEPP frequency is directly related to free intraterminal divalent cation concentration, and the inability of MeHg to alter the rate of decay of MEPP frequency suggests that MeHg may not alter the ability of nerve terminal buffering systems, including Na^+/Ca^{2+} exchange, to clear divalent cations from the cytoplasm.

The inability of increasing $[Ca^{2+}]_e$ to reverse block of slow influx in the absence of Na^+_e is consistent with an irreversible, non-competitive block of the channel portion of slow influx. In the presence of Na^+_e this is relieved by a functional, but reversed Na^+/Ca^{2+} exchange. Thus, block of both fast and slow influx by MeHg occurs via an irreversible block of the channel components which mediate each phase. This is a unique action of MeHg when compared to block of Ca^{2+} channels by divalent cations such as Hg^{2+}, Pb^{2+}, Zn^{2+} and Cd^{2+}, in that block of synaptosomal Ca^{2+} influx by these ions is reversed by increasing $[Ca^{2+}]_e$ (Nachshen, 1984; Suszkiw *et al.*, 1985). Whether this difference is due to the reactivity, increased lipophilicity, or monovalent charge of MeHg is not presently known.

In summary, low concentrations of MeHg block channel-mediated $^{45}Ca^{2+}$ influx into K^+-depolarized synaptosomes in a manner which is only partially antagonized by increasing $[Ca^{2+}]_e$, whereas only high concentrations of MeHg affect Na^+/Ca^{2+} exchange. Furthermore, non-depolarized influx of $^{45}Ca^{2+}$ is only depressed by high concentrations of MeHg. These results suggest a unique pattern of interaction of this monovalent, neurotoxic organic cation with Ca^{2+} channels involved in neurosecretion.

CHAPTER FOUR

CHARACTERIZATION OF THE INTERACTIONS OF METHYLMERCURY WITH Ca^{2+} CHANNELS IN SYNAPTOSOMES AND PHEOCHROMOCYTOMA CELLS: RADIOTRACER FLUX AND BINDING STUDIES

ABSTRACT

The interaction of methylmercury (MeHg) with neuronal Ca^{2+} channels in rat forebrain synaptosomes, and dihydropyridine (DHP)-sensitive Ca^{2+} channels in rat pheochromocytoma (PC12) cells was examined using radiotracer flux assays and radioligand binding analyses. In synaptosomes, the influx of $^{45}Ca^{2+}$ was used to examine the voltage- and state-dependence of block of Ca^{2+} channels by MeHg as well as the effects of MeHg on apparent inactivation of $^{45}Ca^{2+}$ influx. In addition, the differential influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ was used to examine the effect of MeHg on the ionic selectivity of synaptosomal Ca^{2+} channels. The ability of MeHg to block $^{45}Ca^{2+}$ influx via a DHP-sensitive Ca^{2+} channel was examined in PC12 cells. Effects of MeHg on binding of [^{3}H]-nitrendipine in synaptosomes and [^{125}I]-ω-conotoxin GVIA (CgTx) in synaptosomes and PC12 cells were measured. In synaptosomes, MeHg blocked $^{45}Ca^{2+}$ influx in a voltage-dependent manner, as increasing the extracellular K^+ concentration increased the magnitude of block by 100 μM MeHg. When synaptosomes were incubated for 1 s in either a non-depolarizing or a depolarizing solution prior to measuring 1 s of depolarization-induced $^{45}Ca^{2+}$ influx, the potency and efficacy of the block of $^{45}Ca^{2+}$ influx by MeHg were similar. Thus, block of Ca^{2+} channels by MeHg does not appear to be state-dependent. To determine the kinetics of apparent inactivation of $^{45}Ca^{2+}$ influx, synaptosomes were predepolarized in Ca^{2+}-free, high [K^+] solution for intervals varying from 1-10 s prior to measuring 1 s of K^+-induced $^{45}Ca^{2+}$ influx. When compared to control, MeHg (100 μM) altered the rate constant for apparent inactivation and decreased the fraction

of $^{45}Ca^{2+}$ influx which does not inactivate. Influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ during 1 s of depolarization was blocked in a dose-dependent manner by MeHg with estimated IC_{50} values of 125, 150 and > 150 μM for $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$, respectively. In triple-label experiments MeHg decreased influx of all three divalent cations: the effect on $^{85}Sr^{2+}$ influx was significantly greater than on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx. The relative flux of radiolabeled Ca^{2+}:Sr^{2+}:Ba^{2+} was altered from approximately 6:2:3 to 6:1:3 in the presence of 100 μM MeHg. In undifferentiated and nerve growth factor (NGF)-differentiated PC12 cells, K^+-induced $^{45}Ca^{2+}$ influx was blocked by the DHP nifedipine with an approximate IC_{50} value of 5 nM. MeHg reduced $^{45}Ca^{2+}$ influx in PC12 cells with an estimated IC_{50} value of 50 μM, and 125 μM MeHg reduced uptake by >90%. [^{3}H]-nitrendipine bound to synaptosomes with high affinity in normal and elevated [K^+] solutions. In normal [K^+] solutions, Scatchard analysis of the binding data resulted in a K_D value of 630±160 pM and a B_{max} value of 130±40 fmol/mg protein. In the presence of 100 μM MeHg, the values for K_D and B_{max} were 2520±630 pM and 200±30 fmol/mg protein, respectively. Results obtained in the absence and presence of MeHg in elevated [K^+] buffers were not significantly different from those obtained in normal [K^+] buffers. [^{125}I]-ω-conotoxin GVIA bound to a single high affinity site on synaptosomes and PC12 cells with a half saturation occurring in the subnanomolar range. 100 μM MeHg did not significantly alter binding of CgTx in synaptosomes, but did alter CgTx binding in PC12 cells by decreasing the amount of CgTx bound at all concentrations of CgTx tested. These results indicate that MeHg: 1) blocks synaptosomal Ca^{2+} channels in

a voltage- but not state-dependent manner; 2) alters synaptosomal Ca^{2+} channel ionic selectivity and inactivation kinetics; 3) blocks Ca^{2+} influx via DHP-sensitive Ca^{2+} channels in PC12 cells; and 4) alters the binding properties of DHPs in synaptosomes, and CgTx in PC12 cells, but not synaptosomes.

INTRODUCTION

Preliminary experiments demonstrate that MeHg does block Ca^{2+} channels in isolated nerve terminals, and indicate that effects of MeHg on channels are difficult to overcome (Atchison *et al.*, 1986; Shafer and Atchison, 1989). However, these experiments provide little information regarding mechanisms by which MeHg interferes with Ca^{2+} channel function, or the types of Ca^{2+} channels affected by MeHg. However, results of preliminary experiments indicate that there may be interesting differences in the interactions of MeHg and divalent metal cations with neuronal Ca^{2+} channels.

MeHg and divalent metals produce similar disruptions of synaptic transmission at the NMJ and impede the movement of Ca^{2+} through voltage-dependent Ca^{2+} channels. Micromolar concentrations of MeHg (Atchison *et al.*, 1986; Shafer and Atchison, 1989) or inorganic divalent cations (Pb^{2+}, Ni^{2+} and Cd^{2+}) (Nachshen, 1984) block 1 s ("fast" phase) of depolarization-induced influx of $^{45}Ca^{2+}$ into synaptosomes. Increasing $[Ca^{2+}]_e$ concentration readily reverses block of "fast" influx by divalent cations (Nachshen, 1984), but not by MeHg (Atchison *et al.*, 1986; Shafer and Atchison, 1989).

MeHg differs in two important respects from divalent inorganic Ca^{2+} channel blockers: enhanced lipophilicity imparted by the methyl group, and monovalent rather than polyvalent charge. Because data suggest that MeHg disrupts Ca^{2+} entry into the nerve terminal by interacting directly with Ca^{2+} channels, and because chemical differences between MeHg and other heavy metal Ca^{2+} channel blockers

may give rise to differences in action on Ca^{2+} channels, experiments were designed to characterize in detail the nature of the interaction of MeHg with nerve terminal Ca^{2+} channels. Specifically, experiments were designed to determine whether MeHg blocks Ca^{2+} channels in a voltage- and/or state-dependent manner, and whether MeHg is capable of altering the kinetics of apparent inactivation and/or ionic selectivity of synaptosomal Ca^{2+} channels.

The observation that multiple types of Ca^{2+} channels exist in nerve terminals (Penner and Dreyer, 1986; Miller, 1987; Atchison and O'Leary, 1987) raises the question of the identity of Ca^{2+} channels which are blocked by MeHg. As only some types of Ca^{2+} channels have been associated with neurotransmitter release, determination of the types of Ca^{2+} channels affected by MeHg is crucial to determining whether or not effects on Ca^{2+} channels may contribute to the effects of MeHg on transmission at the NMJ. Since the type or types of Ca^{2+} channels in synaptosomal preparations are not well characterized, rat pheochromocytoma (PC12) cells were chosen as an alternative model system for studies of the effects of MeHg on Ca^{2+} channels.

Under normal culture conditions, PC12 cells express predominantly dihydropyridine (DHP)-sensitive Ca^{2+} channels (Takahashi and Ogura, 1983) known as "L" or "long opening" type (Nowycky *et al.*, 1985a,b). When cultured in the presence of NGF, the expression of DHP-insensitive, N-type channels is greatly increased without decreases in the expression of the DHP-sensitive channels. Thus, PC12 cells provide a means by which to examine the effects of MeHg on a

homogenous and/or defined population of Ca^{2+} channel types which are associated with neurotransmitter release.

Finally, the effects of MeHg on the binding of two well-characterized Ca^{2+} channel antagonists were examined. Although the pharmacological binding potency of Ca^{2+} channel antagonists does not necessarily correlate with their functional effects, competitive binding assays nevertheless provide a measure of the ability of MeHg to compete with known Ca^{2+} channel antagonists for their binding sites on nerve membranes. The DHP nitrendipine has been employed in numerous binding and flux studies in synaptosomes (Rampe *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986) and PC12 cells (Toll, 1982) to examine the properties of binding sites which are presumed to be located on or associated with "L"-type Ca^{2+} channels. Therefore, the effects of MeHg on the binding of [^{3}H]-nitrendipine could provide additional information regarding the ability of MeHg to interact with binding sites associated with this type of Ca^{2+} channel.

ω-Conotoxin GVIA (CgTx), a peptide isolated from the venom of the marine snail *Conus geographus*, reduces Ca^{2+} influx in chick synaptosomes (Rivier *et al.*, 1987) and binds to sites on synaptosomes and PC12 cells which appear to be distinct from the DHP binding site (Abe *et al.*, 1986; Cruz and Olivera, 1986; Marqueze *et al.*, 1988; Sher *et al.*, 1988). Therefore, effects of MeHg on binding of CgTx were examined in synaptosomes and PC12 cells to determine whether MeHg may interact with these sites instead of, or as well as, DHP binding sites. Inasmuch as CgTx has been reported to inhibit transmitter release from and $^{45}Ca^{2+}$ influx into isolated nerve

endings, and to bind at distinct sites from the DHPs, its use would permit a differential analysis of binding of MeHg to sites spatially associated with perhaps two different Ca^{2+} channels, or two separate binding sites on Ca^{2+} channels.

MATERIALS AND METHODS

The materials and solutions, procedure for isolation of synaptosomes and general method for measuring radiotracer influx are described in detail in Chapter three. The methods described in this chapter are specific experimental procedures and manipulations required for the experiments in Chapter four. Unless specifically stated, all solutions contained the same constituents and concentrations as those described in Chapter three.

The K^+ dependence of $^{45}Ca^{2+}$ influx was measured in the presence and absence of MeHg (100 μM) by incubating synaptosomes for 2 s in Na-free K^+ solutions containing $^{45}Ca^{2+}$. Choline chloride in normal [K^+] solution was replaced by KCl to achieve the various K^+ concentrations used. To obtain a more accurate estimate of influx at the lower K^+ concentrations used, influx was measured for 2 s at all K^+ concentrations.

The state-dependence of block of the "fast" phase of $^{45}Ca^{2+}$ influx by MeHg was determined by measuring 1 s of $^{45}Ca^{2+}$ influx in synaptosomes which had been exposed to MeHg under conditions in which the population of Ca^{2+} channels would be primarily in the "resting" state, or under conditions in which the population of Ca^{2+} channels would be primarily in the "open" or "inactivated" state. To "open" or "inactivate" Ca^{2+} channels, synaptosomes were predepolarized in 41.25 mM K^+ solution for 10 s by adding 25 μl of synaptosomal suspension (5 mM K^+) to 25 μl of elevated [K^+] (77.5 mM) solution containing MeHg. Ca^{2+} channels in the resting state were exposed to MeHg in 5 mM K^+ solution for 10 s by adding 25 μl of

synaptosomes (5 mM K^+) to 25 μl of normal (5 mM) [K^+] solution containing MeHg. Following the 10 s exposure to MeHg, 1 s of $^{45}Ca^{2+}$ influx was measured by addition of 50 μl of K^+ solution (77.5 or 113 mM K^+) containing 0.06 mM of $^{45}Ca^{2+}$ and MeHg, so that the final K^+ concentration of all solutions was equal to 59.4 mM. One s after the addition of K^+ solution, $^{45}Ca^{2+}$ influx was stopped by the addition of 2 ml of quenching solution. In order to maintain osmolarity conditions equal to those in other experiments and to increase [K^+] to 113 mM, [Na^+] was lowered to 36 mM in all K^+ solutions, being replaced by KCl (113 mM K^+ solution) or choline chloride (physiological saline and 77.5 mM K^+ solutions) on an equimolar basis.

The apparent inactivation of depolarization-induced Ca^{2+} influx was determined by measuring 1 s of $^{45}Ca^{2+}$ influx after various intervals of predepolarization (0-10 s) in nominally Ca^{2+}-free medium to inactivate Ca^{2+} channels (Suszkiw *et al.*, 1986). Briefly, 200 μl of Ca^{2+}-free, elevated (77.5 mM) [K^+] solution with or without MeHg were combined with 200 μl of synaptosomal suspension (5 mM K^+). Following 1 to 10 s of predepolarization, 400 μl elevated [K^+] solution containing 0.02 mM $^{45}Ca^{2+}$ and MeHg were added to the mixture. After an additional 1 s, 3 ml of ice-cold quenching solution were added to stop $^{45}Ca^{2+}$ influx. The final concentration of MeHg before the addition of quenching solution was 100 μM. Non-inactivated influx (1 s) was measured by combining 200 μl of synaptosomal suspension with 600 μl of elevated [K^+] solution containing MeHg (100 μM) and $^{45}Ca^{2+}$. Synaptosomes were retained on Millipore filters and $^{45}Ca^{2+}$ influx was determined as described in Chapter three.

PC12 Cell Experiments. PC12 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere using Dulbecco's modified Eagle medium (pH 7.4, GIBCO) containing 10% horse serum, 5% fetal bovine serum, and 2 mM HEPES. PC12 cells were differentiated by culturing for 7 days in medium containing 100 ng/ml nerve growth factor (NGF) isolated from mouse submaxillary gland. The NGF was a gift from Dr. Steven Heidemann at Michigan State University.

Cells were harvested by suspension in normal (5 mM) [K^+] PC12 medium and centrifugation for 10 min at 5000 x g. This step was repeated twice to wash completely the growth medium from the cells.

The effects of nifedipine (Sigma, St. Louis, MO) and MeHg on depolarization-induced $^{45}Ca^{2+}$ influx were measured using the same method as for synaptosomes, except that elevated [K^+] PC12 medium contained 100 mM K^+ so that the final concentration of K^+ used for depolarization of the PC12 cells was 52.5 mM. Elevated (100 mM) and normal (5 mM) [K^+] PC12 medium used in influx studies with PC12 cells have been described by Stallcup (1979) and are similar to those described for synaptosomes in Chapter three. Influx was measured over 2 min. PC12 cells were not preincubated with nifedipine or MeHg, therefore the only exposure of the PC12 cells to MeHg or nifedipine was the 2 min during which $^{45}Ca^{2+}$ influx was measured. The final concentration of Ca^{2+} was 1 mM. During filtrations, the cells were retained on Whatman GF/B glass fiber filters.

Binding Experiments. The equilibrium binding of [^{3}H]-nitrendipine (specific activity, 70 Ci/mmol) was measured in synaptosomes in the following manner. For

any given experiment, 2 ml of a stock solution containing [^{3}H]-nitrendipine and MeHg (100 μM), when appropriate, was prepared in normal [K^+] solution. Binding of [^{3}H]-nitrendipine was initiated by addition of 20 μl of synaptosomal suspension (250-350 mg protein) to 500 μl of stock solution. After 1 hr of incubation at 25°C, the solution was filtered rapidly through Whatman GF/B glass fiber filters and rinsed with three 5-ml aliquots of quenching solution. To determine total ligand concentration, a 50-μl aliquot was taken from the remaining 500 μl of stock solution. Non-specific binding was measured in the presence of 1 μM nifedipine. Radioactivity remaining on filters was determined as described for $^{45}Ca^{2+}$ influx experiments, except that the scintillation counter contained a ^{3}H quench curve and program to convert cpm to dpm. The average of triplicate values were used for Scatchard analysis (1949) to determine the equilibrium dissociation constant (K_D) value for binding of [^{3}H]-nitrendipine and the density of binding sites (B_{max}). As with all experiments using DHPs, these procedures were carried out in the dark. To measure the binding of ^{3}H-nitrendipine under depolarizing conditions, the exact same procedure was followed using elevated [K^+] solution in place of normal [K^+] solution.

Binding of [^{125}I]-ω-conotoxin GVIA (specific activity 2200 Ci/mmol) to PC12 cells and synaptosomes was measured under non-depolarizing conditions by methods similar to those described for nitrendipine, except that PC12 cell medium was used for binding experiments in PC12 cells. In addition, the effects of pretreatment of synaptosomes with MeHg on CgTx binding were examined in the following manner. Once isolated, synaptosomes were resuspended in 5 ml of physiological saline

containing 100 μM MeHg and incubated at room temperature for 10 min. Following the incubation, synaptosomes were repelleted and washed in MeHg-free physiological saline by centrifugation at 10,000 x g for 10 min and subsequent resuspension. Synaptosomes were then incubated for 25 min at room temperature (25°C) and 10 μl aliquots were used to measure the binding of [^{125}I]-ω-conotoxin GVIA as described above for nitrendipine. In all CgTx binding experiments, non-specific binding was measured in the presence of 1 μM unlabeled CgTx (Peninsula Laboratories, Belmont, CA) and normal [K^+] solution contained 0.1% albumin and no added calcium. After 1 hr, solutions were filtered rapidly over Whatman GF/B filters which had been presoaked in quenching solution containing 0.1% polyethyleneimine (Sigma). Radioactivity remaining on the filters was determined in the Searle model 1197 gamma counter with an efficiency of 53% for ^{125}I. Protein content of synaptosomal and PC12 cell suspensions was determined by the method of Lowry *et al.* (1951).

Statistical analysis. Statistical analysis of results of the inactivation experiments is described in the Results section. For experiments using nifedipine or MeHg to block $^{45}Ca^{2+}$ influx into PC12 cells or to measure the time-course of block by MeHg in synaptosomes, data were analyzed using a randomized block analysis of variance (ANOVA) and, if significant differences were found, individual comparisons were made using Dunnett's one-tailed t-test. For K^+-dependence experiments, a mixed design ANOVA was used to compare influx in control and MeHg-treated synaptosomes; individual comparisons were made using the least significant

difference (lsd) test. To compare the efficiency of block by MeHg at different [K^+], a randomized block ANOVA and Dunnett's one-tailed t-test were used. Data from triple label studies were analyzed using a randomized block ANOVA; individual comparisons were made using Scheffe's test. Comparisons of K_D values for ^{3}H-nitrendipine binding to synaptosomes were made using a mixed design ANOVA and the lsd test. Data from CgTx binding experiments were analyzed using a randomized complete block ANOVA and the lsd test. For all comparisons, statistical significance was defined by $p \leq .05$.

RESULTS

Time Course of Block by MeHg. Block of $^{45}Ca^{2+}$ influx into synaptosomes by MeHg as a function of time is shown in Figure 4.1 (filled circles). MeHg (50 μM) blocked approximately 50% of the $^{45}Ca^{2+}$ influx during the first 1 s of depolarization. There was no statistically significant change in the degree of block by MeHg as a function of time. The influx of $^{45}Ca^{2+}$ into synaptosomes consists of two distinct phases (Nachshen and Blaustein, 1980; Suszkiw *et al.*, 1986): a fast phase, which is mediated by voltage-dependent Ca^{2+} channels, and a slow phase, which is mediated largely by reversed Na^+/Ca^{2+} exchange, but may also contain a residual channel-mediated component (Shafer and Atchison, 1989). As low concentrations of MeHg may not affect reversed Na^+/Ca^{2+} exchange in nerve terminals (Traxinger and Atchison, 1987b; Shafer and Atchison, 1989), uptake via this system may offset the block by MeHg of channel-mediated influx. Therefore, the time-dependent effects of MeHg were examined in medium in which NaCl was replaced by choline chloride on an equimolar basis to prevent uptake via reversed Na^+/Ca^{2+} exchange. Under these conditions, block of $^{45}Ca^{2+}$ influx by MeHg increased as time of exposure during depolarization increased. After 10 or more s of exposure to 50 μM MeHg, block of $^{45}Ca^{2+}$ influx was significantly greater than after only 1 s of exposure (Figure 4.1., open circles). Thus, as the time of exposure to MeHg increased, MeHg blocked a greater portion of channel-mediated, depolarization-induced $^{45}Ca^{2+}$ influx.

K^+(voltage)-Dependence Experiments. In Figure 4.2A, the K^+-dependence of $^{45}Ca^{2+}$ influx into synaptosomes is shown in the absence (solid symbols) and presence

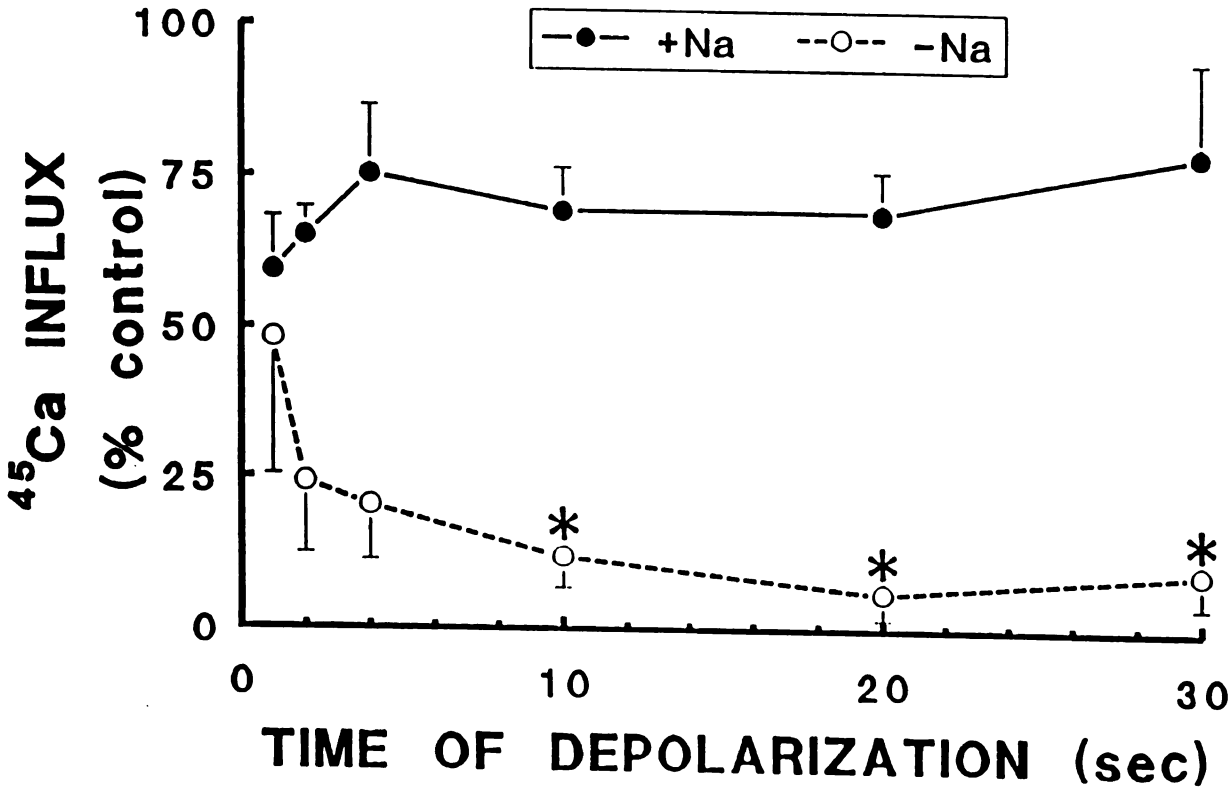

Figure 4.1. Block of $^{45}Ca^{2+}$ influx into synaptosomes vs. time. Block of $^{45}Ca^{2+}$ influx into synaptosomes by MeHg as a function of time of exposure in Na-containing (solid symbols) or Na-free medium (open symbols). $^{45}Ca^{2+}$ influx was measured by the addition of 50 μl of synaptosomes to 50 μl of elevated (77.5 mM) or normal (5 mM) [K^+] solutions containing $^{45}Ca^{2+}$ and 50 μM MeHg. Influx was stopped after various intervals of time by the addition of 2 ml of quenching solution. Influx in normal [K^+] solutions was subtracted to determine depolarization-induced $^{45}Ca^{2+}$ influx. The results are expressed as a percentage of $^{45}Ca^{2+}$ influx in MeHg-free solutions, and are the mean ± SEM of three or four separate experiments, respectively. Values for any particular experiment are the average of three replicates. The asterisk (*) indicates values that are significantly different than ($p < .05$, one-sided t-test) the value at 1 s in Na-containing (significantly greater) or Na-free (significantly less) synaptosomes. After 30 s of depolarization, the values for influx of $^{45}Ca^{2+}$ into synaptosomes in the absence of MeHg were 1.99 ± 0.58 and 7.85 ± 2.22 nmol/μg protein x 10^{-4} in Na-free and Na-containing solutions, respectively.

(open symbols) of 100 μM MeHg. Influx in 5 mM K^+ has not been subtracted from these data. The magnitude of depolarization caused by each K^+ concentration can be estimated by the relationship $\Delta V_m = 60 \log [K^+]_H/[K^+]_L$, where ΔV_m is the change in membrane potential and $[K^+]$ is the K^+ concentration of the normal (L) and elevated (H) $[K^+]$ solutions (Blaustein and Goldring, 1975). Thus, the voltage-dependence of influx can be estimated by this method. The K^+ concentrations used in this experiment will result in depolarizations of 0 to 55 mV from the resting membrane potential, which under similar conditions has been estimated to be approximately -80 mV (Suszkiw *et al.*, 1986). In the absence of MeHg, synaptosomal $^{45}Ca^{2+}$ influx activated at $[K^+]$ above 10 mM, and increased in a linear manner as $[K^+]$ was increased to 41.3 mM. In a separate experiment, higher concentrations did not increase $^{45}Ca^{2+}$ influx further (data not shown). Thus, $^{45}Ca^{2+}$ influx in synaptosomes activates at K^+ concentrations above 10 mM and is maximal at K^+ concentrations of approximately 41 mM. These results are consistent with those of Suszkiw *et al.* (1989). In the presence of 100 μM MeHg, $^{45}Ca^{2+}$ influx increased only gradually as the $[K^+]$ was increased, being significantly less than control values at each respective K^+ concentration. In panel B of Figure 4.2, the percent inhibition of $^{45}Ca^{2+}$ influx by MeHg is plotted as a function of $[K^+]$. At low K^+ concentrations, MeHg blocked approximately 20% of the $^{45}Ca^{2+}$ influx. As the $[K^+]$ was increased, block of uptake by 100 μM MeHg increased to approximately 50% at the highest K^+ concentration tested. The block of $^{45}Ca^{2+}$ influx in 36 mM or

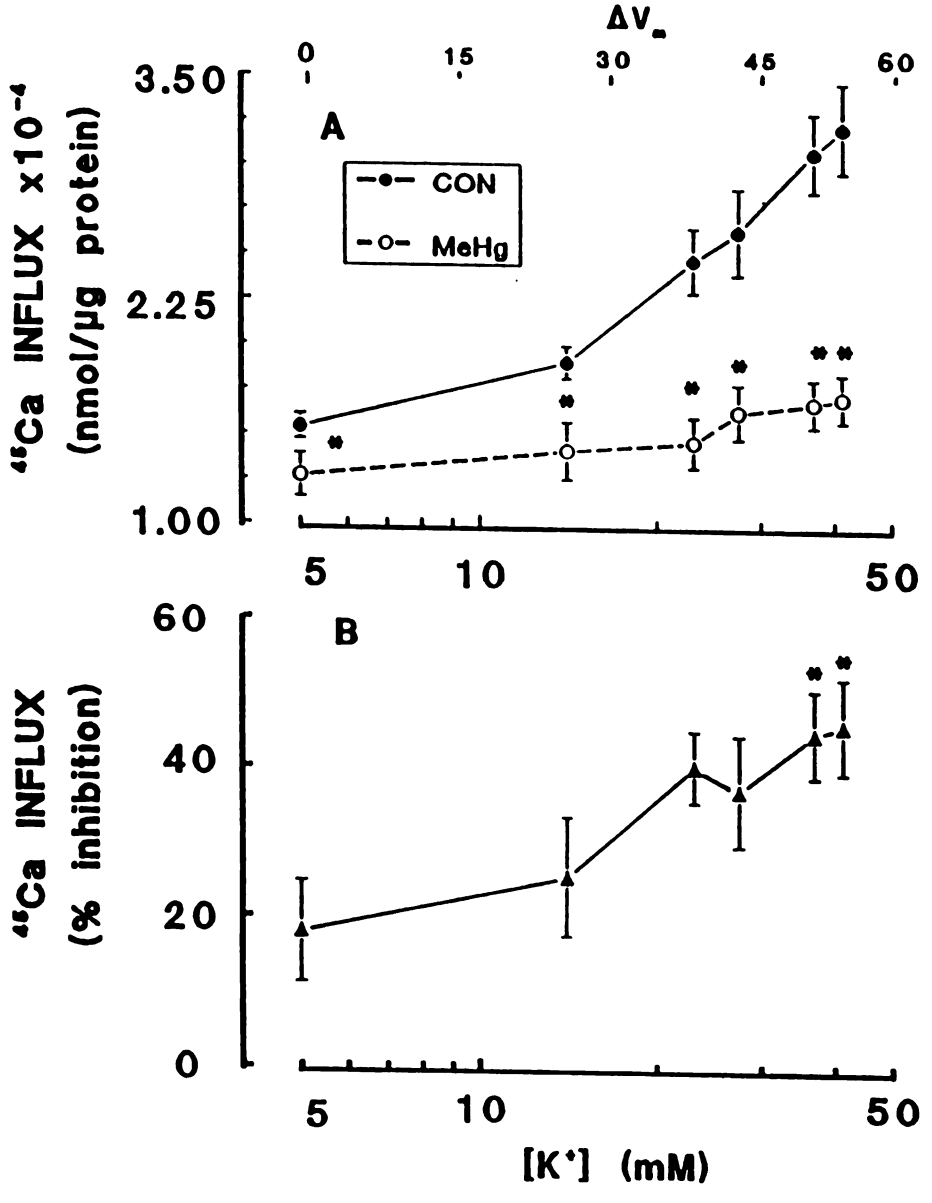

Figure 4.2. Voltage-dependence of $^{45}Ca^{2+}$ influx into synaptosomes and effects of MeHg. A) Effects of $[K^+]$ on influx of $^{45}Ca^{2+}$ into synaptosomes in the absence (solid symbols) and presence of 100 μM MeHg (open symbols). Synaptosomes were incubated for 2 s in Na-free buffers containing $^{45}Ca^{2+}$ and various concentrations of K^+. Influx was stopped by the addition of 2 ml of quenching solution. The asterisk (*) indicates values of influx in the presence of MeHg which are significantly less than ($p<.05$) their respective MeHg-free control value. B) Magnitude of block of $^{45}Ca^{2+}$ influx by 100 μM MeHg at different K^+ concentrations used in part A. The values marked by an asterisk (*) indicate significantly greater block ($p<.0$ 5) by MeHg than the block in 5 mM K^+. All values are the mean ± SEM of four experiments, where each experimental value is the average of three replicates.

higher concentrations of K^+ was significantly greater than that observed in 5 mM K^+ solutions.

State-dependence Experiments. Block of Ca^{2+} channels in excitable membranes frequently exhibits so-called "state-dependence", wherein the blocking affinity of the antagonist is dependent upon the configuration of the channel. Inasmuch as the blocking affinity of certain divalent cations for $^{45}Ca^{2+}$ influx into synaptosomes has been shown to be "state-dependent" (Nachshen, 1985), experiments were designed to determine whether the degree of block by MeHg depended on prior utilization of the channel. This was accomplished by incubating synaptosomes for 10 s in the presence of MeHg in either 5 mM or 41.3 mM K^+ solutions, prior to measuring 1 s of depolarization-induced $^{45}Ca^{2+}$ influx. These K^+ concentrations are below and above the threshold for activation of $^{45}Ca^{2+}$ influx into synaptosomes, respectively. Thus, in synaptosomes incubated in 41.3 mM K^+, MeHg had access to the open and inactivated states of the Ca^{2+} channel, while in synaptosomes incubated in 5 mM K^+ solution, MeHg had access to Ca^{2+} channels in the resting state. After incubation in 5 mM K^+ solution, MeHg caused a dose-dependent suppression of 1 s of $^{45}Ca^{2+}$ influx which reached 84.5±5.0% (mean ± SEM, n=6) reduction with 250 μM MeHg; the estimated IC_{50} value was 75 μM (Figure 4.3, filled circles). When MeHg had access to open or inactivated Ca^{2+} channels prior to measuring 1 s of influx, a dose-dependent suppression of $^{45}Ca^{2+}$ influx was also observed (Figure 4.3, open circles). The estimated IC_{50} value for MeHg was also 75 μM and inhibition was maximum (89.4±4.5%, mean ± SEM, n=5) at 250 μM MeHg. Although $^{45}Ca^{2+}$

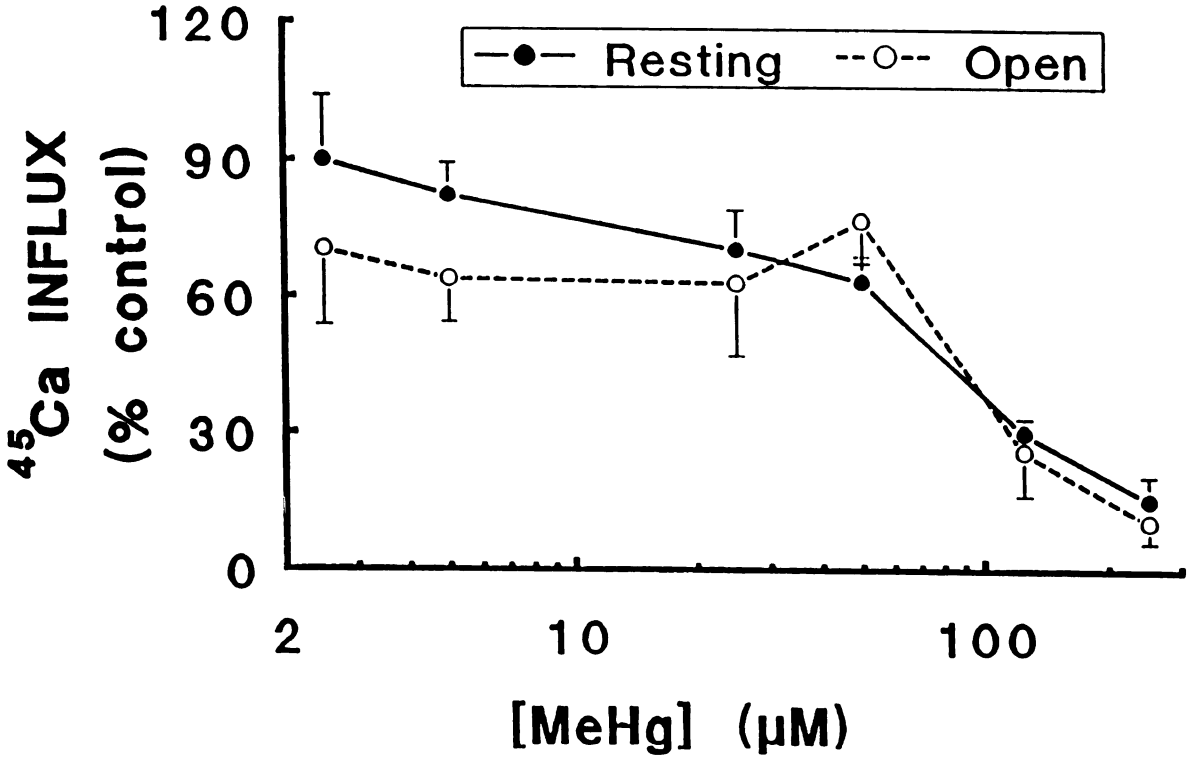

Figure 4.3. State-dependence of the actions of MeHg on $^{45}Ca^{2+}$ influx. Influx measured during 1 s of K^+-induced depolarization following predepolarization for 10 s in the presence of MeHg (open symbols) or following 10s of incubation with MeHg in a non-depolarizing solution (5 mM K^+) (solid symbols). Values are the mean± SEM of six different experiments. For any given experiment, the value was the average of three replicates.

influx at low concentrations of MeHg was reduced to a greater extent in predepolarized synaptosomes than in non-depolarized synaptosomes, there was no significant difference in the reduction of $^{45}Ca^{2+}$ influx under the two conditions. Thus, block of $^{45}Ca^{2+}$ influx by MeHg was not altered when MeHg was allowed access to different states of the Ca^{2+} channel.

Inactivation Experiments. Predepolarization of synaptosomes in the absence of added Ca^{2+} resulted in a time-dependent decrease in $^{45}Ca^{2+}$ influx into synaptosomes when $^{45}Ca^{2+}$-containing solutions were subsequently added (Figure 4.4). This decrease is presumably due to voltage-dependent inactivation of synaptosomal Ca^{2+} channels. The ability of MeHg to alter this process in a quantifiable manner was examined using the following model for apparent inactivation in synaptosomes: $Q = q_1e^{-k_it} + q_2$; where Q is the fraction of Ca^{2+} influx remaining after time t of predepolarization, k is the rate constant for apparent inactivation, and q_1 and q_2 are the fractions of Ca^{2+} influx which do and do not inactivate, respectively. This relationship has been shown previously (Suszkiw *et al.*, 1986) to describe the apparent inactivation of Ca^{2+} influx in synaptosomes under nearly identical experimental conditions. To validate the use of this model, the goodness of fit of these data to the above model was examined by non-linear least squares regression analysis. For control experiments, correlation coefficient values ranged from 0.77 to 0.94. The goodness of fit of these data to several other potential models for inactivation was also examined, including a model for the exponential decay of a voltage-activated current (Belluzzi *et al.*, 1985) which has been used recently to describe the apparent

inactivation of Ca^{2+} influx in synaptosomes incubated in Na^{+}-free medium (Suszkiw *et al.*, 1989). Correlation coefficient values for goodness of fit of these data to other models were less satisfactory than those for the model chosen.

Using the CRUNCH statistical program, non-linear, least-squares regression analysis was performed on the data from each individual experiment to determine k_i, q_1 and q_2. The average values for k_i, q_1 and q_2 for control and MeHg-treated synaptosomes are listed in Table 1 and yield the lines in Figure 4.4 when used in the above function. Significant differences in k_i, q_1 and q_2 in MeHg-treated (n=3) and control (n=5) synaptosomes were tested for using a one-sided Student's t-test ($p \leq .05$). When MeHg-treated synaptosomes were compared to untreated control synaptosomes, the values for k_i, q_1 and q_2 were significantly different. Thus, MeHg alters the kinetics of apparent inactivation of $^{45}Ca^{2+}$ influx in synaptosomes by altering the rate of apparent inactivation and increasing the fraction of influx which inactivates. It should be noted that in the presence of MeHg, inactivation of $^{45}Ca^{2+}$ influx was essentially complete after only 1 s of predepolarization, thus the value for the rate constant k_i only reflects the apparent constant for the rate of inactivation.

Ionic Selectivity Experiments. For dose-response curves, solutions contained a final concentration of 2.4 mM unlabelled Ca^{2+}, Sr^{2+} or Ba^{2+}, and 1 μCi of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ or $^{133}Ba^{2+}$, respectively. MeHg reduced depolarization-induced influx of $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ into synaptosomes in a dose-dependent manner, with estimated IC_{50} values of 125 and 150 μM, respectively (Figure 4.5). Although the IC_{50} value for block of $^{45}Ca^{2+}$ uptake by MeHg is slightly higher than previously reported (Shafer and

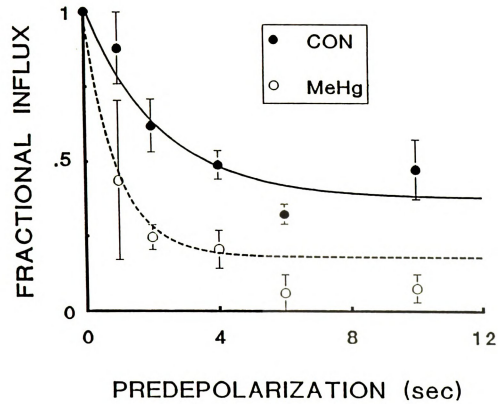

Figure 4.4. Effects of MeHg on apparent inactivation of synaptosomal $^{45}Ca^{2+}$ influx. Effects of MeHg (100 μM) on the rate of apparent inactivation of depolarization-induced $^{45}Ca^{2+}$ influx. Synaptosomes (200 μl) were predepolarized for varying intervals by addition of 200 μl of Ca^{2+}-free, elevated $[K^+]$-solution containing (open symbols) or free of (solid symbols) MeHg. Following predepolarization, 400 μl of elevated $[K^+]$ solution containing MeHg (if appropriate) and 1 μCi of $^{45}Ca^{2+}$ were added. Influx was stopped after 1 s by the addition of 3 ml of quenching solution. Fractional influx is the amount of $^{45}Ca^{2+}$ influx remaining after a designated interval of predepolarization, relative to 1 s of $^{45}Ca^{2+}$ influx in the absence of predepolarization. The values shown are the mean ± SEM of five (MeHg-free) and three (MeHg) different experiments. Values for any particular experiment are the average of three replicates. Curves are drawn using the average non-linear least squares values listed in Table 1.

Table 4.1

Non-Linear least squares regression values
for apparent inactivation of $^{45}Ca^{2+}$ influx into synaptosomes[a]

	k_i (s^{-1})	q_1	q_2
Control[b]	0.50± 0.10	0.66± 0.02	0.38± 0.04
MeHg[c]	6.33± 0.08*	0.74± 0.02*	0.26± 0.2*

[a]Non-linear least squares regression was used to fit data to the function $Q = q_1 e^{-k_i t} + q_2$ where k_i is the rate constants, q_1 is the fraction of influx which inactivates and q_2 is the fraction of influx which does not inactivate.

[b]Data from five different experiments were used to calculate regression values which are given as the mean ± SEM.

[c]Data from three different experiments using 100 μM MeHg were used to calculate regression values which are given as the mean ± SEM.

*Significantly different from control values ($p \leq 0.05$).

Atchison, 1989), the concentration of extracellular calcium used in this experiment was 2.4 mM, compared to 0.05 mM Ca^{2+} in previous experiments. In contrast to Ca^{2+} and Sr^{2+}, the influx of $^{133}Ba^{2+}$ was only slightly inhibited by concentrations of MeHg as high as 250 μM (Figure 4.5). The differential sensitivity of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ fluxes to block by MeHg suggests that MeHg alters the ionic selectivity of the Ca^{2+} channel for these ions. However, the decreased potency of MeHg to block Ba^{2+} influx may not be due to a reduced blocking action of MeHg on Ba^{2+} influx, but to an increased influx of Ba^{2+} into synaptosomes due to lack of Ca^{2+}-induced Ca^{2+} channel inactivation by Ba^{2+} (Eckert and Chad, 1984). In order to control for differences in Ca^{2+} channel inactivation, triple-label studies were designed in which the influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ or $^{133}Ba^{2+}$ into synaptosomes was measured in solutions which contained 0.1 mM Sr^{2+} and Ba^{2+}, 0.02 mM Ca^{2+} and 0.5 mM Mg^{2+}. The effects of MeHg (100 μM) on the relative influx of the three divalent cations are shown in Figure 4.6. To correct for differences in concentration, the values have been normalized by dividing the influx of each ion by its concentration in the extracellular medium (Nachshen and Blaustein, 1982). In the absence of MeHg, the ratio of influx of ^{45}Ca:^{85}Sr:^{133}Ba was approximately 6:2:3. When 100 μM MeHg was present in the medium, the influx of $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ were 67.1$\pm$9.4 and 72.6$\pm$11.6% of their respective control values, but the influx of $^{85}Sr^{2+}$ was only 44.1$\pm$6.9% of its influx into synaptosomes in the absence of MeHg. This was a statistically significant difference when compared to the effects of MeHg on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx into

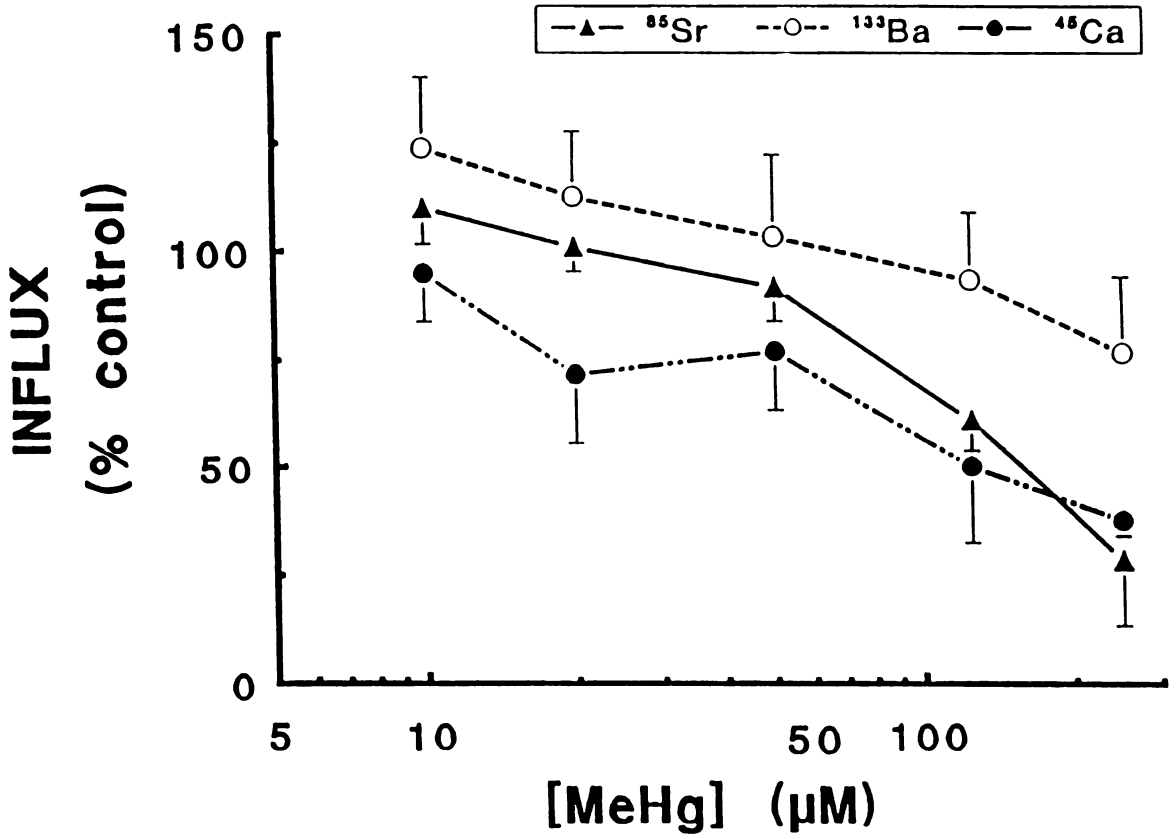

Figure 4.5. Effects of MeHg on the influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ into synaptosomes. Influx was measured by addition of 50 μl of synaptosomal suspension to 50 μl of elevated (77.5 mM) or normal (5 mM) [K^+] solution containing MeHg and 1 μCi of tracer. Influx was stopped after 1 s by the addition of 2 ml of quenching solution. Depolarization-induced influx was determined by subtracting the influx of radiolabel in normal [K^+] solution. The values shown are the mean ± SEM of four ($^{45}Ca^{2+}$), three ($^{85}Sr^{2+}$) and five ($^{133}Ba^{2+}$) different experiments. Values for any particular experiment are the average of three replicates.

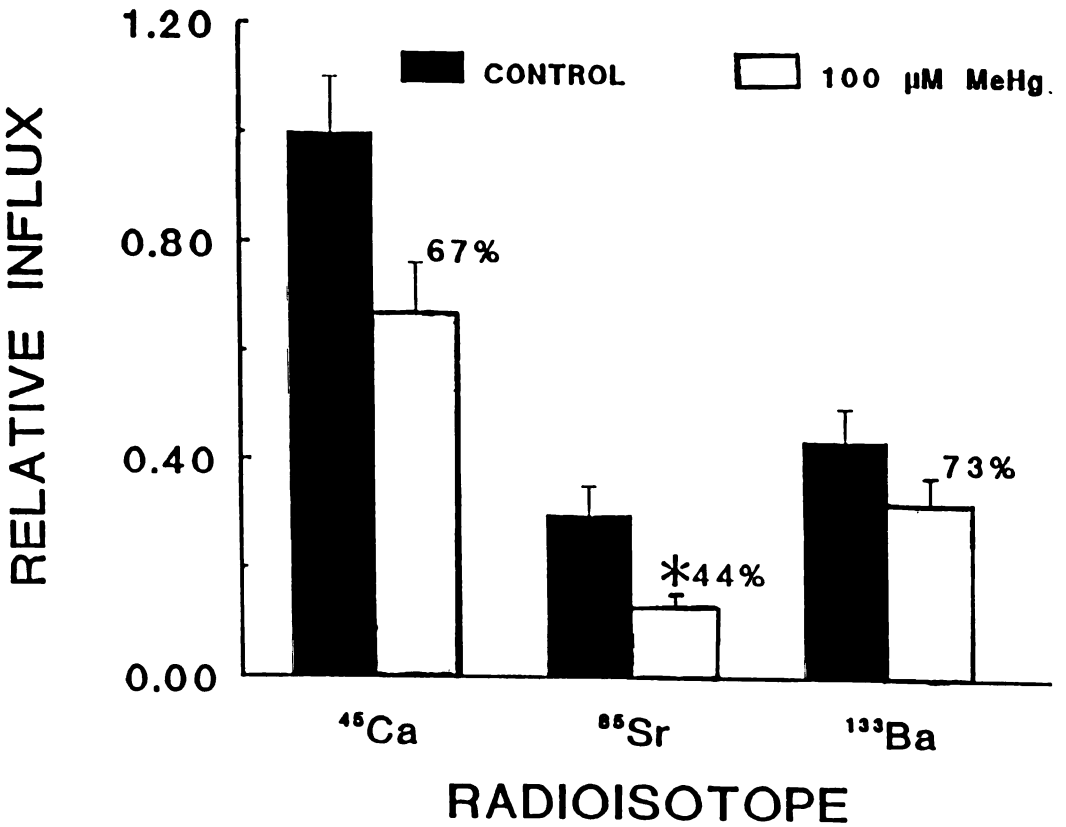

Figure 4.6. Effects of MeHg on the relative influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ into synaptosomes. Influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ or $^{133}Ba^{2+}$ during 1 s of K^+-induced influx was measured in the absence (solid bars) or presence (open bars) of 100 μM MeHg. K^+ solutions contained (mM): $SrCl_2$, 0.1; $BaCl_2$, 0.1; $CaCl_2$, 0.02; $MgCl_2$, 0.05 and 1 μCi of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$. The results have been normalized by dividing the influx of each cation by its external concentration. The values shown are the mean ± SEM of three to five separate experiments. Values for any particular experiment are the average of three replicates. The asterisk (*) indicates an effect of MeHg on $^{85}Sr^{2+}$ influx which is significantly different from the effect of MeHg on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx ($p<.05$).

synaptosomes. Thus, MeHg altered the relative permeability of ^{45}Ca:^{85}Sr:^{133}Ba to 6:1:3.

$^{45}Ca^{2+}$ Influx into PC12 Cells. Influx of $^{45}Ca^{2+}$ into PC12 cells by depolarization in 52.5 mM K^+ gradually increased as time of depolarization was increased, reaching a maximal level after about 1.5 to 2 min of depolarization (Figure 4.7). In addition, influx was a linear function of protein content of the PC12 cell suspension (Figure 4.8). The effects of nifedipine, a Ca^{2+} channel antagonist, on 2 min of $^{45}Ca^{2+}$ influx into PC12 cells are shown in Figure 4.9. In two experiments, the approximate IC_{50} value for block of $^{45}Ca^{2+}$ influx by nifedipine in non-differentiated PC12 cells was 5 nM, and 100 nM nifedipine reduced influx of $^{45}Ca^{2+}$ by greater than 95% (solid symbols). This is consistent with previous studies which have shown that Ca^{2+} influx in undifferentiated PC12 cells is largely DHP-sensitive (Toll, 1982; Takahashi and Ogura, 1983; Kongsamut and Miller, 1986), therefore further repetitions were deemed unnecessary. In NGF-differentiated cells, nifedipine reduced depolarization-dependent $^{45}Ca^{2+}$ influx with an estimated IC_{50} of approximately 5 nM (n=3) (Figure 4.9, open circles). When compared to untreated controls, nifedipine significantly reduced $^{45}Ca^{2+}$ influx at all concentrations tested in differentiated PC12 cells. Although $^{45}Ca^{2+}$ influx was largely DHP-sensitive, 10.4 ± 0.9% of control influx remained in cells treated with 100 nM nifedipine, possibly indicating a DHP-insensitive component. Higher concentrations of nifedipine were not tested.

In undifferentiated PC12 cells, MeHg reduced $^{45}Ca^{2+}$ influx with an IC_{50} of approximately 50 μM, and 250 μM MeHg blocked $^{45}Ca^{2+}$ influx completely (Figure

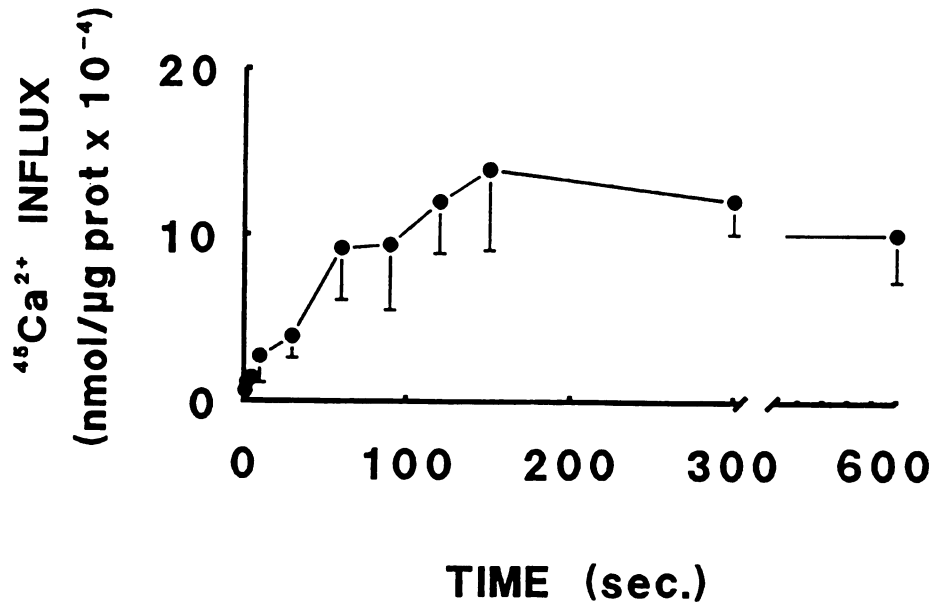

Figure 4.7. Influx of $^{45}Ca^{2+}$ into PC12 cells vs duration of depolarization. $^{45}Ca^{2+}$ influx into PC12 cells induced by depolarization in elevated [K^+] solutions as a function of time. The values shown are the mean ± SEM of three experiments, where the values for any one experiment are the average of three replicates.

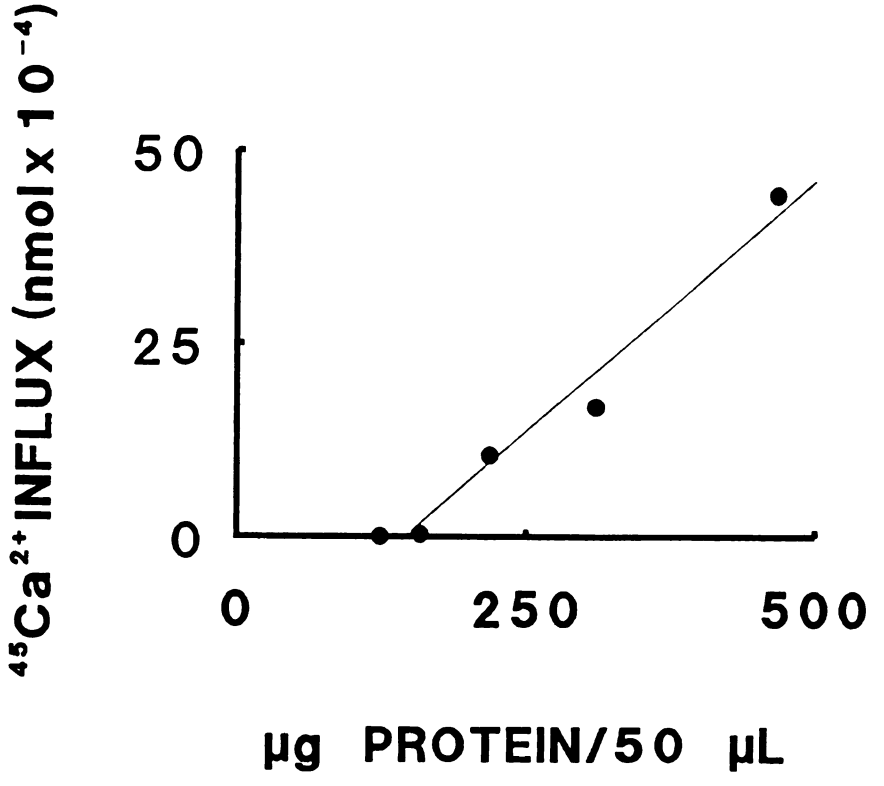

Figure 4.8. Influx of $^{45}Ca^{2+}$ into PC12 cells as a function of protein concentration. Values from a single representative experiment comparing $^{45}Ca^{2+}$ influx with protein content of the PC12 cell suspension. The line is the linear least-squares regression of influx on protein content. Values for influx are the average of three replicates from a single representative experiment.

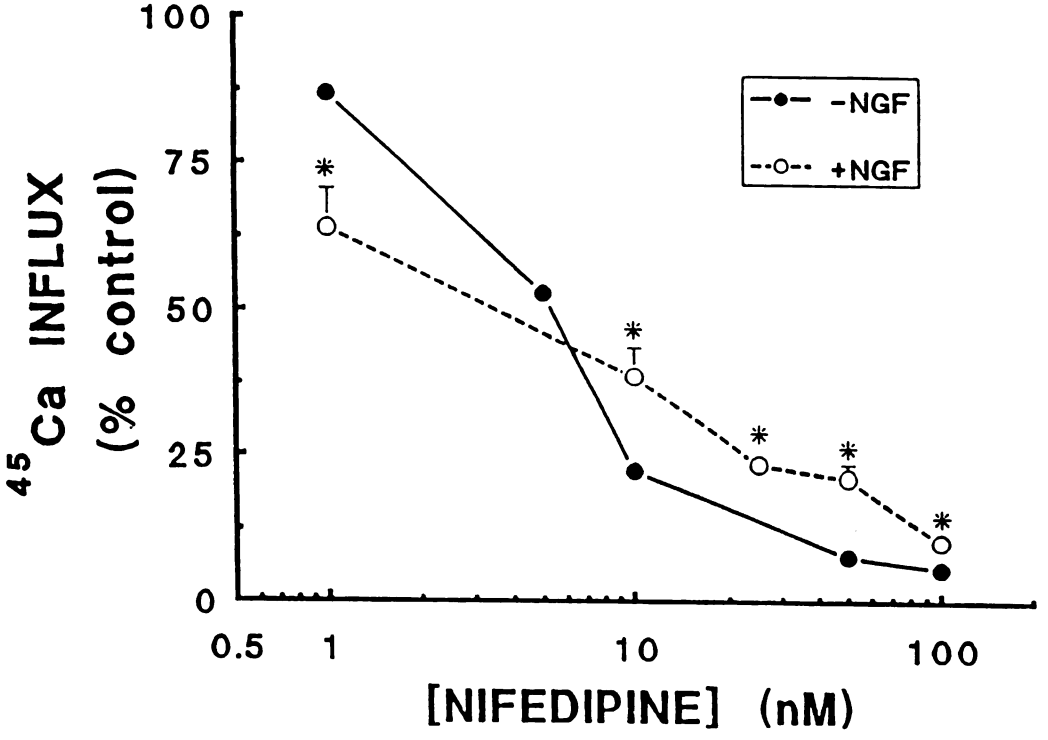

Figure 4.9. Effects of nifedipine on depolarization-induced influx of $^{45}Ca^{2+}$ in PC12 cells. Influx was measured by the addition of 50 μl of non-differentiated (open symbols) and NGF-differentiated (solid symbols) PC12 cell suspension in normal (5 mM) [K^+], PC12 medium to 50 μl of elevated (100 mM) [K^+] or normal [K^+] PC12 medium containing 1 μCi of $^{45}Ca^{2+}$ and various concentrations of nifedipine. The values shown are the mean of two (non-differentiated) and mean ± SEM of three (NGF-differentiated) different experiments. Values for any particular experiment are the average of three replicates. For experiments using NGF-differentiated cells, SE bars are not shown if the SEM was smaller than the symbol size. The asterisk (*) indicates results that are significantly less than control ($p<.05$).

4.10, filled circles). In NGF-differentiated PC12 cells, nearly identical results were obtained (Figure 4.10, open circles). MeHg concentrations of 50 μM and above significantly reduced $^{45}Ca^{2+}$ influx in undifferentiated and NGF-differentiated PC12 cells when compared to untreated controls. Thus, MeHg blocks a DHP-sensitive Ca^{2+} channel in PC12 cells with nearly identical potency to its block of Ca^{2+} entry in synaptosomes. In addition, in two out of three experiments, MeHg completely blocked $^{45}Ca^{2+}$ influx into NGF-differentiated PC12 cells. This may indicate an action of MeHg on a DHP-insensitive Ca^{2+} channel in PC12 cells, but would require electrophysiological experiments to confirm. MeHg did not alter the depolarization-independent entry of $^{45}Ca^{2+}$ into undifferentiated or NGF-differentiated PC12 cells. In addition, concentrations of MeHg as high as 250 μM did not affect the viability of PC12 cells as measured by Trypan Blue exclusion, even after 1 hr of exposure (data not shown). Thus, the effects of MeHg on $^{45}Ca^{2+}$ influx into PC12 cells appear to be due to disruption of voltage-dependent, channel-mediated Ca^{2+} influx and not to overt cytotoxicity to PC12 cells.

Nitrendipine Binding. The binding of [^{3}H]-nitrendipine to synaptosomes reached equilibrium after 10-15 min and was stable for 90 min (results not shown). When the binding of [^{3}H]-nitrendipine (concentrations ranged from 0.18 to 3 nM) to synaptosomes was measured in normal (non-depolarizing) [K^+] solutions, a single, high-affinity binding site was observed (Figure 4.11). Non-specific binding accounted for approximately 50% of total binding. Scatchard analysis of the data (n=3) yielded a K_D value of 630±160 pM and an apparent B_{max} value of 130±40 fmol/mg of

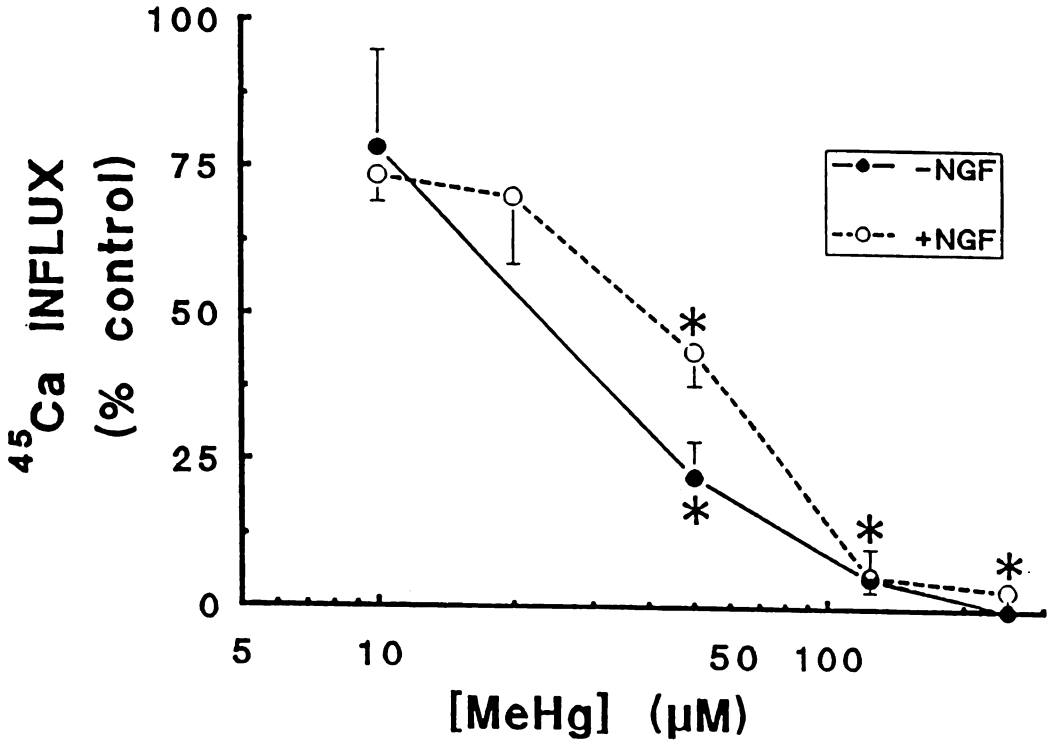

Figure 4.10. Effects of MeHg on depolarization-induced $^{45}Ca^{2+}$ influx into PC12 cells. $^{45}Ca^{2+}$ influx into non-differentiated (solid symbols) and NGF-differentiated (open symbols) PC12 cells was measured as described in Figure 4.9. The values shown are the mean ± SEM for three separate experiments. Values for any particular experiment are the average of three replicates. When SE bars are not shown, the SE is smaller than the size of the symbol. The asterisk (*) indicates results that are significantly less than control ($p<.05$).

protein. Hill slope was approximately equal to 1. These values are consistent with values reported by others (Rampe *et al.*, 1984; Turner and Goldin, 1985; Suszkiw *et al.*, 1986) for [^{3}H]-nitrendipine binding in synaptosomes. In the presence of 100 μM MeHg, the binding of [^{3}H]-nitrendipine was also linear, however, the value for K_D was increased significantly over the control K_D value to 2520±630 pM (n=3). The apparent B_{max} was 200±30 fmol/mg of protein in the presence of 100 μM MeHg. A valid determination of B_{max} could not be made due to the nitrendipine concentration range used, therefore the B_{max} value obtained in the presence of MeHg is only an estimate and little direct information about the effects of MeHg on the number of binding sites can be gained by comparison to control B_{max} values. Under depolarizing conditions, similar results were obtained. In the absence of MeHg, the K_D value for the binding of [^{3}H]-nitrendipine was 1100±300 pM. Although K_D was higher under depolarizing conditions, it was not significantly different from the K_D value in normal [K^+] solutions. In the presence of MeHg, the K_D value in elevated [K^+] solutions was 5330±2100 pM and was significantly higher than the respective control value. Apparent B_{max} values in the absence and presence of 100 μM MeHg were 200±23 and 320±74 fmol/mg, respectively. Thus, MeHg decreased the affinity of binding of [^{3}H]-nitrendipine to synaptosomes, indicating that it may act at or near the nitrendipine binding site. As depolarization did not alter the characteristics of [^{3}H]nitrendipine binding to synaptosomes, the results of these experiments are reported here, but are not illustrated in Figure 4.11.

ω-Conotoxin Binding. The specific binding of [^{125}I]-ω-conotoxin GVIA to synaptosomes in the absence and presence of MeHg is shown in Figure 4.12A. Non-specific binding accounted for approximately 20-30% of total binding. In the absence of MeHg, CgTx bound to synaptosomes with an apparent half-saturation of 0.1 to 0.2 nM. The maximum density of binding sites estimated by extrapolation of the saturated component to the Y-axis is between 300 and 450 fmol/mg protein. These results are similar to results for CgTx binding to synaptosomal membranes from rat and chick brain (Abe *et al.*, 1986; Cruz and Olivera, 1986; Marqueze *et al.*, 1988). In the presence of 100 μM MeHg, binding of CgTx to synaptosomes was not significantly different from that of control. Because the actions of MeHg at the neuromuscular junction (Traxinger and Atchison, 1987b) and in synaptosomes are not readily reversible (Shafer and Atchison, 1989), synaptosomes were pretreated with MeHg prior to measuring CgTx binding. This pretreatment did not change the characteristics of CgTx binding (results not shown).

In undifferentiated PC12 cells, specific binding of [^{125}I]-ω-conotoxin in the absence of MeHg was saturable (Figure 4.12B). The apparent half saturation was less than 0.1 nM CgTx. The apparent B_{max} was approximately 25-50 pmol/mg protein. In the presence of 100 μM MeHg, the amount of [^{125}I]-ω-conotoxin specifically bound was reduced significantly when binding was measured at CgTx concentrations between 10 and 500 pM. These results indicate that MeHg interacts with the receptor for ω-CgTx on PC12 cells, but not on synaptosomes.

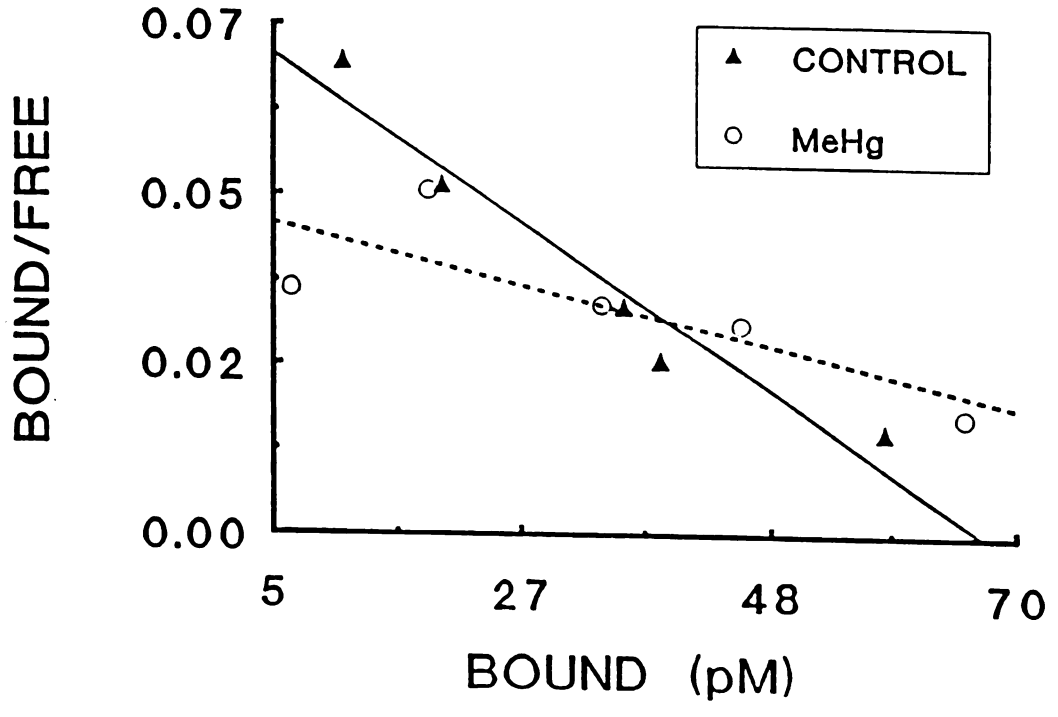

Figure 4.11. Effects of MeHg on nitrendipine binding to synaptosomes. Specific binding of [^{3}H]-nitrendipine to synaptosomes in the absence (solid triangles) and presence (open circles) of 100 μM MeHg in normal [K^+] solutions. Values shown are the average of three replicates from a single representative experiment.

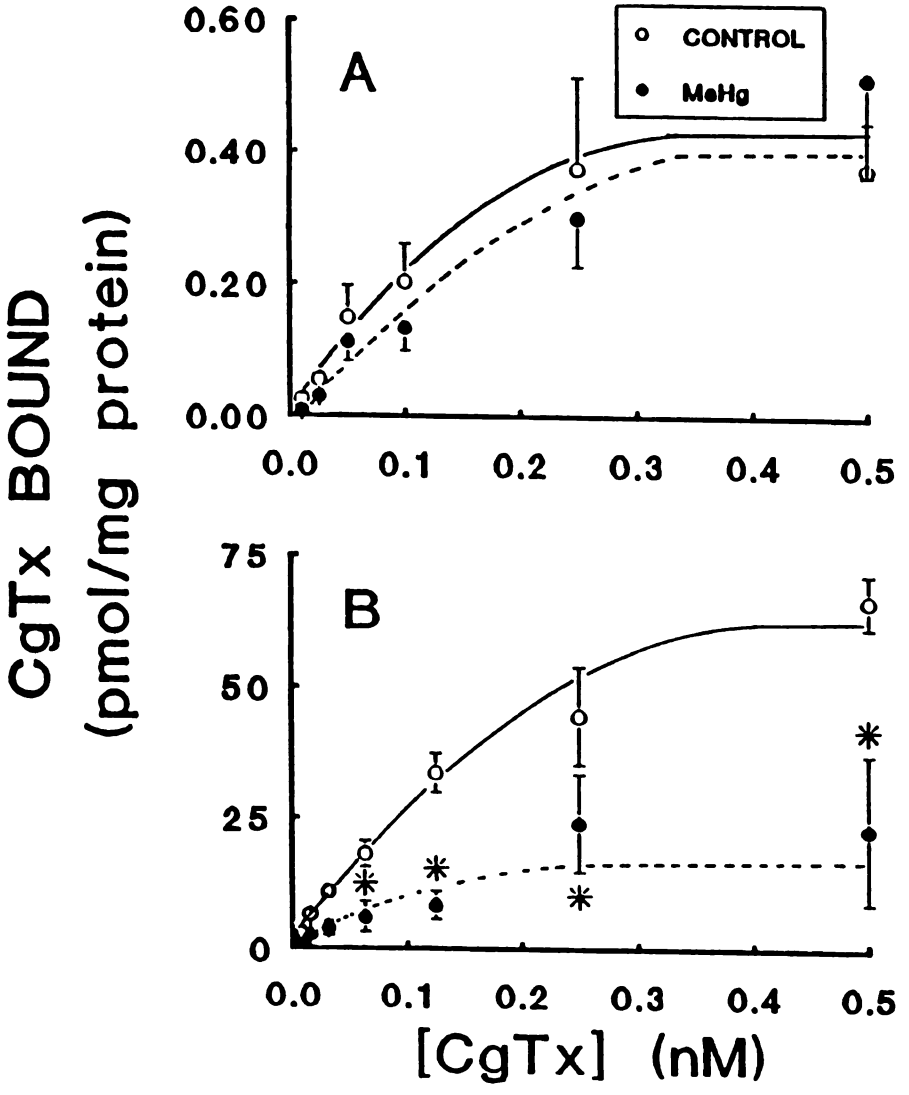

Figure 4.12. Effects of MeHg on binding of conotoxin to synaptosomes and PC12 cells. Specific binding of [^{125}I]-ω-conotoxin GVIA to synaptosomes (A) and PC12 cells (B) in the absence (open symbols) and presence (solid symbols) of 100 μM MeHg. Values shown are the mean ± SEM of three experiments, where the values for any given experiment are the average of three replicates. When SE bars are not shown, the SE is smaller than the size of the symbol. The asterisks (*) indicate values which are significantly different from their respective MeHg-free control ($p < .05$).

DISCUSSION

The goal of these experiments was to characterize the effects of MeHg on nerve terminal Ca^{2+} channel properties using synaptosomes and to attempt to identify the type or types of neuronal Ca^{2+} channels with which MeHg interacts in a defined population using PC12 cells. These results suggest that MeHg: 1) blocks $^{45}Ca^{2+}$ influx into synaptosomes in a voltage-dependent manner; 2) blocks Ca^{2+} influx through synaptosomal Ca^{2+} channels in a manner which does not depend on the configuration (open, resting or inactivated) of the Ca^{2+} channel; 3) acts on synaptosomal Ca^{2+} channels to alter both the kinetics of apparent inactivation and ionic selectivity; 4) is capable of blocking a DHP-sensitive Ca^{2+} channel in PC12 cells, and may also block a DHP-insensitive Ca^{2+} channel in NGF-differentiated PC12 cells; and 5) alters nitrendipine and CgTx binding to high-affinity sites on synaptosomes and PC12 cells, respectively.

The block of $^{45}Ca^{2+}$ influx by MeHg increases as a function of time of exposure in Na-free medium, but not in Na^+-containing meduim. Under the former conditions, Na^+/Ca^{2+} exchange does not contribute significantly to $^{45}Ca^{2+}$ influx; therefore influx occurs predominantly via Ca^{2+} channels (Suszkiw *et al.*, 1986; Shafer and Atchison, 1989). These results indicate that MeHg may block more Ca^{2+} channels as the time of exposure increases or alter the inactivation rate of Ca^{2+} channels or both.

MeHg differs from inorganic, divalent Ca^{2+} channel blockers such as Cd^{2+}, Pb^{2+}, Hg^{2+} in that it is monovalent and has increased lipophilicity imparted by the

methyl group. The ability of MeHg to block $^{45}Ca^{2+}$ influx in a voltage-dependent manner implies that the monovalent charge on MeHg may be important for the interaction of this compound with Ca^{2+} channels. More recent results suggest however, that lipophilicity may be critical for voltage-dependent actions of mercurials, as only organic mercurials block synaptosomal $^{45}Ca^{2+}$ influx in a voltage-dependent manner (Hewett and Atchison, 1990). Voltage-dependent block of cardiac Ca^{2+} channels by inorganic divalent cations is thought to arise due to binding of the cation to sites within the pore of the channel (Hess *et al.*, 1986; Lansman *et al.*, 1986). Thus, these results suggest that MeHg may act at a site within the channel.

It should be noted that the voltage-dependence of block could be due to non-specific interactions of MeHg with the membrane rather than specific interaction with Ca^{2+} channels. Sulfhydryl reactive agents such as mercuric chloride and parahydroxymercuribenzoate depolarize the membrane (Huneeus-Cox *et al.*, 1966). If MeHg were to cause significant depolarization, it might also give rise to a "voltage-dependent" block of influx, as it may antagonize the block by MeHg at lower K^+ concentrations by causing depolarization and opening more Ca^{2+} channels than in the control synaptosomes. In squid axon membranes, concentrations of MeHg less than 100 μM do not cause depolarization (Shrivastav *et al.*, 1976) and in neuroblastoma cells 40 μM MeHg results in only 4 mV of depolarization after 6 min of exposure (Quandt *et al.*, 1982). Thus, MeHg does not cause significant depolarization of the membrane in these preparations. However, in guinea pig synaptosomes, 100 μM MeHg caused a depolarization of the membrane of 36 mV

after 1 min of exposure (Kauppinen *et al.*, 1989). Lower concentrations of MeHg may be less effective at depolarizing the synaptosomal membrane, as 10 μM MeHg produces a maximal depolarization of 15 mV (Hare and Atchison, 1990). In the experiments reported in the present study, exposure to MeHg was extremely short (2 s). Therefore, although it is possible that MeHg causes some membrane depolarization, it is unlikely that the magnitude of depolarization would be great enough to affect the results significantly.

Access to the resting or open and inactivated states of synaptosomal Ca^{2+} channels did not affect the potency or efficacy of block of $^{45}Ca^{2+}$ influx by MeHg. Different association and dissociation rates from the resting, open and inactivated states of ionic channels is thought to underlie state-dependent block of I_{Na} by local anesthetics (Strichartz, 1973; Courtney, 1975; Hille, 1977) and the action of certain Ca^{2+} channel blockers (Lee and Tsien, 1983). Results of the present study suggest that MeHg does not have an increased affinity for any particular state of the Ca^{2+} channel, as its blocking effects were equivalent following access to the channel in the presence and absence of predepolarization. This is distinct from the blocking action of DHPs which block I_{Ca} in a state-dependent manner in cardiac myocytes (Uehara and Hume, 1985) and the possible state-dependence of Ni^{2+}, La^{3+} and verapamil in synaptosomes (Nachshen, 1985). It should be noted that synaptosomal preparations may contain multiple types of Ca^{2+} channels which may have different rates of inactivation. Thus, it is possible that these experiments have examined effects of MeHg on two or more subpopulations of Ca^{2+} channels and that MeHg has equal

affinity for each subpopulation. However, apparent inactivation in these experiments was best described by a single exponential process, which is consistent with previous results (Suszkiw *et al.*, 1986; 1989). Therefore, the results of the state-dependent experiments have been interpreted as though the synaptosomal Ca^{2+} channel population responded to depolarization in a homogeneous manner.

Predepolarization of synaptosomes in the presence of MeHg significantly increased the rate of apparent inactivation of $^{45}Ca^{2+}$ influx. Inactivation of synaptosomal Ca^{2+} channels with time results in decreased $^{45}Ca^{2+}$ influx; MeHg may decrease influx further by blocking open/active Ca^{2+} channels, giving rise to an increase in the rate of apparent inactivation. Intuitively, one might expect that the rate constants for normal inactivation and MeHg-induced block of Ca^{2+} channels would differ, resulting in two exponential components of inactivation, unlike the case observed in the present study. Nevertheless, the results of the present experiment cannot rule out this hypothesis definitively. Alternatively, these results may indicate an ability of MeHg to hasten the process of Ca^{2+} channel inactivation. MeHg may act directly on the Ca^{2+} channel to increase the rate of apparent inactivation or may act indirectly to increase inactivation of Ca^{2+} channels by causing release of Ca^{2+} from intracellular stores (Kauppinen *et al.*, 1989), resulting in increased $[Ca^{2+}]_i$ and Ca^{2+}-dependent inactivation of Ca^{2+} channels. This latter mechanism, although possible, is unlikely to contribute largely to the increased rate of apparent inactivation of $^{45}Ca^{2+}$ influx in this experiment. It requires that MeHg diffuse through the synaptosomal membrane and interact with the mitochondria to cause Ca^{2+} release.

Ca^{2+} must then diffuse back to the membrane and interact with Ca^{2+} channels to cause inactivation. This sequence of events would have to take place extremely rapidly, as apparent inactivation in the presence of MeHg occurs in less than 1 s. In addition, the synaptosomes have been prepared, incubated in and predepolarized in nominally Ca^{2+}-free buffers. A redistribution of Ca^{2+} from the mitochondria would be expected, lowering the overall mitochondrial Ca^{2+} content.

In addition to increasing the rate of apparent inactivation, MeHg also decreases the fraction of $^{45}Ca^{2+}$ influx which does not inactivate during the predepolarization period. Non-inactivating $^{45}Ca^{2+}$ influx in synaptosomes is comprised of both a channel-mediated component and a reversed Na^+/Ca^{2+} exchange component (Coutinho *et al.*, 1984; Suszkiw *et al.*, 1986; Shafer and Atchison, 1989). Nerve terminal Na^+/Ca^{2+} exchange appears to have decreased sensitivity to block by MeHg (Traxinger and Atchison, 1987b; Shafer and Atchison, 1989); therefore, if MeHg caused significant Ca^{2+} release from mitochondria, it could decrease this phase of uptake by decreasing the driving force for exchange, or reversing exchange. Alternatively, the effect of MeHg on the non-inactivating portion of Ca^{2+} influx suggests an action of MeHg on a non-inactivating population of Ca^{2+} channels. This decrease may be the result of either a direct block of these channels, or increased Ca^{2+}-dependent inactivation due to MeHg-induced Ca^{2+} release from intracellular stores.

MeHg also altered the relative influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$ into synaptosomes via Ca^{2+} channels. $^{85}Sr^{2+}$ influx through Ca^{2+} channels was more

sensitive to block by MeHg than was influx of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$. This represents a unique action by MeHg on Ca^{2+} channels when compared with other divalent cations such as Ni^{2+}, which will block the influx of Ca^{2+}, Sr^{2+} and Ba^{2+} without altering the relative influx of these three cations (Nachshen and Blaustein, 1982). Although these experiments do not define the site of action of MeHg, they suggest that MeHg may affect the function of the selectivity filter of the Ca^{2+} channel. It has been suggested that ionic selectivity in cardiac Ca^{2+} channels is due to differences in the affinity of divalent cations for binding sites within the pore of the channel, rather than a physical structure which "filters" ions which can enter the channel (Hess *et al.*, 1986; Lansman *et al.*, 1986). If this were the case for synaptosomal Ca^{2+} channels as well, then these results suggest that Sr^{2+} is less effective at displacing MeHg from this site than Ca^{2+} or Ba^{2+}. In addition, these results would be consistent with voltage-dependent effects of MeHg.

The ability of nifedipine to block the influx of $^{45}Ca^{2+}$ into PC12 cells is consistent with the action of other DHP antagonists on Ca^{2+} influx in this cell line (Toll, 1982; Takahashi and Ogura, 1983; Kongsamut and Miller, 1986) and indicates that depolarization-induced $^{45}Ca^{2+}$ influx into these cells is mediated largely by DHP-sensitive or "L"-type Ca^{2+} channels. In addition, in the present study, approximately 10% of $^{45}Ca^{2+}$ influx in differentiated PC12 cells remained after exposure to 100 nM nifedipine, indicating a DHP-insensitive portion of $^{45}Ca^{2+}$ influx. Although it would require electrophysiological experiments to confirm, the remaining influx may represent influx via an "N"-type conductance in PC12 cells. MeHg blocked $^{45}Ca^{2+}$

influx into both non-differentiated and NGF-differentiated PC12 cells at concentrations similar to those which block $^{45}Ca^{2+}$ influx into synaptosomes (Shafer and Atchison, 1989). In two out of three experiments, MeHg completely blocked $^{45}Ca^{2+}$ influx into NGF-differentiated PC12 cells. This indicates that MeHg may be capable of blocking a DHP-insensitive Ca^{2+} channel as well as a DHP-sensitive channel. The ability of MeHg to block DHP-sensitive or "L"-type Ca^{2+} channels in PC12 cells is important in light of recent studies which indicate that these channels may be responsible for, or at least modulate Ca^{2+} entry associated with release of some neurotransmitters from neurons (Perney *et al.*, 1986; Atchison and O'Leary, 1987; Rane *et al.*, 1987; Holz *et al.*, 1988; Atchison, 1989) and PC12 cells (Takahashi *et al.*, 1985; Kongsamut and Miller, 1986). In addition, DHP-insensitive Ca^{2+} conductances have been reported to mediate in part release of [^{3}H]-norepinephrine from NGF-differentiated PC12 cells (Kongsamut and Miller, 1986). Thus, MeHg is capable of blocking at least one and possibly two types of Ca^{2+} channels which are associated with neurotransmitter release. This action may contribute in large measure to the observation that MeHg depresses Ca^{2+}-dependent transmitter release (Traxinger and Atchison, 1987b).

High affinity binding of DHPs has been demonstrated in synaptosomes (Turner and Goldin, 1985; Suszkiw *et al.*, 1986; Dooley *et al.*, 1987b; Dunn, 1988). However, the relationship between binding of DHPs and effects of DHPs on $^{45}Ca^{2+}$ influx into synaptosomes is unclear. Several studies (Daniell *et al.*, 1983; Suszkiw *et al.*, 1986; 1989; Reynolds *et al.*, 1986; Rivier *et al.*, 1987) indicate that Ca^{2+} influx into

synaptosomes is insensitive to block by DHP antagonists, whereas, other studies (Turner and Goldin, 1985; Dunn, 1989; Martinez-Serrano *et al.*, 1989) indicate that a portion of Ca^{2+} influx in synaptosomes is sensitive to DHP antagonists. In either case, the concentrations of DHP required to block $^{45}Ca^{2+}$ influx are in great excess of those at which DHPs bind specifically to synaptosomes. In 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 values observed by others for nitrendipine binding in synaptosomes (Turner and Goldin, 1985; Suszkiw *et al.*, 1986). DHP agonists and antagonists are thought to bind to Ca^{2+} channels more readily when the channel is in the open or inactivated state, respectively (Uehara and Hume, 1985). However, depolarization of the synaptosomes did not readily affect the affinity of nitrendipine binding or the maximum number of binding sites. The binding affinity of the DHP antagonist (+)-PN200-110 to synaptosomes was decreased by K^+-induced depolarization (Dunn, 1989). Although other explanations are possible, the lack of effect of depolarization on the affinity of DHP binding would be consistent with the suggestion that DHP binding sites in synaptosomal preparations are associated with fragments of postsynaptic membrane which remain attached to the synaptosomes (Suszkiw *et al.*, 1989). These fragments could not maintain a membrane potential and hence would not respond to changes in K^+ concentration. The affinity of nitrendipine binding to synaptosomes was decreased in the presence of a concentration of MeHg which readily blocks $^{45}Ca^{2+}$ influx in synaptosomes (Shafer and Atchison, 1989) and DHP-sensitive influx in PC12

cells. The ability of MeHg to compete with nitrendipine for a high-affinity binding site in synaptosomes is consistent with its ability to block DHP-sensitive channels in PC12 cells. Experiments to demonstrate specific binding of nitrendipine to intact PC12 cells were unsuccessful, as appreciable specific binding was not observed. The author is unaware of any reports of specific binding of nitrendipine to intact PC12 cells.

Caution must be observed in correlating the results of binding studies in synaptosomes with flux studies in PC12 cells and concluding that MeHg blocks a DHP-sensitive Ca^{2+} channel in synaptosomes. According to one model for the movement of Ca^{2+} through DHP-sensitive cardiac Ca^{2+} channels, higher concentrations of Ca^{2+} are necessary to observe a Ca^{2+} current (Hess and Tsien, 1984) than are normally employed in experiments with synaptosomes. Thus, fluxes measured in these experiments may not necessarily be mediated by DHP-sensitive Ca^{2+} channels. As shown in Figure 4.5, MeHg will block $^{45}Ca^{2+}$ influx into synaptosomes at concentrations of Ca^{2+} high enough to mediate flux via L-type channels. However, the contribution, if any, of $^{45}Ca^{2+}$ flux into synaptosomes mediated by L-type channels under these conditions has not been determined.

The polypeptide, ω-conotoxin GVIA, binds to Ca^{2+} channels at a site which is distinct from the DHP binding site (Abe *et al.*, 1987; Cruz and Olivera, 1986; Marqueze *et al.*, 1988; Sher *et al.*, 1988) and in some axon terminals is thought to be associated with transmitter release. As opposed to nitrendipine, MeHg did not alter the binding of CgTx to synaptosomes. This indicates that MeHg does not interact

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with this site in synaptosomes, or that CgTx displaces MeHg from this site. The effects of MeHg on $^{45}Ca^{2+}$ uptake in synaptosomes are only partially reversible by increasing $[Ca^{2+}]_e$ (Shafer and Atchison, 1989), and at neuromuscular junctions are only reversible under certain conditions (Traxinger and Atchison, 1987b). Thus, it seemed likely that MeHg is tightly or irreversibly bound to receptors on the cell surface. Consequently, experiments were designed to test whether pretreatment with MeHg might alter CgTx binding to its receptor in synaptosomes. Results of these experiments suggest that MeHg does not interact with the CgTx binding site in synaptosomes, as CgTx binding was not altered by pretreatment with MeHg or in the presence of MeHg.

In contrast to the results of synaptosomal experiments, MeHg did alter the binding of CgTx to PC12 cells. The amount of CgTx bound to PC12 cells was decreased significantly in the presence of MeHg at concentrations which effectively block $^{45}Ca^{2+}$ entry into this cell line. Thus, MeHg apparently interacts with the CgTx receptor in PC12 cells but not on synaptosomes. This suggests that the CgTx receptor, and hence perhaps the Ca^{2+} channels, in synaptosomes and PC12 cells are different. ω-Conotoxin is thought to interact with "N" and possibly "L" type channels in mammalian neurons (Reynolds *et al.*, 1986). In both synaptosomes and PC12 cells, experimental results indicate the presence of only a single class of conotoxin receptor, thus it is possible that CgTx binds to one channel type in synaptosomes and the other type in PC12 cells. However, it is difficult to interpret the results of conotoxin binding experiments, as the type(s) of Ca^{2+} channel(s) in synaptosomes are

unknown, and, in PC12 cells, only "N" type conductances are sensitive to block by CgTx (Plummer *et al.*, 1989). While the latter indicates that ω-conotoxin GVIA may not block "L"-type channels in PC12 cells, it does not preclude binding to this channel type from taking place. Thus, it is to determine which Ca^{2+} channel subtype CgTx is binding to in these experiments. Alternatively, CgTx may be binding to a site in synaptosomes and/or PC12 cells which is not associated with Ca^{2+} channels. Much higher concentrations of CgTx are required to block $^{45}Ca^{2+}$ influx in mammalian synaptosomes than in avian or amphibian synaptosomes (Suszkiw *et al.*, 1987), in spite of the presence of high affinity binding sites for CgTx. Thus, interpretation of these data beyond the conclusion that MeHg interacts with the CgTx receptor in PC12 cells, but not synaptosomes, would be purely speculative.

MeHg is a very reactive heavy metal and alters a number of neuronal functions. Although effects of MeHg on Ca^{2+} channels are probably not solely responsible for the neurotoxic actions of MeHg, the rapid action of MeHg on Ca^{2+} influx into synaptosomes indicates that nerve terminal Ca^{2+} channels may be an initial site of interaction of MeHg with neurons. In summary, the results of this study suggest that MeHg interacts with nerve terminal Ca^{2+} channels in a manner unique from inorganic divalent cations or DHPs. MeHg clearly interacts with a DHP-sensitive Ca^{2+} channel in PC12 cells, but the relationship of this interaction to MeHg's effects on $^{45}Ca^{2+}$ influx in synaptosomes, and synaptic transmission at the neuromuscular junction is unclear. In addition, MeHg does not block Ca^{2+} channels in a state-dependent manner as do DHPs. MeHg is also unique from other

neurotoxic heavy metals in its ability to alter both the apparent inactivation kinetics and ionic selectivity of fluxes through the Ca^{2+} channel. Differences in lipophilicity and charge may underlie the unique blocking action of MeHg on Ca^{2+} channels.

EFFECTS OF THE NEUROTOXICANT METHYLMERCURY ON NEURONAL Ca^{2+} CHANNELS: EFFECTS ON Ca^{2+} CHANNELS INVOLVED IN TRANSMITTER RELEASE

Volume II

By

Timothy Joseph Shafer

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology
and Institute for Environmental Toxicology

1991

CHAPTER FIVE

METHYLMERCURY BLOCKS N- AND L-TYPE Ca^{2+} CHANNELS IN NERVE GROWTH FACTOR-DIFFERENTIATED PHEOCHROMOCYTOMA (PC12) CELLS

ABSTRACT

Effects of methylmercury (MeHg) on whole cell Ba^{2+} currents in rat pheochromocytoma (PC12) cells were examined. Based on biophysical characteristics and sensitivity to ω-conotoxin GVIA (CgTx) and dihydropyridine (DHP) agonists and antagonists, voltage-activated Ba^{2+} currents in PC12 cells were mediated by N- and L-type Ca^{2+} channels. Addition of MeHg (10 μM) to the extracellular solution caused a rapid and complete block of current carried by 20 mM Ba^{2+}. The rate of block of I_{Ba} by MeHg increased in a concentration-dependent manner between 1 and 20 μM. Increasing the frequency of stimulation from 0.1 to 0.4 Hz facilitated block of I_{Ba} by MeHg. A 2 min application of 10 μM MeHg in the absence of stimulation also reduced I_{Ba} by approximately 80%. Thus, block of I_{Ba} by MeHg is not state-dependent. Additionally, MeHg blocked I_{Ba} when the membrane holding potential was -40, -70 and -90 mV, indicating that both N- and L-type Ca^{2+} channels are blocked by MeHg. Block of I_{Ba} by MeHg was voltage-dependent at a membrane holding potential of -40 mV, but not at holding potentials of -70 and -90 mV. Decreasing the extracellular concentration of Ba^{2+} ($[Ba^{2+}]_e$) from 20 mM to 10 mM increased the magnitude of block by MeHg from 45.6 to 77.3%. Increasing $[Ba^{2+}]_e$ to 30 mM caused no further antagonism of block. Block of I_{Ba} by MeHg was not reversed by washing with MeHg-free solution. The ionic permeability of PC12 cell Ca^{2+} channels was $Ca^{2+} = Sr^{2+} > Ba^{2+}$. In the presence of MeHg, all three divalent cations were equally permeant. These results indicate that: 1) MeHg blocks N- and L-type Ca^{2+} channels in PC12 cells at low micromolar concentrations; 2) block of

Ca^{2+} channels by MeHg occurs regardless of the state of the Ca^{2+} channel or membrane holding potential; 3) block of L-type Ca^{2+} channels by MeHg is voltage-dependent; 4) decreasing the extracellular [Ba^{2+}] exacerbates the block by MeHg, but washing cells in MeHg-free solution did not reverse block of Ca^{2+} channels by MeHg; 5) the ionic permeability of Ca^{2+} channels in PC12 cells is altered by MeHg.

INTRODUCTION

Results of experiments presented in previous chapters confirm important differences in the action of MeHg on synaptosomal $^{45}Ca^{2+}$ influx and neuromuscular transmission when compared to divalent cations. While increasing $[Ca^{2+}]_e$ readily reverses block by divalent cations of $^{45}Ca^{2+}$ influx into synaptosomes (Nachshen, 1984; Suszkiw *et al.*, 1985) and nerve-evoked transmitter release at the neuromuscular junction (Nilson and Volle, 1976; Kober and Cooper, 1976; Cooper and Manalis, 1983), the effects of MeHg on synaptosomes (Atchison *et al.*, 1986; Shafer and Atchison, 1989) and at the neuromuscular junction (Traxinger and Atchison, 1987b) are only partially antagonized by increasing $[Ca^{2+}]_e$. MeHg also alters the ionic selectivity and apparent rate of inactivation of $^{45}Ca^{2+}$ influx in synaptosomes and blocks synaptosomal $^{45}Ca^{2+}$ influx in a manner which is voltage-, but not state-dependent (Shafer *et al.*, 1990). Such effects are not typically observed with inorganic divalent cations.

Furthermore, MeHg blocks depolarization-induced influx of $^{45}Ca^{2+}$ into PC12 cells and synaptosomes with similar potency and efficacy (Shafer *et al.*, 1990) and alters binding of specific Ca^{2+} channel antagonists to their receptors on synaptosomes and PC12 cells. These data provide preliminary indications that MeHg interacts with Ca^{2+} channel types which are associated with neurotransmitter release. However, as noted in the previous chapter, the results of binding experiments do not allow for unambiguous determinations of channel types due to occasional lack of correlation of functional effects with specific binding. In addition, the kinetic resolution of

putative channel-mediated events using flux measurements is markedly slower than for electrophysiological measurements of channel activity in single cells. Thus, experiments were designed to make unambiguous determinations of Ca^{2+} channel types affected by MeHg and to examine the effects of MeHg on channel function using techniques with more physiologically relevant time resolution.

Calcium or barium currents from PC12 cells cultured in nerve growth factor-(NGF) containing medium are mediated by separate channel entities with distinct kinetics and pharmacological sensitivities resembling the L- and N-type Ca^{2+} channels (Plummer *et al.*, 1989; Usowicz *et al.*, 1990) described by Fox *et al.* (1987a,b). These two channel types have been associated with neurotransmitter release in several different preparations (Atchison and O'Leary 1987; Rane *et al.*, 1987; Holz *et al.*, 1988; Atchison, 1989; Lemos and Nowycky, 1989). In the present study, PC12 cells and the patch voltage-clamp technique (Hamill *et al.*, 1981) were used to examine directly the ability of MeHg to interact with N- and/or L-type Ca^{2+} channels and to examine characteristics of Ca^{2+} channel block by MeHg such as reversibility of block, state and voltage-dependence of block, and effects of MeHg on Ca^{2+} channel inactivation and ionic permeability.

MATERIALS AND METHODS

Chemicals and solutions. Stock solutions (500 μM) of methylmercuric chloride (ICN Biomedicals, Plainview, NY) were prepared weekly in extracellular solution containing the proper concentration and species of divalent cation, from which test solutions were prepared daily. Stock solutions of nifedipine (Sigma, St. Louis, MO) and (+)-(S)-202-791 (generously donated by Sandoz Ltd., Switzerland) in 95% ethanol were diluted in extracellular solution to the required concentrations (less than 0.05% ethanol after addition to extracellular solution). ω-Conotoxin GVIA was purchased from Peninsula Laboratories (Belmont, CA). Unless otherwise noted, extracellular solution contained (mM): HEPES (20), $MgCl_2$ (1.0), d-glucose (10), NaCl (115), and TEACl (20) pH 7.3. This solution was mixed with appropriate volumes of 100 mM $BaCl_2$, $CaCl_2$ or $SrCl_2$ to yield the final concentration of divalent cation indicated in the text (usually 20 mM $BaCl_2$). Tetrodotoxin (TTX, 5 μM) purchased from Sigma (St. Louis, MO) was added to block voltage-sensitive Na^+ channels (Narahashi *et al.*, 1964). Internal (pipette) solution contained (mM): HEPES (10), $MgCl_2$ (1), d-glucose (10), TEACl (10), EGTA (5), CsCl (125) and ATP (Sigma, St. Louis, MO) (2), pH 7.3. TEA^+, Ba^{2+} (Hagiwara *et al.*, 1978; Armstrong *et al.*, 1982) and Cs^+ were used in external and/or internal solutions to suppress K^+ current in PC12 cells. ATP was added to internal solutions to prevent time-dependent rundown of current amplitude (Bean, 1985).

Culture of PC12 cells. PC12 cells were cultured at 37°C in a humidified 10% CO_2 atmosphere using Dulbecco's modified Eagle medium (pH 7.3, GIBCO) containing 10% horse serum, 5% fetal bovine serum and 2 mM HEPES. Inasmuch as certain antibiotics have been shown to be capable of blocking Ca^{2+} channels (Atchison *et al.*, 1988), antibiotics were omitted from the culture medium. PC12 cells were differentiated by addition of 100 ng/ml of 7s NGF. To maintain consistency from experiment to experiment, cells from the 15th passage from our receipt of the cell line were used for recording Ba^{2+} currents.

Current recordings. Cells were plated into 35 mm polystyrene dishes and cultured for 5-7 days in 2 ml of medium containing NGF. Prior to recording Ba^{2+} current, the culture medium was removed and cells were washed 2-3 times and then covered with 1 ml of extracellular solution. Test compounds were prepared in extracellular solutions such that addition of a 500 μl aliquot to the dish would result in the final concentrations of test compound indicated in the text and figure legends. This method ensured rapid mixing of solutions and maintained osmolarity of the extracellular solution. Voltage-activated Ba^{2+} currents in PC12 cells were recorded using the whole cell patch voltage-clamp method (Hamill *et al.*, 1981). Resistance of patch electrodes (1.2 mm glass, World Precision Instruments, New Haven, CT) was between 2-6 MΩ. The patch clamp circuit consisted of an Axon Instruments CV-1 headstage and Axopatch 1B patch clamp. Pulse protocols were generated and current responses recorded on-line using a Compaq 386 micro computer and the pClamp™ program interfaced to the Axopatch 1B via a Axon Instruments TL-1 interface board.

For analysis of current-voltage relationships, a 255 ms pulse to each indicated test potential was applied from a holding potential of -40, -70 or -90 mV. Leak current was measured at the appropriate holding potential by pulses which were equal in duration, but opposite in polarity and ½ the magnitude of the test potentials. To examine the reversibility of MeHg-induced block of I_{Ba} by washing in MeHg-free solutions, inward (holding potential -90 mV, test potential +10 mV, 255 ms pulses) and leak currents were recorded from PC12 cells in the absence of MeHg. MeHg was then added to the extracellular solution for 1 min and the currents were recorded again. All but approximately 100 - 200 μl of extracellular solution were then removed from the dish and replaced with a 1 ml aliquot of MeHg-free extracellular solution. This was repeated 3 times to wash the cells and current was recorded again. Current responses were filtered at 10 KHz using a 4-pole lowpass Bessel filter. Following scaling, linear leak and capacitive currents were subtracted from gross current using the pClamp™ program. Pulse protocols and filter settings for other experimental protocols are described in the Results section. Series resistance remaining after compensation was less than 2 MΩ, as 80% or greater compensation was commonly achieved. Recordings which contained indications of inadequate space clamp, such as pronounced shoulders during current activation, were not included in analysis. All recordings were made at room temperature (22-25°C). A minimum of 3 cells were recorded from for any given experiment and dishes of cells were discarded after addition of test solutions.

RESULTS

Description of I_{Ba} in PC12 cells. The pharmacological and biophysical properties of Ba^{2+} current in differentiated PC12 cells were characterized in order to confirm the identity of current components presumably mediated by N- and/or L-type Ca^{2+} channels (Plummer *et al.*, 1989; Usowicz *et al.*, 1990). Ba^{2+} currents elicited by a test potential to +10 mV from holding potentials of -40 (Figure 5.1A), -70 (Figure 5.1B), or -90 mV (Figure 5.1C) contained non-inactivating and inactivating components (-70 and -90 mV only). The time constant, τ, for the inactivating component of currents at a test potential of +10 mV averaged 225±86 ms (n=8) and 175±39 (n=15) ms in cells held at -90 and -70 mV, respectively. A family of Ba^{2+} currents is illustrated in Figure 5.2. The holding potential was -90 mV and test potentials began at -50 mV and were increased in 10 mV increments to +70 mV.

Components of Ba^{2+} current in PC12 cells are sensitive to block by the N-channel antagonist CgTx (Figure 5.3A). The inactivating component of current was apparently eliminated, while a non-inactivating component remained. Nifedipine (20 nM), a DHP Ca^{2+} channel antagonist, substantially reduced I_{Ba} (data not shown), whereas (+)-(S)-202-791 (<5 μM), a pure DHP Ca^{2+} channel agonist (Hof *et al.*, 1985), enhanced I_{Ba} during voltage steps (Figure 5.3B) and induced tail currents upon repolarization (Figure 5.3, inset). Thus, Ba^{2+} currents recorded from differentiated PC12 cells cultured in this laboratory have pharmacological and biophysical profiles consistent with currents mediated by N- and L-type Ca^{2+} channels, respectively.

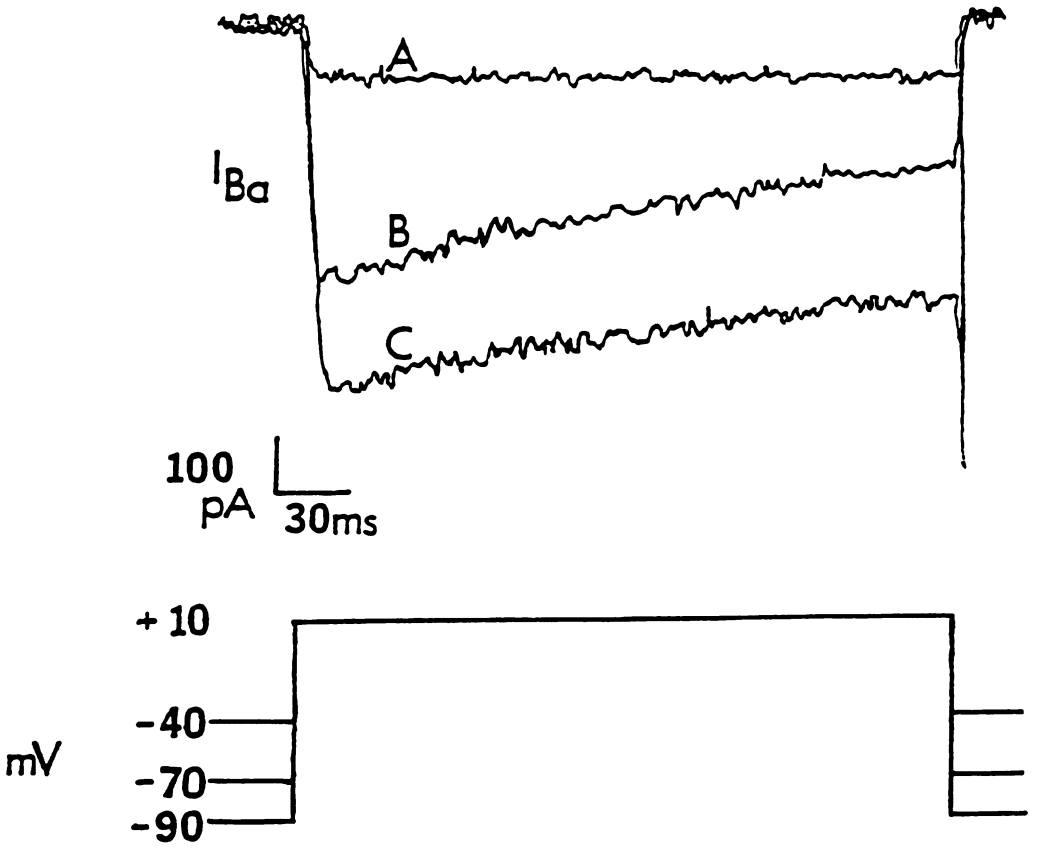

Figure 5.1. Inactivating and non-inactivating components of Ba^{2+} current in PC12 cells. Ba^{2+} current in differentiated PC12 cells in response to a voltage step to +10 mV from membrane holding potentials of -40 (A), -70 (B) or -90 (C) mV. Command potentials shown below current traces were 255 ms long. Linear components of leak and capacitance have been subtracted. Current responses were filtered at 10 KHz.

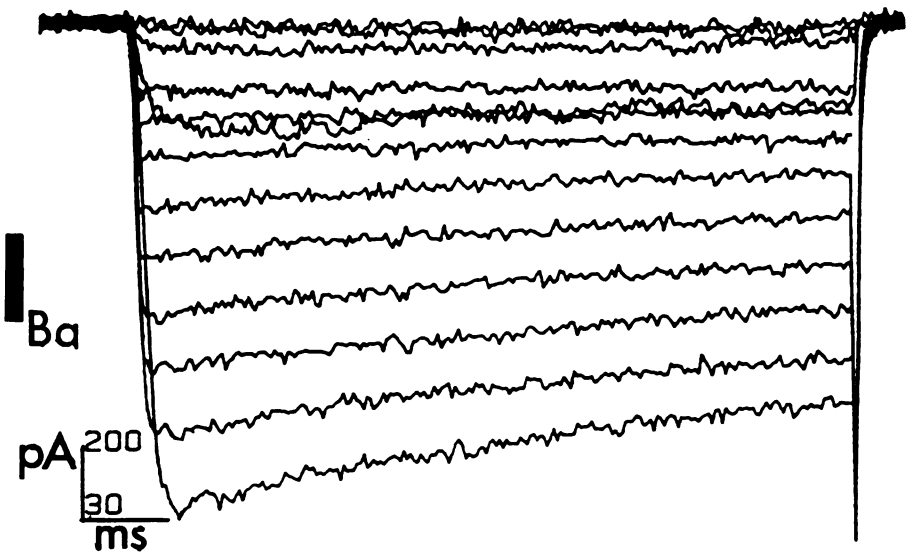

Figure 5.2. Current responses to a series of test potentials. Current responses in a differentiated PC12 cell to test potentials between -50 and +70 mV when the magnitude of the test potential is increased in 10 mV increments. Membrane holding potential was -90 mV and test potentials were 255 ms in duration. Current responses were filtered at 10 KHz and linear components of leak and capacitance current have been subtracted.

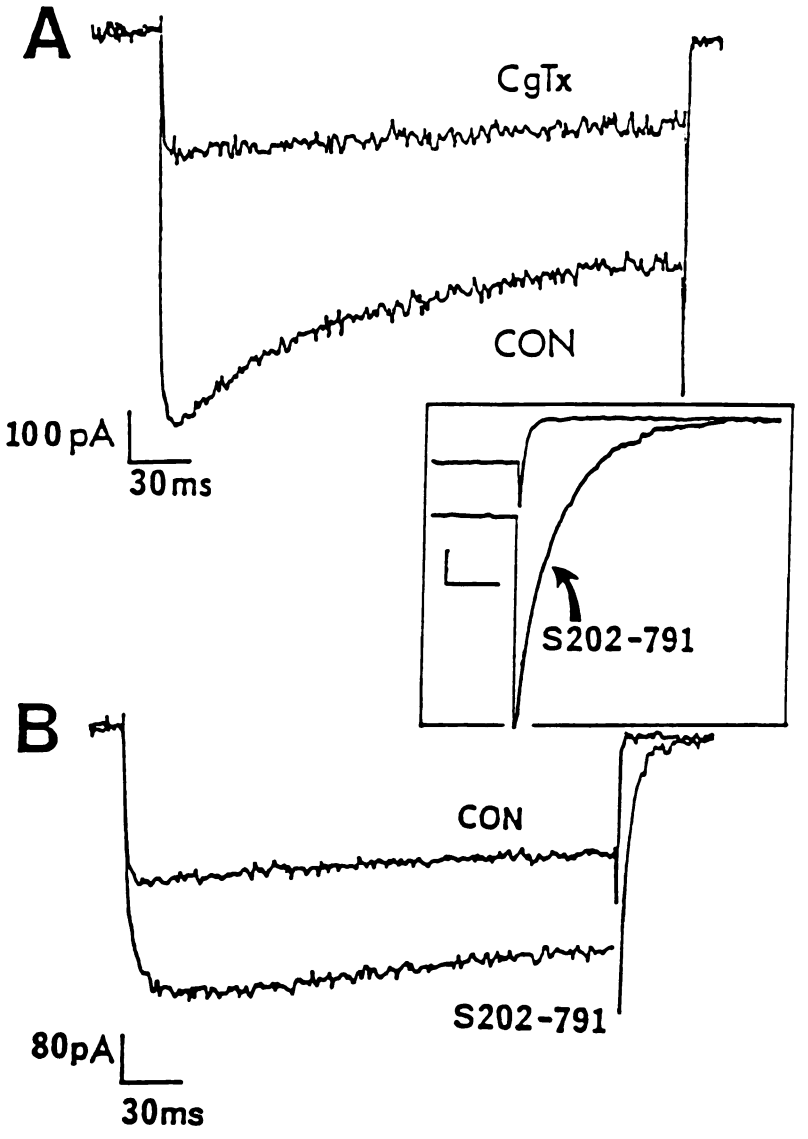

Figure 5.3. Effects of ω-conotoxin and (+)-S202-791 on Ba^{2+} current in PC12 cells. A) Effect of CgTx (16 μM) on I_{Ba} in a differentiated PC12 cell. The current response is to a 255 ms step to +10 mV from a holding potential of -90 mV. Current response is filtered at 10 KHz. B) Effect of (+)-(S)-202-791 (< 5 μM) on I_{Ba} in a differentiated PC12 cell. The current response is to a 255 ms step to +10 mV from a holding potential of -70 mV. Inset: Tail currents recorded upon repolarization to -70 mV from a 20 ms step to +10 mV prior to and after addition of (+)-(S)-202-791. Scale bars are 400 pA and 7 ms. All current responses were filtered at 10 KHz and linear components of leak and capacitance were subtracted. Responses shown are the average of 5 trials prior to and after addition of CgTx or (+)-(S)-202-791.

Effect of MeHg on I_{Ba} in PC12 cells. The effects of MeHg on membrane properties and currents were examined using the pulse protocol shown in Figure 5.4A. A depolarizing pulse was delivered to elicit inward current, followed by a hyperpolarizing pulse so that the leak current in the cell could be measured. Linear components of leak and capacitive current were not subtracted from these records. Thus, effects of MeHg on inward current, presumably mediated by Ba^{2+}, leak current and capacitive current can be examined in consecutive current traces. Addition of MeHg (10 μM) to the extracellular solution resulted in a rapid block of inward current (Figure 5.4A), but did not alter membrane leak current nor membrane capacitance, indicating that the effect of MeHg is not due simply to disruption of membrane electrotonic properties. When this depolarizing pulse was repeated once every 5 s, addition of increasing concentrations of MeHg to the extracellular solution resulted in a concentration-dependent increase in the rate and magnitude of block of I_{Ba} following leak subtraction (Figure 5.4B).

Ba^{2+} current amplitude in PC12 cells often increased during the first 1-1.5 min after adjusting whole cell capacitance and series resistance compensation controls (Figures 5.4B and 5.5, data prior to MeHg addition), after which time current amplitude remained stable for beyond 10 min. This initial increase in current may be associated with exchange of cytosolic constituents and pipette solutions, resulting in block of K^+ channels by Cs^+ and TEA^+ and/or phosphorylation of Ca^{2+} channels by ATP-dependent mechanisms. To ensure that effects of MeHg were examined on stable Ba^{2+} current, cells were held at rest for 2 min before recording I_{Ba} in all

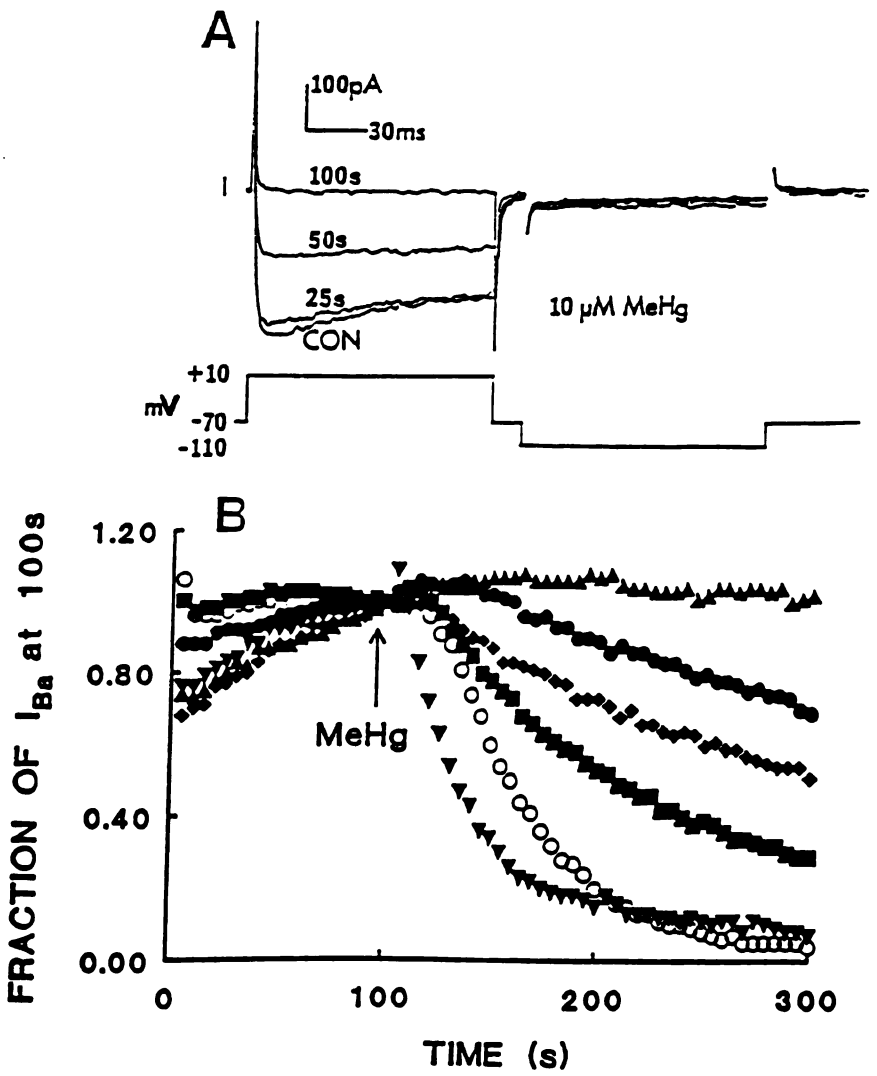

Figure 5.4. Effects of MeHg on currents in PC12 cells. A) Effect of 10 μM MeHg on gross inward and leak current measured using whole cell patch voltage-clamp techniques on a differentiated PC12 cell. As shown in the command potential protocol below the current traces, membrane potential was clamped at -70 mV and current responses to the designated voltage steps (100 ms ea.) were recorded. This voltage protocol was repeated once every 5 s. Current traces shown were recorded immediately before addition of MeHg (CON), and 25, 50 and 100 s after addition of MeHg to the recording solution. Current responses were filtered at 1 KHz and leak current has not been subtracted. B) Effect of increasing concentrations of MeHg on peak Ba^{2+} current measured in response to the pulse protocol in A after leak subtraction. Pulses were repeated once every 5 s and recording medium containing MeHg was added after 100 s, as indicated by the arrow. Final concentrations of MeHg were: 0 (upright triangles), 1 (solid circles), 2.5 (solid diamonds), 5 (solid squares), 10 (open circles) or 20 (inverted triangles) μM. Peak current in each trace was normalized to peak current measured prior to the addition of MeHg. Values shown are the average of peak current from 4-8 different cells and average standard error values were 23% of mean values throughout the experiment.

subsequent experiments, except for those designed to examine frequency-dependence of block. In addition, all experiments were completed within 10 min of adjusting patch circuit controls.

Use-dependence of block of I_{Ba} by MeHg. The ability of MeHg to interact with Ca^{2+} channels in PC12 cells in a use-dependent manner was assessed by examining effects of MeHg on Ba^{2+} currents elicited by stimulating PC12 cells at frequencies between 0.1 and 0.4 Hz. At the fastest rate of stimulation, the time required for MeHg (5 μM) to block approximately 70% of the current (approximately 3 min) was less than the time required for MeHg to block current at slower rates (approximately 6 min) of stimulation (Figure 5.5). Thus, increasing the frequency of stimulation, and hence use of Ca^{2+} channels, appears to facilitate slightly their block by MeHg.

Although increasing the frequency of stimulation appears to facilitate block of I_{Ba} by MeHg, Ba^{2+} current was reduced appreciably in MeHg-containing solutions even at relatively low rates of stimulation (0.1 Hz). In addition, block of synaptosomal $^{45}Ca^{2+}$ influx by MeHg is not state-dependent (Shafer *et al.*, 1990). Thus, experiments were designed to determine if activation of Ca^{2+} channels is essential for the blocking action of MeHg. Accordingly, effects of MeHg on I_{Ba} in PC12 cells were examined following a period of inactivity during which MeHg was added to the extracellular solution. The protocol was as follows: a series of 10 voltage steps from -70 to +10 mV (255 ms duration) was delivered at 3 s intervals. Leak current, which was subtracted after scaling and averaging, was then measured by a series of 20 steps from -70 to -110 mV. After measurement of leak current, 5

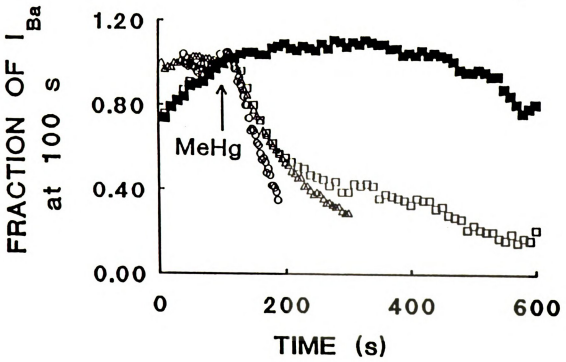

Figure 5.5. Use-dependent effects of MeHg on I_{Ba} in PC12 cells. Effect of increasing the rate of stimulation on peak inward current in the absence (solid symbols) or presence of 5 μM MeHg (open symbols) in differentiated PC12 cells. The pulse protocol used in Figure 5.4A was repeated at frequencies of 0.01 (squares), 0.2 (triangles) and 0.4 (circles) Hz. Peak current in each trace was normalized to peak current measured prior to the addition of MeHg. Values shown are the mean of 4-8 different cells and average standard error values were 24% of mean values throughout the experiment. Current responses were filtered at 1 KHz and leak current has been subtracted. Control values for 0.2 and 0.4 Hz were not different from values for 0.1 Hz and are not illustrated.

or 10 μM MeHg was added to the extracellular solution, and 2 min later the voltage protocol described above was repeated. When no MeHg was added to the extracellular solution, no significant changes were observed in peak or end current (results not shown). After a 2 min exposure to 5 μM MeHg in the absence of stimulation, the average amplitude of the first current response was reduced when compared with the average amplitude of the first current response in the absence of MeHg. Peak and end currents were reduced by an average of 32% and 26%, respectively (Figure 5.6, bottom panel). Addition of 10 μM MeHg resulted in significant reductions (paired t-test, $p < 0.05$) of 77 and 70% for peak and end currents, respectively, when compared to control values (Figure 5.6, bottom panel). In addition, 10 μM MeHg caused complete elimination of the inactivating component of whole-cell current (Figure 5.6, top panel). In none of four cells to which 10 μM MeHg was added could an estimate of τ be obtained for the first current response in the presence of MeHg, and no current responses containing inactivating components were observed.

Holding Potential- and Voltage-Dependence of Block by MeHg. Current-voltage relationships for I_{Ba} in PC12 cells were obtained from holding potentials of -40, -70, and -90 mV in the presence and absence of 10 μM MeHg (Figure 5.7). At all three holding potentials, a 2 min exposure to MeHg decreased the magnitude of peak Ba^{2+} current elicited at each test potential. There was no readily apparent holding potential-dependence of the action of MeHg. Currents elicited by depolarizing steps from a holding potential of -40 mV were blocked in a voltage-

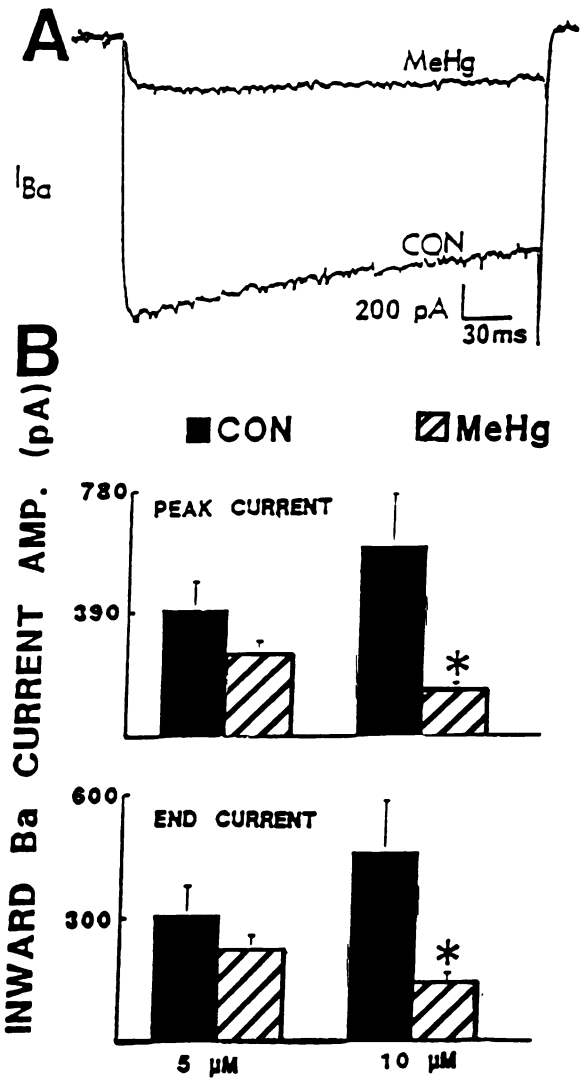

Figure 5.6. Resting block of I_{Ba} by MeHg in PC12 cells. A) I_{Ba} in a differentiated PC12 cell recorded prior to (CON) and after MeHg (10 μM) was present in the recording solution for 2 min. B) Amplitude of inward Ba^{2+} current recorded before (solid bars) and after (striped bars) a 2 min. exposure to 5 or 10 μM MeHg. The asterisk (*) indicates a significant reduction in current amplitude in the presence of MeHg when compared to control values. (Paired t-test, $P < .05$). Values shown are the mean ± SEM of 7 and 4 different cells exposed to 5 or 10 μM MeHg, respectively. Current responses are to a 255 ms voltage step to +10 mV from a membrane holding potential of -70 mV and were filtered at 10 KHz. Linear components of leak and capacitance current have been subtracted.

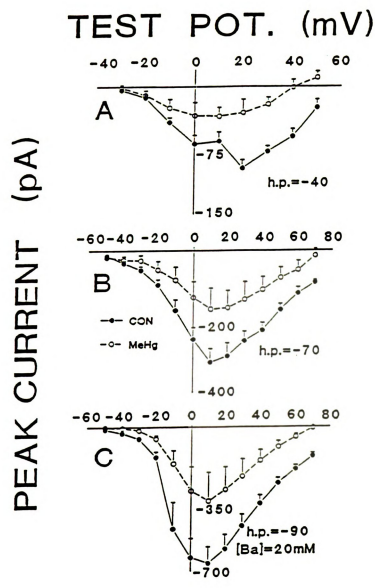

Figure 5.7. Effects of MeHg at different holding and test potentials in PC12 cells. Current-voltage relationships for I_{Ba} in differentiated PC12 cells recorded in the absence (filled symbols) and presence (open symbols) of 10 μM MeHg. Voltage steps (255 ms) to the test potentials (TEST POT.) indicated in the figure were applied from holding potentials of -40 (A), -70 (B) and -90 (C) mV. Current responses were filtered at 10 KHz and following subtraction of leak and capacitance current, peak current was measured. Results shown are the mean± SEM of peak current from 4 to 8 cells, each cell received all test potentials.

dependent manner by MeHg. The magnitude of block by MeHg increased from 23 to 98% as the test potential was increased from -20 to +40 mV.Block of I_{Ba} by MeHg was not strongly voltage-dependent at holding potentials of -70 and -90 mV. At the holding potential of -90 mV, the magnitude of reduction of I_{Ba} by MeHg at the most weakly and most strongly depolarizing test potentials (-30 and +70 mV) was greater than the magnitude of reduction of I_{Ba} by MeHg at test potentials which elicit peak I_{Ba} (0 and +10 mV). MeHg did not alter the test potentials at which activation of I_{Ba} or peak I_{Ba} were observed. However, MeHg caused a negative shift in the apparent reversal potential of I_{Ba} at all holding potentials.

To determine whether irreversible, voltage-dependent block of Ca^{2+} channels by MeHg occurred, block of I_{Ba} by 5 and 10 μM MeHg was examined using a holding potential of -40 mV; test pulses (255 ms) were increased by 10 mV increments from -30 to +50 mV and then decreased in 10 mV increments back to -30 mV (Figure 5.8). If MeHg blocks Ca^{2+} channels in an irreversible, voltage-dependent, manner, one would expect a greater magnitude of block as test pulses are decreased from +50 to -30 mV than that observed when test potentials were increased from -30 to +50 mV. Current amplitudes and effects of MeHg were similar in each case, indicating that MeHg does not bind irreversibly to Ca^{2+} channels in a voltage-dependent manner.

Effects of $[Ba^{2+}]_e$ on block of I_{Ba} by MeHg. Previous studies in synaptosomes indicated that at non-saturating $[Ca^{2+}]_e$, increasing $[Ca^{2+}]_e$ antagonizes somewhat the magnitude of block of $^{45}Ca^{2+}$ influx by MeHg (Atchison *et al.*, 1986; Shafer and Atchison, 1989). Therefore, it seemed prudent to determine if the action of MeHg

on I_{Ba} depended on $[Ba^{2+}]_e$. Decreasing $[Ba^{2+}]_e$ resulted in decreases in the control current as well as currents measured after 2 min exposure to 10 μM MeHg (Figure 5.9). At a test potential of +10 mV, MeHg caused a 77.3% reduction when compared to control current for solutions containing 10 mM Ba^{2+} and a 45.6% for those containing 20 mM Ba^{2+}. In 30 mM Ba^{2+} solutions, inward currents were not significantly larger than in 20 mM Ba^{2+} solutions, indicating that these concentrations saturate Ca^{2+} channels in PC12 cells. In 30 mM Ba^{2+} solutions, exposure to 10 μM MeHg reduced I_{Ba} by 49.8% (results not shown). Thus, $[Ba^{2+}]_e$ antagonizes the blocking action of MeHg, but is not capable of overcoming completely the effects of MeHg. As in the above current-voltage relationships, MeHg also caused a negative shift in the apparent reversal potential for I_{Ba}, and an outward current was observed in the presence of MeHg when $[Ba^{2+}]_e = 10$mM.

Reversibility of MeHg-induced Block by Wash. To determine if MeHg acted on Ca^{2+} channels in a fashion similar to inorganic divalent cations or if MeHg acted on Ca^{2+} channels in an irreversible fashion, the reversibility of MeHg-induced block of I_{Ba} in PC12 cells by washing cells with MeHg-free solutions was examined. Effects on I_{Ba} of exposure to 10 μM MeHg were not reversed by washing with MeHg-free solutions in 4 attempts (results not shown). In one cell, block of I_{Ba} by 5 μM MeHg was also not reversed by washing with MeHg-free solutions (results not shown). In contrast, block of I_{Ba} by 1 μM Cd^{2+} was reversed readily by washing with Cd^{2+}-free solutions (results not shown). The ability of micromolarconcentrations of Cd^{2+} to block I_{Ba} is consistent with previous results in cultured cells (Boland and Dingledine,

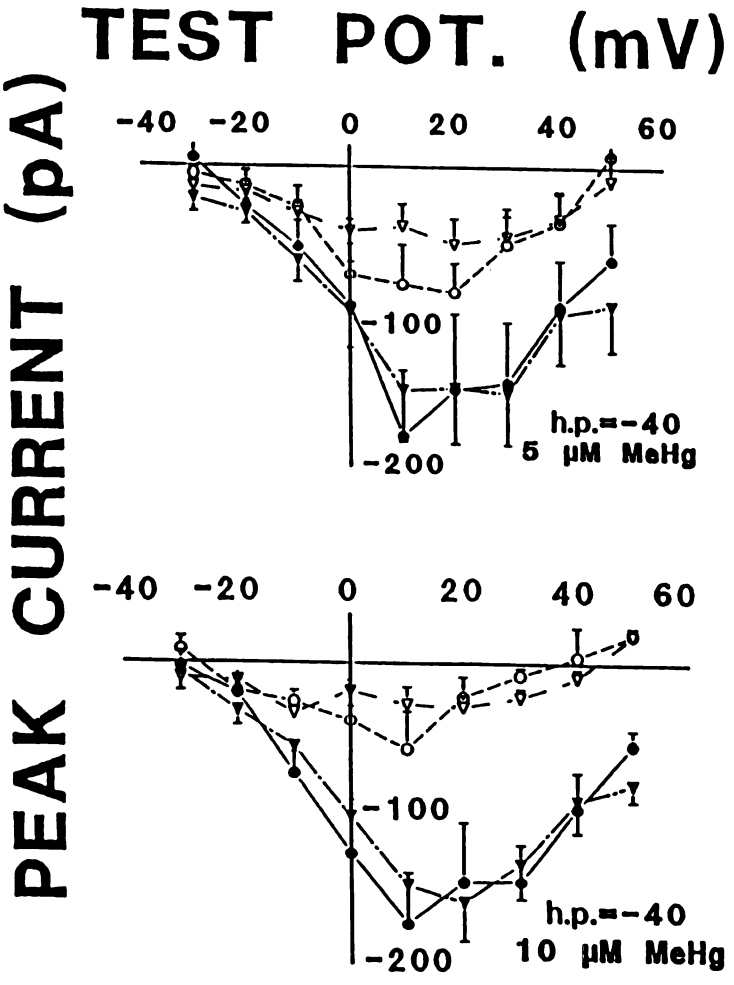

Figure 5.8. Voltage-dependent block by MeHg of I_{Ba} in PC12 cells is not irreversible. Current-voltage relationship for I_{Ba} in differentiated PC12 cells in the absence (filled symbols) and presence (open symbols) of 5 or 10 μM MeHg. From a holding potential of -40 mV, test pulses (255 ms) were delivered in increasing increments (circles) of 10 mV from -30 to +50 mV, followed by decreasing increments (triangles) of 10 mV from +50 to -30 mV. Current responses were filtered at 10 KHz. Following subtraction of leak and capacitance current, peak current was measured. Results shown are the mean ± SEM of peak current from 4 cells.

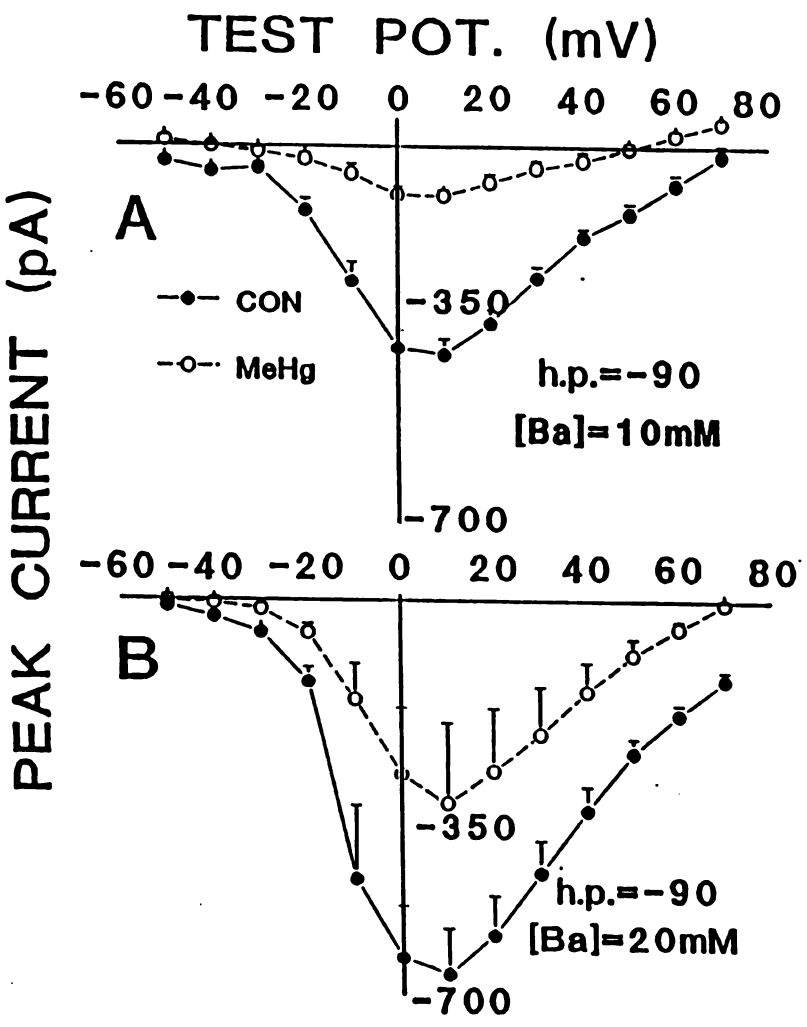

Figure 5.9. Ba^{2+} can antagonize block of Ca^{2+} channels by MeHg. Current-voltage relationships for I_{Ba} in differentiated PC12 cells recorded in the absence (solid circles) and presence (open circles) of 10 M MeHg in recording solutions containing 10 (A) or 20 mM $BaCl_2$ (B). Voltage steps (255 ms) to the test potentials (TEST POT.) indicated in the figure were applied from a holding potential of -90 mV. Current responses were filtered at 10 KHz and following subtraction of leak and capacitance current, peak current was measured. Results shown are the mean ± SEM of peak current from 4 to 8 cells, each cell received all test potentials.

1990), as is the ability of washing with Cd^{2+}-free solutions to reverse the block of I_{Ba} in cultured cells (Boland and Dingledine, 1990) and block of $^{45}Ca^{2+}$ influx into synaptosomes (Nachshen, 1984) by Cd^{2+}. Thus, effects of MeHg on Ca^{2+} channels are not readily reversible.

Ionic permeability of Ca^{2+} channels in PC12 cells. Currents mediated by Ba^{2+} (Plummer *et al.*, 1989; Usowicz *et al.*, 1990) and Ca^{2+} (Garber *et al.*, 1989) have been measured in NGF-differentiated PC12 cells. However, the author is unaware of any previous reports comparing the permeability of PC12 cell Ca^{2+} channels for divalent cations (Ca^{2+}, Ba^{2+} and Sr^{2+}). MeHg altered the ionic selectivity of synaptosomal Ca^{2+} channels by decreasing influx of $^{85}Sr^{2+}$ to a greater extent than influx of radiolabeled Ca^{2+} and/or Ba^{2+} (Shafer *et al.*, 1990). Thus, currents mediated by Ca^{2+} channels using 10 mM Ca^{2+}, Ba^{2+} or Sr^{2+} as the charge carrier in extracellular solutions were examined and effects of MeHg on ionic permeability of PC12 cell Ca^{2+} channels were characterized. To prevent differences in cell size from influencing the results, recordings were made using solutions of Ca^{2+}, Ba^{2+} or Sr^{2+} which were labelled as unknowns, and results from recordings made from 46 cells were pooled. Only after current records had been analyzed was the identity of the divalent cation in the extracellular solution revealed. The permeability sequence of Ca^{2+} channels in differentiated PC12 cells was $Ca^{2+} = Sr^{2+} > Ba^{2+}$ when the holding potential was -90 mV (Figure 5.10). This observation was consistent throughout several cell passage numbers (10, 11 and 15) and days in culture (4, 5, 7 and 12) in the presence of NGF. MeHg (10 μM) reduced current mediated by all three divalent cations. However, the

apparent ionic selectivity of Ca^{2+} channels for Ca^{2+} and Sr^{2+} over Ba^{2+} was eliminated in the presence of MeHg.

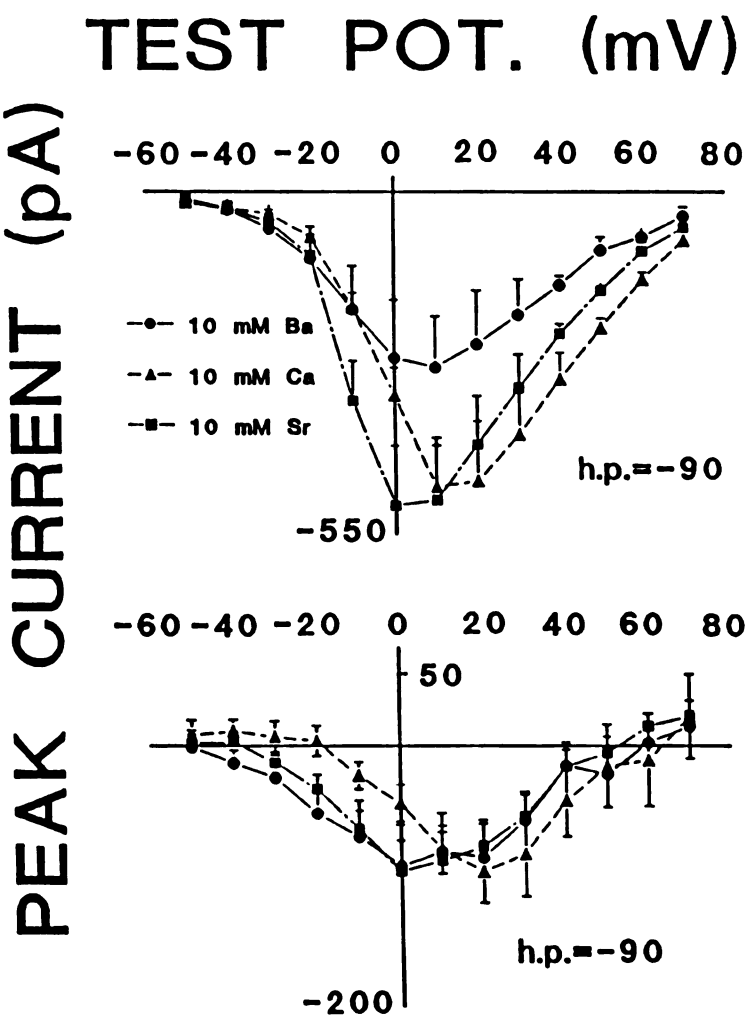

Figure 5.10. MeHg alters ionic permeability of Ca^{2+} channels in PC12 cells. Current-voltage relationships for current mediated by 10 mM Ca^{2+} (triangles), Sr^{2+} (squares) or Ba^{2+} (circles) in the absence (upper panel) or presence (lower panel) of 10 μM MeHg. Voltage steps (255 ms) to the test potentials (TEST POT.) indicated in the figure were applied from holding potentials of -90 mV. Current responses were filtered at 10 KHz and following subtraction of leak and capacitance current, peak current was measured. Results shown are the mean± SEM of peak current from 14-15 and 6 cells in the absence and presence of MeHg, respectively, each cell received all test potentials.

DISCUSSION

Results of the present study indicate that 1) NGF-differentiated PC12 cells cultured in this laboratory contain both N- and L-type Ca^{2+} channels; 2) low micromolar concentrations of MeHg block I_{Ba} in PC12 cells and the rate of block is concentration-dependent; 3) increasing the frequency of channel stimulation may facilitate block of I_{Ba} by MeHg, but channel use is not required for the blocking action of MeHg; 4) block of I_{Ba} by MeHg is not holding potential-dependent; 5) block of I_{Ba} by MeHg is antagonized, but not completely overcome, by increasing $[Ba^{2+}]_e$; 6) block of I_{Ba} by MeHg is voltage-dependent when the holding potential is -40 mV, but is not strongly voltage-dependent when the holding potential is -70 or -90 mV, 7) block of I_{Ba} by MeHg is not reversed by washing with MeHg-free solutions; 8) the apparent ionic selectivity of whole cell current mediated by Ca^{2+} channels in PC12 cells is $Ca^{2+} = Sr^{2+} > Ba^{2+}$, and MeHg alters the apparent ionic selectivity of Ca^{2+} channels in PC12 cells. These results suggest that block of I_{Ba} in differentiated neuron-like cells mirrors block of radiotracer flux into synaptosomes. Moreover, the blocking properties of MeHg appear to be unique from inorganic divalent metals.

NGF-differentiated PC12 cells express both N- and L-type Ca^{2+} channels (Plummer *et al.*, 1989; Usowicz et al, 1990). The presence of T-type channels in PC12 cells has also been reported (Garber *et al.*, 1990). Due to variability between PC12 subclones it is important to characterize the biophysical and pharmacological properties of Ca^{2+} channel types present prior to making conclusions about effects of uncharacterized chemicals on Ca^{2+} channels in these cells. Ba^{2+} current from cells

cultured in the presence of NGF in this laboratory contained no inactivating component when held at -40 mV, and contained inactivating and non-inactivating components when held at more hyperpolarized membrane potentials. The average time constant for decay of the inactivating portion of the current was somewhat slower than that reported previously for N-type channels and current in PC12 cells (Plummer *et al.*, 1989), although many individual cells contained currents with τ values of around 95 ms. Furthermore, Ba^{2+} current from cells used in these experiments exhibited characteristic responses to DHP L-channel type agonists and antagonists and were blocked by ω-conotoxin GVIA, which affects exclusively N-type channels in PC12 cells (Plummer, *et al.*, 1989; Usowicz *et al.*, 1990). Thus, based on both the biophysical and pharmacological characteristics of whole cell current, these results clearly indicate the presence of N- and L-type Ca^{2+} channels in PC12 cells cultured in this laboratory.

Block of Ba^{2+} current in PC12 cells is rapid and complete upon addition of 10 μM MeHg to the extracellular solution. Leak and capacitative currents in PC12 cells were not affected by MeHg, indicating that effects of MeHg are relatively specific for channel function. Both N- and L-type channels in PC12 cells are affected by MeHg, as MeHg blocks Ba^{2+} current at all holding potentials tested. These results confirm results of previous binding experiments which indicated that MeHg interacts with N- and L-type channels in PC12 cells and synaptosomes by interfering with CgTx and DHP binding, respectively (Shafer *et al.*, 1990). The concentration-dependency for block of I_{Ba} occurred at lower concentrations (1-20 μM) of MeHg than did block of

$^{45}Ca^{2+}$ influx (50-100 μM) by MeHg into PC12 cells (Shafer et al, 1990) and synaptosomes (Shafer and Atchison, 1989). Although this discrepancy does not appear extremely large, one must consider differences in divalent cation concentration used for electrophysiological (10-20 mM Ba^{2+}) compared to flux (0.05 - 1 mM Ca^{2+}) measurements. There are small but significant differences in the techniques used to measure Ca^{2+} influx and Ba^{2+} current; namely continuous and comparatively long (1-10 s) intervals of depolarization yet brief exposure (only 1-10 s) to MeHg for flux studies as opposed to short (100-250 ms), repeated step depolarizations during a several minute period of exposure to MeHg for Ba^{2+} current measurements. Higher concentrations of MeHg may be necessary to block Ca^{2+} channels due to the much shorter overall lengths of exposure to MeHg during flux assays.

Block of Na^+ channels by local anesthetics (Strichartz, 1973; Courtney, 1975; Hille, 1977) and Ca^{2+} channels by organic Ca^{2+} channel antagonists (Uehara and Hume, 1985) has been described as "use-dependent", indicating that these compounds bind preferentially to the open or inactivated state of the channel. The results of the present experiments suggest that MeHg effectively blocks Ca^{2+} channels in the resting state, yet the blocking action can be facilitated by channel use. Thus, the action of MeHg on Ca^{2+} channels is not state-dependent. This is consistent with results obtained using synaptosomes (Shafer *et al.*, 1990). DHP Ca^{2+} channel blockers substantially reduce I_{Ca} in the absence of prior stimulation and have use-dependent actions only during high-frequency stimulation (Lee and Tsien, 1983; Uehara and

Hume, 1985). By contrast, the actions of verapamil and D-600 are strongly use-dependent, whereas diltiazem exhibits both resting and use-dependent block of I_{Ca} (Lee and Tsien, 1983; Uehara and Hume, 1985). Furthermore, multivalent inorganic cations (Nachshen, 1985) act on synaptosomal Ca^{2+} channels in a use-dependent manner. MeHg, like the organic Ca^{2+} channel blockers, is lipophilic, but it is also similar to divalent cations in size and is charged. Thus, MeHg may have access to the Ca^{2+} channel by more than one route, including diffusion through the membrane and entry through the channel pore. Perhaps the combination of charge and lipophilicity of MeHg may account for its ability to block Ca^{2+} channels in the resting state, while increasing frequency of stimulation facilitates the block.

Addition of MeHg in the absence of stimulation consistently eliminated the inactivating component of current. This action of MeHg is unique compared to that of Cd^{2+} and Ni^{2+}, which do not alter the time course of decay of I_{Ba} in a dorsal root ganglion cell line (Boland and Dingledine, 1990). N-type Ca^{2+} channels in PC12 cells may be more sensitive to block by MeHg than L-type channels. Alternatively, MeHg may impair the ability of N-type channels to inactivate during the voltage step. This latter mechanism would also require a simultaneous reduction in single channel conductance or number of open Ca^{2+} channels (open probability) by MeHg to account for the decreased current amplitude when MeHg is present. Either mechanism is consistent with the results of the present experiments; single channel recordings would be required to distinguish between them. MeHg altered the inactivation kinetics of synaptosomal $^{45}Ca^{2+}$ influx; the rate constant for inactivation

was increased and the amount of non-inactivating influx was decreased (Shafer *et al.*, 1990). Thus, MeHg produces analogous actions on inactivating and non-inactivating current in PC12 cells and synaptosomes.

MeHg blocked Ba^{2+} currents elicited by depolarizing steps to positive potentials from holding potentials of -40, -70 and -90 mV. The blocking action of MeHg was not strongly voltage-dependent at holding potentials of -70 and -90 mV, except that the degree of block was less at test potentials near the peak of the current-voltage relationship when the holding potential was -90 mV. This may be related to the ability of Ba^{2+} to antagonize the blocking action of MeHg, as the driving force for Ba^{2+} entry through Ca^{2+} channels and the number of channels in the open state is greatest at these test potentials. Block of I_{Ba} was voltage-dependent at -40 mV. Presumably, there is very little N-type current at this holding potential due to steady-state inactivation of N channels (Fox *et al.*, 1987a,b). Thus, MeHg appears to block L-type Ca^{2+} channels in a voltage-dependent manner, a result consistent with voltage-dependent block of MeHg on synaptosomal $^{45}Ca^{2+}$ influx (Shafer *et al.*, 1990). The hydrophobic nature of MeHg may be important in this regard, as organic, but not inorganic mercurials block $^{45}Ca^{2+}$ influx into synaptosomes in a voltage-dependent manner (Hewett and Atchison, 1990).

Additionally, MeHg caused a negative shift in the apparent reversal potential of I_{Ba} at all holding potentials and for all Ba^{2+} concentrations tested. Under the experimental conditions used, the reversal potential for I_{Ba} is theoretically positive to +80 mV. An actual outward Ba^{2+} current is not expected under the present

experimental conditions, due to the presence of EGTA in the internal solution. We cannot eliminate the possibility that MeHg interferes with block of K^+ channels by TEA^+ or Cs^+, but does not block K^+ channels itself, thus giving rise to outward currents in the presence of MeHg. Outward currents have also been observed in isolated cardiac myocytes (Lee and Tsien, 1982) and in bovine adrenal chromaffin cells (Fenwick *et al.*, 1982) when K^+ or Cs^+ permeate Ca^{2+} channels at highly positive test potentials. However, Ca^{2+} channel blockers such as D600 or Cd^{2+} effectively block these outward currents (Lee and Tsien, 1982). Thus, any potential outward Cs^+ currents should be blocked by MeHg. The present data do not suggest any particular mechanism underlying this effect of MeHg.

Washing PC12 cells in MeHg-free solutions did not reverse the block of I_{Ba} by MeHg in any of the cells tested, although block of I_{Ba} by Cd^{2+} was reversed readily by washing. Furthermore, extracellular Ba^{2+} can antagonize, but not completely overcome the blocking actions of MeHg. This is consistent with effects of increasing $[Ca^{2+}]_e$ on the effects of MeHg on synaptosomal $^{45}Ca^{2+}$ influx (Shafer and Atchison, 1989). These results are also consistent with previous experiments at the neuromuscular junction which indicate that the effects of MeHg on synaptic transmission are not readily reversed by washing with MeHg-free solutions and can only be partially reversed by increasing $[Ca^{2+}]_e$ (Traxinger and Atchison, 1987b). Block of neuromuscular transmission by Pb^{2+} (Cooper and Manalis, 1983; Atchison and Narahashi, 1984; Pickett and Bornstein, 1984), Cd^{2+} (Nilson and Volle, 1976; Cooper and Manalis, 1984a,b), or Co^{2+} (Weakly, 1973) is reversed by increasing

$[Ca^{2+}]_e$ or washing with normal, metal-free solutions. This represents a fundamental difference in the action of MeHg when compared to divalent heavy metals. The mechanism by which MeHg interacts with Ca^{2+} channels in an essentially irreversible manner is unknown, although reactivity of MeHg with sulfhydryl groups on membrane proteins and/or the enhanced lipophilicity of MeHg due to the presence of the methyl group could be involved.

Ionic permeability ratios in PC12 cells have not been previously reported. Based on whole cell currents, the permeability of PC12 cell Ca^{2+} channels was $Ca^{2+} = Sr^{2+} > Ba^{2+}$. This ionic permeability sequence was somewhat unexpected, as Ba^{2+} usually is not the least permeant of the three cations based on single channel recordings (Tsien *et al.*, 1988). Addition of MeHg to the extracellular solution resulted in a loss of apparent ionic selectivity of Ca^{2+} channels, a result consistent with the ability of MeHg to alter ionic selectivity of synaptosomal $^{45}Ca^{2+}$ influx (Shafer *et al.*, 1990).

In conclusion, MeHg interacts rapidly with N- and L-type Ca^{2+} channels in PC12 cells at low micromolar concentrations. Given the ability of MeHg to interfere with binding of Ca^{2+} channel antagonists (Shafer *et al.*, 1990), pharmacological methods alone cannot be used to examine the effects of MeHg on individual Ca^{2+} channels subtypes. Further characterization of the effects of MeHg on the individual Ca^{2+} channel subtypes will require single channel recording techniques. Concentrations of MeHg which block I_{Ba} in PC12 cells are consistent with those which block evoked endplate potentials at the neuromuscular junction (Atchison and

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Narahashi, 1982). The ability of MeHg to block both N- and L-type channels is important in this respect. At the neuromuscular junction, L-type Ca^{2+} channels can have at least a modulatory role in neurotransmitter release (Atchison and O'Leary, 1987), but do not contribute to nerve-evoked release under normal conditions (Atchison, 1989). L-type channels have also been implicated in release of substance P from chick dorsal root ganglion cells (Rane *et al.*, 1987; Holz *et al.*, 1988) and ^{3}H-norepinephrine release from undifferentiated PC12 cells (Kongsamut and Miller, 1986). In rat sympathetic neurons (Hirning *et al.*, 1988), peripheral neurons (Perney *et al.*, 1986), NGF-differentiated PC12 cells (Kongsamut and Miller, 1986), various areas of the central nervous system (Dooley *et al.*, 1987a) and presumably rat motor nerve terminals (Atchison, 1989), N-type Ca^{2+} channels are implicated as the predominant type involved in neurotransmitter release. Given the ability of MeHg to block channel types which are associated with neurotransmitter release, block of Ca^{2+} channels at the nerve terminal may be an important component of its action to disrupt synaptic transmission.

CHAPTER SIX

EFFECTS OF METHYLMERCURY ON Na^+ CHANNELS IN NEUROBLASTOMA CELLS

ABSTRACT

Effects of MeHg on whole-cell Na^+ currents (I_{Na}) in neuroblastoma N1E-115 and NG-108 cells were examined using patch-voltage clamp techniques. In neuroblastoma cells, 10 μM MeHg reduced I_{Na} by approximately 50% after a 2 min exposure. MeHg did not alter the activation time of Na^+ current, nor the time course of whole-cell current inactivation. Na^+ currents could still be recorded after 8 min of exposure to MeHg. Effects of MeHg on NG-108 cells did not differ from those of MeHg on N1E-115 cells. These results suggest that MeHg interacts readily with Na^+ channels in neuroblastoma cells.

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INTRODUCTION

Results of Traxinger and Atchison (1987b) indicate that MeHg disrupts action potential conduction as well as Ca^{2+} channel function at the NMJ. The focus of this dissertation has been directed towards possible effects of MeHg on Ca^{2+} channels. However, propagation of the action potential in mammalian motor nerves, the stimulus for Ca^{2+} channels to open, depends on the function of Na^+ and K^+ channels in the plasma membrane. MeHg blocks the action potential in squid giant axon (Huneeus-Cox *et al.*, 1966) and increases its threshold for excitation (Shrivistav *et al.*, 1976). Na^+ currents in neuroblastoma cells are decreased by MeHg while only small reductions in K^+ current are observed (Quandt *et al.*, 1982). Furthermore, the sulfhydryl agents *p*-chloromercuriphenylsulfonic acid and inorganic mercury reduced Na^+ current in crayfish axons (Shrager, 1977). Thus, in addition to Ca^{2+} channels, Na^+ channels are also a potential target for MeHg action in mammalian motor nerves. Alterations in Na^+ channel function by MeHg may therefore indirectly affect Ca^{2+} channel function by altering patterns of cellular excitability.

In previous investigations of the effects of MeHg on action potential propagation and Na^+ channel function, concentrations of MeHg between 25 and 100 μM were required to alter Na^+ channel function (Shrivistav *et al.*, 1976; Quandt *et al.*, 1982), whereas concentrations between 5 and 20 μM were required to block Ba^{2+} current in PC12 cells (Shafer and Atchison, 1991). However, studies of effects of MeHg on Na^+ channel function have employed primarily intracellular voltage-clamp techniques, while effects of MeHg on Ca^{2+} channel function has been examined using

whole-cell patch voltage-clamp techniques. Thus, it is difficult to compare these results directly due to the differences in techniques employed to study channel function. Therefore, experiments were designed to examine effects of MeHg on Na^+ currents measured using the whole-cell configuration of the patch voltage-clamp technique. By doing so, effects of MeHg on Na^+ channels will be described under conditions very similar to those for which effects of MeHg on Ca^{2+} channels have been well characterized. This will provide additional information concerning the ability of MeHg to affect a second type of voltage-gated channel in neuronal cells.

MATERIALS AND METHODS

Na^+ current recordings. Voltage-activated Na^+ currents were recorded from undifferentiated mouse neuroblastoma N1E-115 cells (provided by Dr. P. Cobbett of Michigan State University) or cAMP-differentiated (7 days) NG-108 cells using the whole cell configuration of the patch voltage-clamp technique (Hamill *et al.*, 1981). These cell lines were used to examine effects of MeHg on Na^+ current rather than PC12 cells because consistent expression of Na^+ current could not be achieved from available stocks of PC12 cells. External solution contained (mM): HEPES (20), $MgCl_2$ (1.0), d-glucose (10), NaCl (115), and TEACl (20) pH 7.3. Internal or pipette solution contained (mM): HEPES (10), $MgCl_2$ (1), d-glucose (10), TEACl (10), EGTA (5), CsCl (125) and ATP (2), pH 7.3.

To record Na^+ currents, resistance of patch electrodes (1.2 mm glass) was between 2-6 megohms and the patch clamp circuit consisted of an Axon Instruments CV-1 headstage and Axopatch 1B patch clamp. Pulse protocols were generated and current responses recorded on-line using a Compaq 386 personal computer and the pClamp™ program interfaced to the Axopatch 1B via an Axon Instruments TL-1 interface board. Current responses were filtered at 10 kHz. Linear leak and capacitive current components were subtracted using a computer-generated protocol. This protocol functions by using a series of small amplitude subpulses to compute leak and capacitive current expected during a larger test pulse. Ideally, the subpulses elicit no voltage-gated current. Hence the leak and capacitive current measured during the subpulses can be averaged and scaled to that expected from the test pulse.

The magnitude of the subpulses is determined by dividing the magnitude of the test pulse by the number of subpulses. For these experiments, a series of 10 subpulses was used to measure leak. MeHg was added to 1 ml of bath solution in 500 μl aliquots to yield the final concentration indicated in the text.

RESULTS

Effects of MeHg on Na^+ current in neuroblastoma cells. Addition of MeHg to the extracellular solution rapidly reduced Na^+ current in neuroblastoma cells. A 2 min resting exposure of neuroblastoma cells to 10 μM MeHg reduced peak inward Na^+ current by 20 - 50% at all test potentials examined (Figure 6.1A). However, in contrast to effects of MeHg on whole-cell Ba^{2+} current (Shafer and Atchison, 1991), MeHg did not alter the inactivation kinetics of whole-cell Na^+ current. This is clearly apparent if current recordings obtained from the same cell in the absence and presence of MeHg are compared after scaling the MeHg-treated current response to the same amplitude as the control response. (Figure 6.1, inset). MeHg did not affect τ, the rate constant for inactivation, at test potentials between -40 and +40 mV (Figure 6.1B). Also, MeHg did not alter significantly the time to peak amplitude of Na^+ current in neuroblastoma cells (Figure 6.2). This is consistent with effects of other sulfhydryl reagents on Na^+ channels (Shrager, 1977). Na^+ current could be recorded from neuroblastoma cells after 8 min of exposure to 10 μM MeHg (not shown). This concentration of MeHg completely blocks I_{Ba} in PC12 cells after 3-5 min exposure. Effects of MeHg on Na^+ currents in NG-108 cells were not different from those on N1E-115 cells (results not shown). Blocking actions of MeHg may be altered by administration of hyperpolarizing prepulses (results not shown).

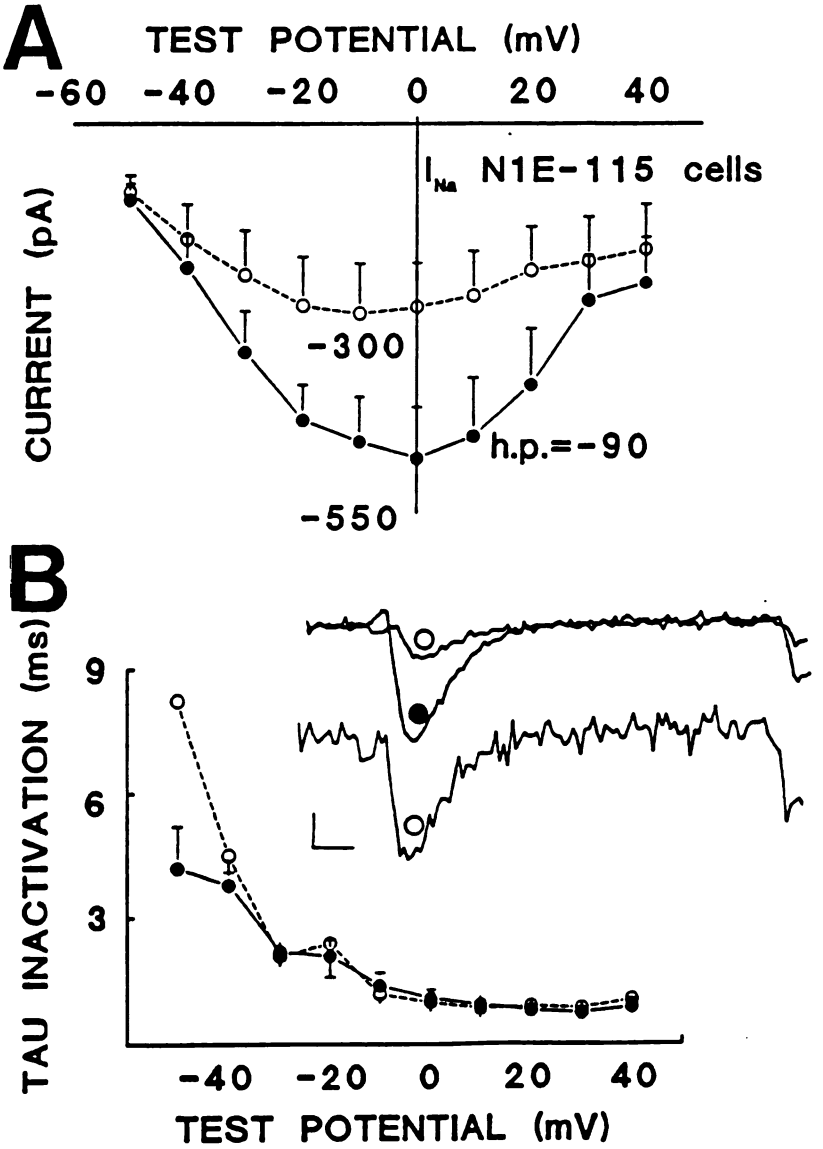

Figure 6.1. Effects of MeHg on Na^+ channels in N1E-115 cells. A. Peak inward Na^+ current-voltage relationship in N1E-115 cells recorded prior to (filled circles) and following a 2 min exposure to 10 μM MeHg (open circles). B. Time constant (tau) for inactivation of whole-cell Na^+ current at test potentials used in A measured prior to (filled circles) and following a 2 min exposure to 10 μM MeHg (open circles). All current responses were recorded using 10 ms test potentials from a holding potential of -90 mV. Responses were filtered at 10 kHz and values are the mean ± SEM of six cells, each of which was treated with MeHg. Inset: Top pair of traces are current responses to a test potential of -10 mV prior to (filled circles) and following a 2 min exposure to 10 μM MeHg (open circles). The lower trace is the MeHg-treated response scaled to the same magnitude as the control response. Note that the kinetics of decay do not appear to be altered by MeHg. Scale bars for the top pair of traces are 200 pA and 1 ms. The current responses shown are the average of ten responses.

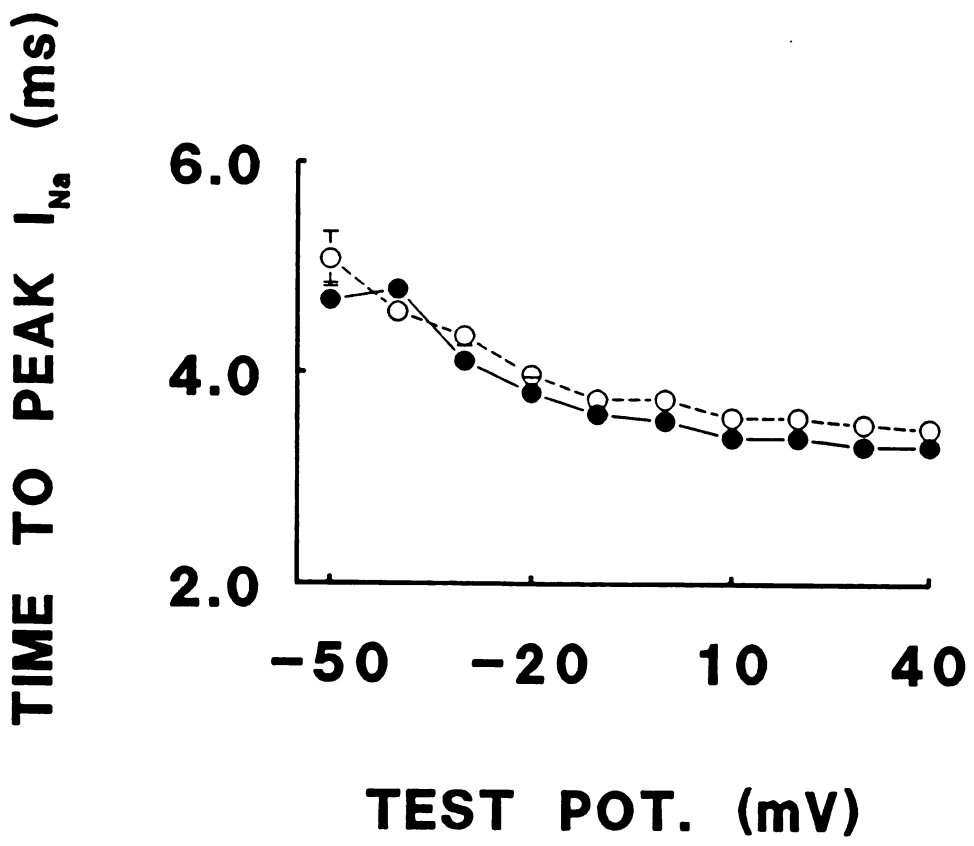

Figure 6.2. Effects of MeHg on time to peak Na^+ current. The time required for inward Na^+ current to reach peak amplitude following a depolarizing stimulus. The values are the mean ± SEM of 5 (solid circles, control) or 3 cells treated with 10 μM MeHg.

DISCUSSION

Results of these experiments indicate that low micromolar concentrations of MeHg disrupts Na^+ channel function in clonal cells. Several previous studies indicated that MeHg disrupts action potential propagation and Na^+ channel function (Shrivistav *et al.*, 1976; Quandt *et al.*, 1982), but effects of MeHg on Na^+ channel function had not been examined using patch-voltage clamp techniques. Shrivistav *et al.*, (1976) reported that concentrations of 25-100 μM MeHg decreased Na^+ current by 50% in squid axons after 15 min of exposure in the bath solution. Quandt *et al.*, (1982) reported that a 3 min exposure to 20 μM MeHg reduced Na^+ current by approximately 10% in N1E-115 cells. Higher concentrations of MeHg (60 μM) produced greater reductions (80 - 100%) in Na^+ current (Quandt *et al.*, 1982). Results of present experiments indicate that 10 μM MeHg rapidly and effectively blocks Na^+ current in neuroblastoma cells. It is possible that the lower temperatures (10°C) employed by Quandt *et al.* (1982) may account for some of the differences in the effects of MeHg on neuroblastoma cells. Changes in temperature may alter channel gating (Hille, 1984) or may influence fluidity of the cell membrane; should target sites important for MeHg-disruption of Na^+ channel function be located within the membrane, changes in fluidity may alter the ability of MeHg to reach these sites.

In addition to Na^+ current, MeHg also blocks Ca^{2+} current in PC12 cells at concentrations between 1-20 μM (Shafer and Atchison, 1991). This is unique when compared to inorganic Ca^{2+} channel antagonists, in that MeHg affects two different channel types with equal avidity. Although the present data (including effects of

MeHg on Ca^{2+} channels) do not suggest any particular mechanism responsible for this ability of MeHg, at least three mechanisms could account for this effect. First, like Na^+, MeHg is monovalent. However, the Na^+ channel is not permeanated to positively charged methylated amines (Hille, 1984), and therefore may not be permeanated by MeHg. In addition, mercury is a transition metal; this class of metals includes Hg^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (Nachshen, 1984), which block Ca^{2+} channels by interactions with binding sites within the channel pore (Lansman *et al.*, 1986; Hess *et al.*, 1986). Thus, MeHg may enter the pore of both Na^+ and Ca^{2+} channels, resulting in block. Second, other Ca^{2+} channel antagonists, including the DHPs, have also been shown to block Na^+ channels in cardiac myocytes (Yatani and Brown, 1985), although the concentrations of DHP required to block Na^+ channels were higher than those which block Ca^{2+} channels. PD 122860, is a DHP compound which stimuluates Na^+ channel function and blocks Ca^{2+} channel function (Haleen *et al.*, 1989). In addition, veratridine and grayanotoxin block Ca^{2+} and Na^+ channels in the same concentration range in N1E-115 cells (Romey and Lazdunski, 1982). This has led to the suggestion that similarities in the molecular structures of Na^+ and Ca^{2+} channels may underlie these interactions (Hosey and Lazdunski, 1988). There is a high degree of sequence homology between the α subunit of the voltage-dependent Na^+ channel and the α_1 subunit of the DHP-sensitive Ca^{2+} channel from skeletal muscle. The greatest area of homology exists within four repeated domains, each containing six transmembrane segments, one of which presumed to be responsible for voltage-sensitivity (Campbell *et al.*, 1988). Perhaps MeHg, which is

lipophilic, interacts with these regions in both Na^+ and Ca^{2+} channels. The possible effect of MeHg on steady-state inactivation of Na^+ channels is interesting in this regard. Recently, four putative Ca^{2+} channel cDNA molecules which are homologous to the α_1 subunit of the DHP-sensitive Ca^{2+} channel from heart and skeletal muscle have been isolated from rat brain (Snutch *et al.*, 1990). Finally, ethanol has also been reported to block both Na^+ (Harris and Bruno, 1985) and Ca^{2+} channels (Greenberg *et al.*, 1987a; Marks *et al.*, 1989) in different systems. Thus, disruption of lipid environment may also reduce channel function.

MeHg alters both Na^+ and Ca^{2+} channel function, however, there appear to be differences in the characteristics of block of Na^+ and Ca^{2+} channels by MeHg. The most striking difference is the lack of effect of MeHg on inactivation kinetics of whole-cell Na^+ current. In PC12 cells, 10 μM MeHg consistently eliminated the inactivating component of Ba^{2+} current (Shafer and Atchison, 1991). Second, the time courses may be different; block of Na^+ channels by MeHg was not complete after 8 min, whereas block of Ca^{2+} channels by 10 μM MeHg was complete after 3-5 min. Although this difference was not statistically significant, the phenomenon was consistent.

In conclusion, MeHg interacts readily with Na^+ channels in neuroblastoma cells at concentrations which also block Ca^{2+} channels under similar conditions. The ability of MeHg to interact with Na^+ channels at low micromolar concentrations is consistent with previous reports (Huneeus-Cox *et al.*, 1966; Shrivistav *et al.*, 1976; Quandt *et al.*, 1982) as well as effects of MeHg at the neuromuscular junction

(Traxinger and Atchison, 1987b). The ability of MeHg to interact with more than one channel type at similar concentrations is unique when compared to inorganic Ca^{2+} channel antagonists. Furthermore, in contrast to effects on whole-cell Ba^{2+} current, MeHg reduced whole cell Na^{+} current without producing apparent alterations of Na^{+} channel inactivation kinetics. Thus, in addition to Ca^{2+} channels, Na^{+} channels may also be an important site of MeHg action in neuronal membranes.

CHAPTER SEVEN

EFFECTS OF METHYLMERCURY ON Na^+ AND Ca^{2+} CURRENTS IN INTACT NEUROMUSCULAR JUNCTIONS

ABSTRACT

The ability of MeHg to block presynaptic Ca^{2+} and Na^{+} channels in intact neuromuscular junctions was examined in mouse *triagularis sterni* motor nerves. Potential changes arising from Na^{+} and Ca^{2+} channel function could be recorded from the perineurial sheath surrounding motor neurons when K^{+} channels were blocked by TEA and 3, 4-diaminopyridine. Two components of the Ca^{2+}-dependent potential were observed; a fast component of approximately 60 ms duration and a long-lasting component of approximately 100 - 800 ms duration. MeHg (100 μM) blocked both Na^{+} and Ca^{2+} components rapidly. In 2 of 5 preparations exposed to 50 μM MeHg, the Ca^{2+} channel-mediated component was blocked prior to block of the Na^{+} channel-mediated component. In the remaining three preparations, both Na^{+}- and Ca^{2+}-dependent potentials were blocked at similar times. Following block by MeHg, perfusing the preparation in MeHg-free solutions did not result in recovery of Na^{+} or Ca^{2+} channel activity; nor did increases in intensity and/or duration of stimulus to the intercostal nerves. In the presence of K^{+} channel blockers, repetitive firing of nerves in response to a single stimulus was observed in 20 - 30% of the *triangularis* preparations; in two preparations treated with MeHg, repetitive firing decreased prior to block of the stimulus-induced Na^{+}/Ca^{2+} potentials. These results suggest that MeHg blocks Na^{+} and Ca^{2+} channels in intact neuromuscular preparations.

INTRODUCTION

The results of experiments presented in previous chapters provide strong evidence that MeHg disrupts function of Ca^{2+} channels in nerve terminals and blocks Ca^{2+} channel types which are thought to be involved in neurotransmitter release. Effects of MeHg on synaptosomal $^{45}Ca^{2+}$ influx and on Ba^{2+} currents through somatic N- and L-type Ca^{2+} channels in PC12 cells are comparable in many respects; MeHg alters ionic selectivity and inactivation, blocks Ca^{2+} channels in a voltage- but not state-dependent manner, and still exerts blocking effects in spite of increasing $[Ca^{2+}]_e$. These results suggest either that the same types of Ca^{2+} channels are present in each preparation, or that MeHg interacts with a variety of Ca^{2+} types in a similar fashion. Regardless, these results support the hypothesis that disruption of nerve-evoked neurotransmitter release by MeHg is in part the result of block of Ca^{2+} channels at the presynaptic terminal by MeHg. However, experiments performed previously have not measured effects of MeHg on Ca^{2+} channel function in intact motor nerve terminals. Thus, one cannot be certain that MeHg is indeed blocking Ca^{2+} channels in intact motor nerve terminals. Furthermore, MeHg also blocks Na^+ channels in cultured cells (Chapter 6, Quandt *et al.*, 1982) and may alter Na^+ channel function in intact nerves (Traxinger and Atchison, 1987b). Therefore, experiments were designed to examine effects of MeHg on Na^+ and Ca^{2+} channel function in intact motor nerves by measuring effects of MeHg on Na^+- and Ca^{2+}-dependent potentials in the perineurial sheath of mouse motor nerves.

The perineurium is a layer of flattened cells which surrounds mammalian nerve trunks, forming a relatively impermeant barrier to small molecules and ions. Because of this property, the perineurium functions as an electrical insulator around the nerve trunk (Figure 7.1). Axial currents which move from the most distal nodes of Ranvier into the nerve terminal form local circuits in the perineurium by returning to their node of origin *via* the perineurial space (Gundersen *et al.*, 1982; Mallart, 1985). By placing a microelectrode inside the perineurial sheath near motor nerve terminal regions, voltage changes resulting from inward Na^+ currents in preterminal regions and inward Ca^{2+} currents in nerve terminals can be recorded simultaneously (Mallart, 1985; Penner and Dreyer, 1986). Furthermore, since the perineurium contains multiple nerve fibers, the signal to noise ratio is increased over "loose patch" recordings (Brigant and Mallart, 1982; Mallart and Brigant, 1982) of Na^+ and Ca^{2+} currents from individual terminals (Penner and Dreyer, 1986). Thus, effects of MeHg on Na^+ and Ca^{2+} channels were examined simultaneously by using perineurial recording techniques in intact motor nerve terminals of mouse *triangularis sterni* muscle.

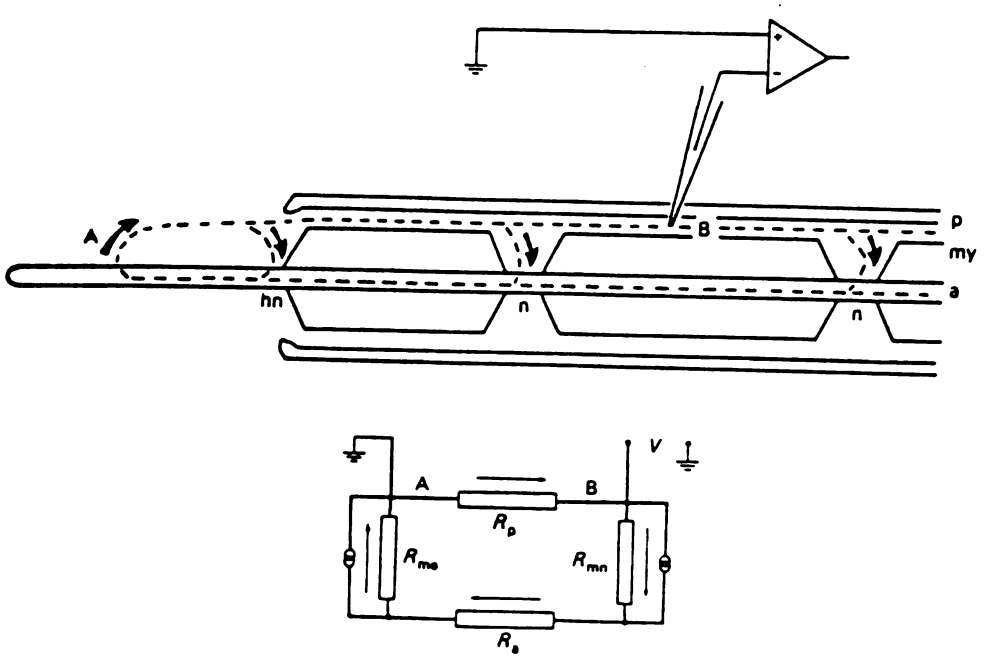

Figure 7.1. Local circuits in the perineurial sheath of motor neurons. Top, simplified diagram of a motor nerve terminal, with the terminal on the left. The current pathways are indicated by the dashed lines. hn, heminodal region; n, node of Ranvier; p, perineurium; my, myelin sheath; a, axon. Bottom, simplified equivalent electrical circuit. A corresponds to the external medium, B indicates the perineurial space. R_{me}, membrane transverse resistance of the terminal; R_{mn}, membrane transverse resistance at the nodes; R_p and R_a are the longitudinal resistances of the perineurial space and the axon, respectively. The arrows indicate direction of current flow.

Modified from: Mallart, *J. Physiol.* 368:565-575 (1985).

MATERIALS AND METHODS

Na^+ and Ca^{2+} voltage recordings from the perineurial sheath. Potential changes arising from Na^+ and Ca^{2+} channel function were recorded from the perineurial sheath of motor neurons in mouse *triangularis sterni* muscle (M^{c}Ardle *et al.*, 1981). The muscle and intercostal nerves were dissected from the ribcage of adult male Swiss-Webster mice (30-50 g). Following cervical dislocation, the ribcage was separated from the spinal column and bathed in oxygenated physiological solution consisting of (mM): NaCl(135); KCl (5); $CaCl_2$ (2); $MgCl_2$ (1); HEPES (14) and d-glucose (11), pH 7.4. This solution was replaced completely every 3-5 min while the overlying intercostal muscles and ribs were removed. Upon completion of the dissection (Figure 7.2), the muscle was immediately transferred to a perfusion chamber and perfused at a rate of 1-2 ml/min with oxygenated physiological solution.

To measure Na^+- and Ca^{2+} (Ba^{2+})-dependent potentials in the perineurial sheath, the muscle was perfused with solution containing (mM): NaCl (118); TEACl (20); $BaCl_2$ (5); $MgCl_2$ (1); HEPES (14); d-glucose (11); 3,4-diaminopyridine (0.5) and d-tubocurarine chloride (0.05), pH 7.4. The solution was saturated with 95/5% O_2/CO_2. TEACl and 3,4-diaminopyridine were added to suppress K^+ channel activity and d-tubocurarine was added to block post-synaptic ACh receptors, thereby eliminating muscle twitch. The intercostal nerves were stimulated at 0.1 Hz by a suction electrode; typical threshold values were 15-30 mV and 15-30 μs duration. Potential changes in the perineurial sheath were measured by glass microelectrodes (1.2 mm O.D., 10 MΩ) filled with physiological solution. Signals were recorded using

a CV-1 headstage and an Axopatch 1D patch clamp amplifier in the current clamp mode. Voltage responses were stored on FM tape (Vetter Instruments) for later analysis using a chart recorder (Gould Instruments) and/or by digitization at a rate of 1 point every 20 μs and subsequent storage on a Compaq personal computer.

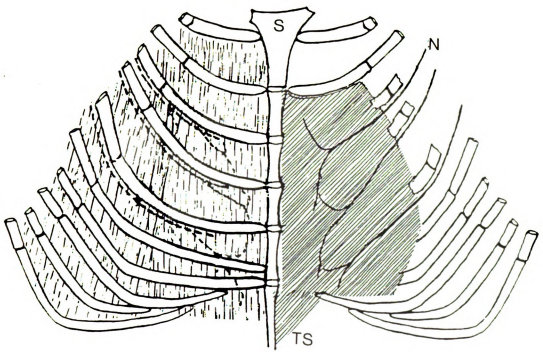

Figure 7.2. Diagram of mouse *triangularis sterni* muscle. The muscle, located on the inner surface of the ribcage inserts into the sternum and the intercalations of the ribs. The overlying ribs and intercostal muscles have been removed. Branches of the 2nd, 3rd, and 4th intercostal nerves, which innervate the *triangularis*, are visible.

RESULTS

Effects of MeHg on Na^+ and $Ca^{2+}(Ba^{2+})$-dependent voltage changes in the perineurial sheath. Placing a glass microelectrode inside the perineurial sheath of nerves in the *triangularis sterni* muscle resulted in a negative potential of -2 to -5 mV, consistent with previous reports (Mallart, 1985; Penner and Dreyer, 1986; Anderson and Harvey, 1988). When perfusing the muscle with normal saline solution, stimulation of the nerve was followed closely (approximately 0.8 ms) by a negative deflection in voltage (Figure 7.3A); a small fast component (arrow) slightly preceded a larger component of longer duration. Previously, it has been demonstrated that the first component arises from Na^+ currents in the nodal and heminodal portions of the nerve, whereas the second component arises from outward K^+ current in the nerve terminal (Brigant and Mallart, 1982; Mallart, 1985; Penner and Dreyer, 1986; Anderson and Harvey, 1988).

When the solution perfusing the muscle contained 20 mM TEA and 500 μM 3,4-diaminopyridine, the potential consisted of a fast negative deflection followed by a longer-lasting positive deflection (Figure 7.3B): demonstrated to arise from nodal Na^+ and terminal Ca^{2+} currents, respectively (Brigant and Mallart, 1982; Mallart, 1985; Penner and Dreyer, 1986; Anderson and Harvey, 1988). Addition of TTX (5 μM) to the perfusing solution blocked the Na^+ component of the potential (results not shown). Two components of positive potential were observed: a fast transient of approximately 60 ms in duration (Figure 7.3C), and a long-lasting (100 - 800 ms) potential of varying duration (Figure 7.3D,E). These results are consistent with those

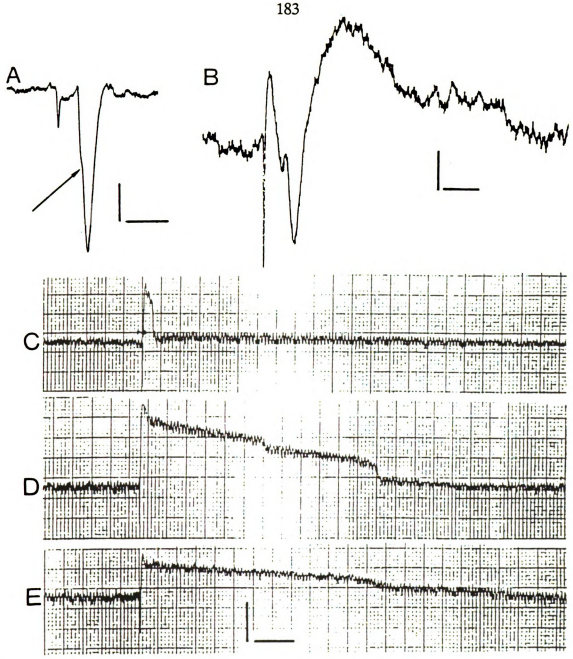

Figure 7.3. Voltage responses recorded from perineurial sheaths. A) Na^+/K^+ voltage potential recorded from perineurial sheaths near nerve terminal regions. The arrow indicates the shoulder arising from Na^+ current in the nodal and heminodal regions. B) Na^+/Ca^{2+} voltage potential recorded in the presence of TEA and 3,4-diaminopyridine in 5 mM Ba^{2+} solutions. C-E) Na^+/Ca^{2+} voltage potentials from the same preparation demonstrating rapid (C) and long-lasting (D, E) Ca^{2+} responses. Responses are not averaged or summed and were recorded in response to intercostal nerve stimulation at a rate of 0.1 Hz. Scale bars are 0.35 mV and 2 ms (A), 0.5 mV and 4 ms (B), 0.5 mV and 200 ms (C-E).

of Penner and Dreyer (1986) in which two types of Ca^{2+} potentials were demonstrated in mouse motor nerve terminals; presumably due to the presence of two different Ca^{2+} channel subtypes. Microscopic resolution was limited (150X) to an extent which prevented placement of electrodes within the finest branches of the perineurial sheath, which contain 3-4 nerves (Penner and Dreyer, 1986). Under these conditions the number of nerves contributing to potentials cannot be determined. Therefore, quantitative assessment of effects of MeHg on potential amplitude provide little definitive information concerning effects of MeHg on individual nerves or terminals. Amplitude scale bars have been included in some figures to provide information concerning relative amplitudes. The amplitude of the Ca^{2+} channel-dependent potential typically was between 0.5 and 1.5 mV in the absence of MeHg, depending on the preparation.

The positive portion of the potential was dependent on the presence of divalent cations in the perfusing solution. Preparations which were dissected in 2 mM Ca^{2+} and then perfused with nominally Ca^{2+}- and Ba^{2+}-free solution exhibited small and short duration Ca^{2+} potentials, presumably due to residual Ca^{2+} in the muscle tissue (Figure 7.4A,B, left trace). Addition of 5 mM Ba^{2+} to the perfusing solution increased both the magnitude and duration of the Ca^{2+} potential (Figure 7.4A,B, right trace). Furthermore, "puffing" 1 mM cobalt into the perfusion chamber decreased the amplitude and duration of only the positive potential in a reversible manner (Figure 7.4B). These results are consistent with results of Mallart (1985) and Penner and Dreyer (1986) in which these potentials were demonstrated to arise from

Ca^{2+} channel function in nerve terminals. Addition of MeHg (100 μM) to the perfusing solution caused a reduction in amplitude and eventual block of both the Na^+ and fast Ca^{2+} potentials after a delay of 3-4 min (Figure 7.5). Both Na^+ and fast Ca^{2+} potentials were decreased simultaneously in three preparations. In contrast to the results of Traxinger and Atchison (1987b), washing with MeHg-free solution at the time of block did not reverse effects of MeHg; even after 15-20 min. In addition, increasing stimulus duration (up to 5 times threshold) and/or intensity (up to 10 times threshold) at the time of block and/or during wash also were ineffective in reversing block of either the Na^+ or the Ca^{2+} channel-mediated response by MeHg.

50 μM MeHg also rapidly decreased both Na^+ and fast Ca^{2+} potentials in 4 out of 5 preparations. However, in 2 preparations, the fast Ca^{2+} component appeared to be affected prior to the Na^+ component. In three preparations (one had been exposed to 100 μM MeHg), the long-lasting component of the positive potential was decreased prior to block of Na^+ and fast Ca^{2+} potentials. These results suggest a somewhat earlier or more specific effect of MeHg on nerve terminal Ca^{2+} channels. Thus, MeHg is capable of blocking Ca^{2+} currents in intact motor nerve terminals. In addition, effects of MeHg on specific components of perineurial waveforms indicate that the decreases in signal amplitude are not due to disruption of the insulating properties of the sheath by MeHg. As with 100 μM MeHg, these effects were not reversible by washing the preparation in MeHg-free solutions or by increasing stimulus intensity and/or duration. Thus, block by MeHg of Na^+ channels, and possibly of Ca^{2+} channels, appears to be irreversible under these conditions.

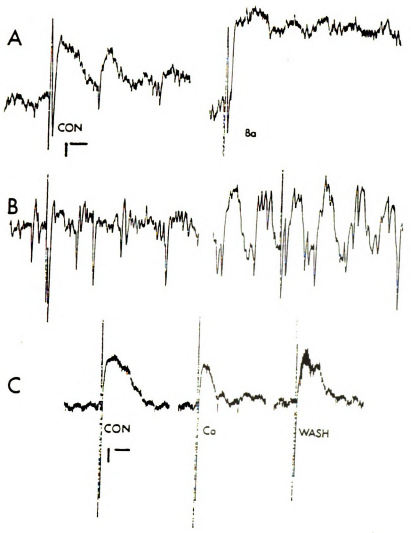

Figure 7.4. Ca^{2+} channel-mediated component of voltage potentials in the perineurial sheath. When perfusing with solutions containing no added Ca^{2+} or Ba^{2+}, the Ca^{2+} channel-mediated component of the potential is small and of short duration (A and B, left traces). When 5 mM Ba^{2+} is added to the perfusing solution (A), or "puffed" into the perfusion chamber (B), the Ca^{2+} channel-mediated component of the potential increases in amplitude as well as duration (right traces). Scale bars are 9 ms and 0.25 mV in A and 0.125 mV in B. C) While perfusing in 5 mM Ba^{2+} solution, 1 mM Co^{2+} was "puffed" over the muscle. The voltage traces were recorded prior to addition of Co^{2+} (CON), in the presence of Co^{2+} (Co), and during washout of Co^{2+} (WASH). Scale bars are 0.25 mV and 20 ms.

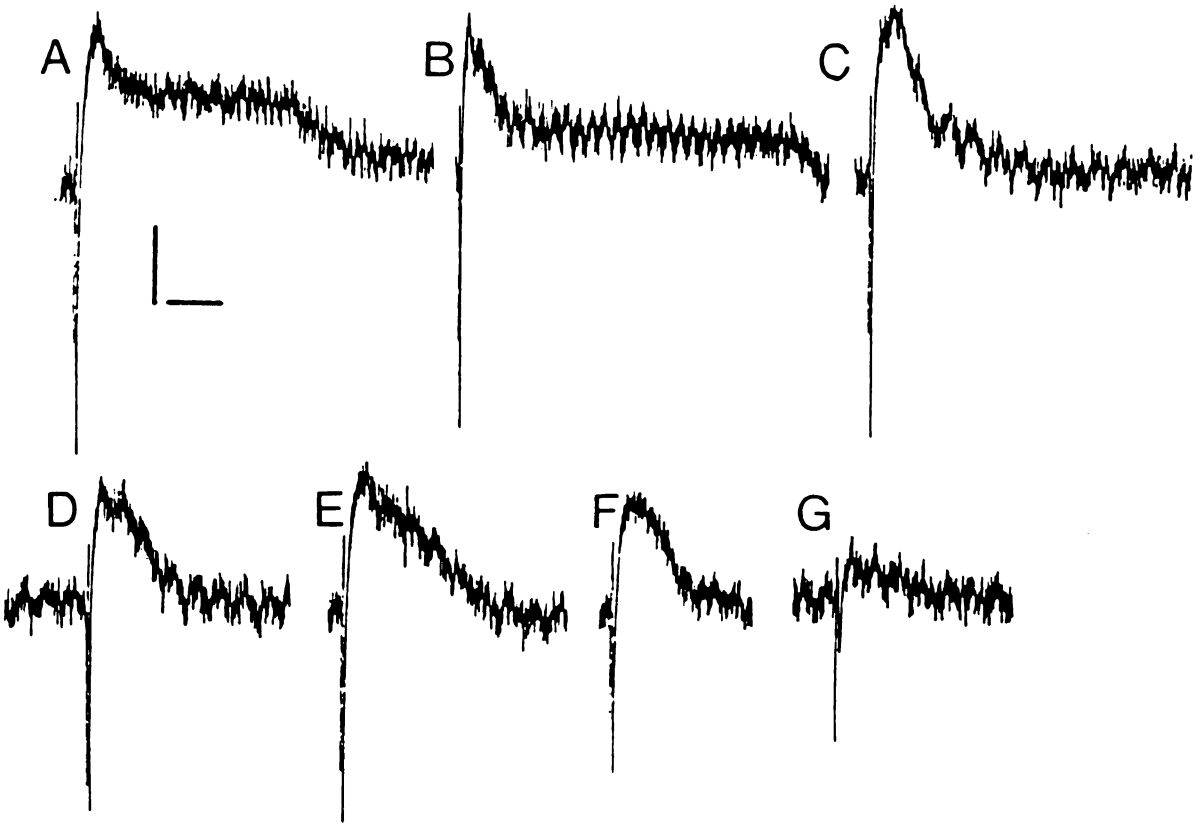

Figure 7.5. Effects of MeHg on Na^+/Ca^{2+} potentials in the perineurial sheath. Na^+/Ca^{2+} potentials recorded prior to (A) and 0.16 (B), 1 (C), 2 (D), 3 (E) 4 (F) and 5 (G) min after addition of 100 μM MeHg. Scale bars are 0.5 mV and 40 ms, except for B; for which the temporal bar is 80 ms. Note the loss of the long-lasting component of the Ca^{2+} potential after 1 min.

When using K^+ channel blocking agents to unmask Ca^{2+} channel activity in the nerve terminal, repetitive firing of neurons often occurs due to lack of repolarization of the neuronal membrane by outward K^+ currents. Other investigators have used small concentrations of local anesthetics to prevent repetitive firing (Brigant and Mallart, 1982; Mallart, 1985; Penner and Dreyer, 1986; Anderson and Harvey, 1987; 1988). Since MeHg may affect axonal Na^+ channels and decrease excitability (Shrivistav *et al.*, 1976; Quandt *et al.*, 1982; Traxinger and Atchison, 1987b), use of local anesthetics might mask or confound possible effects of MeHg on Na^+ channel function in intact nerves. Thus, no pharmacological manipulations were undertaken in these experiments to suppress repetitive firing of motor neurons. Repetitive firing was observed in approximately 20 to 30% of *triangularis sterni* preparations (Figure 7.6A,B), only 2 of which were treated with MeHg. Addition of MeHg (50 μM) to these preparations resulted in a suppression of repetitive firing prior to block of stimulus-evoked Na^+ and fast Ca^{2+} potentials (Figure 7.6C-F).

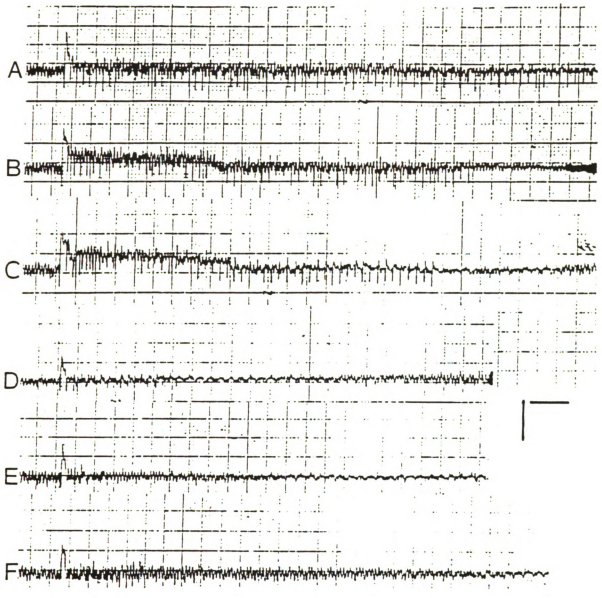

Figure 7.6. Effects of MeHg on repetitive firing of motor nerves. An example of repetitive firing in a motor nerve recorded prior to (A,B) and 20 (C), 70 (D), 80 (E), and 90 (F) s after the addition of 50 μM MeHg. Note also the loss of the long-lasting portion of the Ca^{2+} response in this example. Scale bar is 200 ms.

DISCUSSION

Results of these experiments indicate that MeHg: 1) appears to block both Na^+ and Ca^{2+} currents in intact neuromuscular preparations, and 2) under the conditions used in this study, blocks presynaptic Na^+ currents in a manner which is not reversible by increasing stimulus intensity or duration.

At concentrations of 50 and 100 μM, MeHg blocked the inward Na^+-dependent component of the voltage potential. In addition, MeHg suppressed repetitive firing of motor nerves prior to significant reductions of stimulus-induced Na^+ or Ca^{2+} potentials. Repetitive firing of the nerve is a function of membrane excitability, and may occur under conditions of these experiments due to lack of repolarization of the nerve by outward K^+ current before Na^+ channels recover from inactivation. The ability of MeHg to suppress repetitive firing is consistent with the results of Traxinger and Atchison (1987b) which indicated that MeHg decreased neuronal excitability. Block of a portion of the population of Na^+ channels by MeHg may prevent repetitive firing by decreasing the number of Na^+ channels available for opening immediately after an action potential propagates through a given region of the nerve. Alternatively, MeHg has been demonstrated to depolarize neuronal membranes (Shrivistav *et al.*, 1976; Hare and Atchison, 1990). Should this occur in intact axons as a result of MeHg exposure, one might expect a decrease in nerve excitability; depolarization might result in steady-state inactivation of Na^+ channels. Therefore, fewer channels would be available for activation.

In addition to blocking the Na^+ potential, MeHg also reduced the fast Ca^{2+} potential recorded from the perineurial sheath. Because of effects on Na^+ potentials, decreases in Ca^{2+} potentials might simply have been due to failure of the action potential to invade the terminal and activate Ca^{2+} channels. However, in two preparations, the Ca^{2+} channel-mediated potentials were reduced prior to block of the Na^+ channel-mediated potentials. In addition, the long-lasting component of the Ca^{2+} potential was blocked prior to block of the Na^+ and fast Ca^{2+} potential in three preparations. Under these conditions, the action potential successfully invaded nerve terminals, since the fast Ca^{2+} potential was unaffected. These results indicate that in addition to Na^+ channels, Ca^{2+} channels in the presynaptic terminal are blocked by MeHg. Effects of MeHg on neurotransmitter release may be due to effects on both Na^+ and Ca^{2+} channels in the presynaptic nerve; consistent with predictions of Traxinger and Atchison (1987b).

The fast component of the Ca^{2+} potential is presumed to be associated with neurotransmitter release (Mallart, 1985; Penner and Dreyer, 1986), although the Ca^{2+} channel type(s) which contribute to this component have not yet been determined. This component is not affected by organic Ca^{2+} channel blockers (Penner and Dreyer, 1986) or ω-conotoxin GVIA (Anderson and Harvey, 1987), but is blocked by millimolar concentrations of divalent cations (Penner and Dreyer, 1986). Thus, the pharmacological characteristics of this component cannot be classified by the T, N, and L nomenclature system (Nowycky *et al.*, 1985a). The long-lasting component of the potential is not thought to be coupled directly to

neurotransmitter release (Penner and Dreyer, 1986). However, no evidence exists to preclude this mechanism from contributing to nerve-evoked release. This component is sensitive to verapamil and diltiazem but not DHPs (Penner and Dreyer, 1986). Thus, the Ca^{2+} channel types mediating this component are unknown. Although MeHg blocks both N- and L-type Ca^{2+} channels in PC12 cells, the ambiguity over the types of Ca^{2+} channels present in the motor nerve terminal makes it difficult to determine whether or not MeHg is acting on N- and L-type channels in this preparation.

Traxinger and Atchison (1987b) reported that block of EPPs in rat hemidiaphram preparations could be reversed by washing in MeHg-free solutions and increasing the intensity and/or duration of the stimulus to the phrenic nerve. In addition, following reversal of initial MeHg-induced block, a subsequent block by MeHg was relieved by increasing $[Ca^{2+}]_e$. In the present experiments, the effects of MeHg on Na^+ and Ca^{2+} potentials were not relieved by washing in MeHg-free solutions and/or by increasing stimulus intensity or duration. The disparity of results from this study with those of Traxinger and Atchison (1987b) may be due to the conditions employed in this study to unmask Ca^{2+} channel-mediated components of voltage changes in the perineurial sheath. Block of K^+ channels by TEA and 3,4-diaminopyridine will result in depolarization of the axonal membrane; should MeHg further depolarize the membrane, decreases in excitability may result from Na^+ channel inactivation. In addition, the high concentration of Ba^{2+} used to measure Ca^{2+} channel function may also stabilize the axonal membrane (Frankenheuser and

Hodgkin, 1957; reviewed by Hille, 1984). Indeed, Traxinger and Atchison (1987b) reported that increasing $[Ca^{2+}]_e$ along with addition of 4-aminopyridine prevented reversal of MeHg-induced block of EPPs when stimulus intensity was increased. Under the conditions of this study, therefore, it might be expected that MeHg-induced decreases in nerve excitability may be difficult to overcome.

In conclusion, effects of MeHg on Na^+ and Ca^{2+} channel function in intact motor nerves and terminals have been examined for the first time. Consistent with predictions from *in vitro* systems, the results indicate that MeHg disrupts function of both channel types in intact nerve terminals. The ability of MeHg to block Ca^{2+} channels in intact motor nerve terminals supports the hypothesis that effects of MeHg on Ca^{2+} channel function may contribute to block of neurotransmitter release by MeHg.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

This dissertation has presented results of experiments designed to examine the following hypothesis: block of Ca^{2+} channels by MeHg may contribute to MeHg-induced block of synaptic transmission at the motor nerve terminal. This hypothesis was based on the experimental observations that block of synaptic transmission at the neuromuscular junction (Traxinger and Atchison, 1987b), and of depolarization-induced $^{45}Ca^{2+}$ influx into synaptosomes by MeHg could, under certain conditions, be antagonized by extracellular calcium (Atchison *et al.*, 1986). Specifically, experiments were designed to examine how MeHg disrupts Ca^{2+} channel function and to determine types of Ca^{2+} channels affected by MeHg: particular emphasis has been placed on Ca^{2+} channels located in nerve terminals and on Ca^{2+} channel types which are presumed to mediate neurotransmitter release from central and/or peripheral terminals. The small size and close anatomical association of the motor nerve terminal with muscle fibers severely restrict the ability to measure directly Ca^{2+} channel function in intact NMJ preparations. Thus, out of necessity, some experiments were performed on isolated nerve terminals or cultured cell lines. Based on results of these experiments, one putative mechanism by which MeHg effects neurotransmitter release at the NMJ is now better understood.

Preliminary experiments characterized the ability and nature of block by MeHg of various components of Ca^{2+} influx into isolated nerve terminals. MeHg blocked voltage-sensitive Ca^{2+} channels in synaptosomes; the component of $^{45}Ca^{2+}$ influx which has been associated specifically with neurotransmitter release from synaptosomes (Blaustein, 1975; Floor, 1983; Leslie *et al.*, 1985) was dramatically

reduced by MeHg (Atchison *et al.*, 1986; Shafer and Atchison, 1989) Block of this component was only partially relieved by increasing $[Ca^{2+}]_e$ (Shafer and Atchison, 1989). Thus, in isolated nerve terminals, MeHg disrupts, in a potent and efficacious manner, function of Ca^{2+} channels demonstrated to mediate Ca^{2+} influx responsible for neurotransmitter release. The ability of MeHg to block Ca^{2+} channels in a heterogeneous preparation may represent a common action of MeHg at all nerve terminals. Furthermore, elucidation of the mechanisms underlying MeHg neurotoxicity will require more information regarding effects of MeHg in the central nervous system. Inasmuch as alterations in Ca^{2+}influx may contribute to acute effects of MeHg on neurotransmitter release at the NMJ, these data suggest that alterations of Ca^{2+} channel function should be considered in future studies of effects of MeHg in the central nervous system.

Further characterization of MeHg effects on synaptosomal Ca^{2+} influx demonstrated that MeHg altered Ca^{2+} channel functions including ionic selectivity and inactivation. In addition, block of Ca^{2+} channels by MeHg was demonstrated to be voltage- but not state-dependent. MeHg also altered binding of specific Ca^{2+} channel ligands to their receptor sites in synaptosomes and PC12 cells (Shafer *et al.*, 1990). These effects of MeHg were unique when compared to effects of divalent inorganic cations such as Cd^{2+}, Co^{2+} and Pb^{2+} (Nachshen, 1984; Suszkiw *et al.*, 1985). The ability of MeHg to alter binding of ω-conotoxin GVIA and the DHP nitrendipine suggested that MeHg may interact with receptor sites associated with N- and L-type Ca^{2+} channels, respectively.

The role of voltage-sensitive Ca^{2+} channels in mediating neurotransmitter release is crucial. To date, there are three well characterized Ca^{2+} channel types; N, L and T (Tsien, 1988). The N- and L-type channels have been proposed to regulate neurotransmitter release from a variety of central, peripheral and clonal nerve cells. The patch-voltage clamp recording technique was used to examine the ability of MeHg to block N- and L-type Ca^{2+} channels in the clonal cell line PC12; both channel types were blocked rapidly by low micromolar concentrations of MeHg (Shafer and Atchison, 1991). These experiments demonstrated two important aspects of block of Ca^{2+} channels by MeHg; that MeHg blocks Ca^{2+} channel types which are thought to mediate neurotransmitter release from central (Dooley *et al.*, 1987a) and peripheral nerve terminals (Rane *et al.*, 1987; Holz *et al.*, 1988; Atchison, 1989), and that characteristics of block of somatic and synaptosomal Ca^{2+} channels by MeHg are similar in many respects.

The ability of MeHg to affect Ca^{2+} channels in intact motor nerve terminals was confirmed by examining effects of MeHg on voltage changes in the perineurial sheath arising from Ca^{2+} channel function in mouse motor nerve terminals. MeHg blocked Ca^{2+} potentials as well as Na^{+} potentials recorded from the perineurium. This result confirms directly that MeHg disrupts Ca^{2+} channel function in intact motor nerve terminals, consistent with predictions from experiments in synaptosomes and PC12 cells. Thus, results of experiments within this dissertation clearly support the hypothesis: MeHg disrupts function of Ca^{2+} channel types which are associated with neurotransmitter release (Shafer and Atchison, 1991) and alters function of Ca^{2+}

channels located in the terminals of central (Atchison *et al.*, 1986; Shafer and Atchison, 1989; Shafer *et al.*, 1990) and motor neurons (chapter 7).

While no direct evidence has been presented htat Mehg disrupts channel function by entering the channel pore, the facilitation of block of I_{Ba} by MeHg by increasing the frequency of stimuluation as well as the voltage-dependence of block suggest that MeHg may interact within the channel pore. The ionic radii of ions which carry current through Ca^{2+} channels range from 1.06 Å for Ca^{2+} to 1.43 Å for Ba^{2+} while Hg^{2+} has an ionic radius of 0.93 Å. The hydration energies are 382, 316 and 441 Kcal/mol, respectively (Cooper *et al.*, 1984). Divalent mercury (Hg^{2+}) has an atomic raduis of 1.49 Å (Miller, 1984). Given that ionic radius will decrease by addition of a positive charge and hydration energy will decrease, one might expect that the -Hg^{+} portion of MeHg would be on the order of ionic size and hydration to allow it to enter the Ca^{2+} channel pore. The Na^{+} channel is permeable to organic ions such as guanidium and aminoguanidium, which are much larger in ionic radii than Na^{+}. Interestingly, methylated ions cannot pass through the Na^{+} channel (Hille, 1984). Thus, it is not inconceivable that MeHg may enter the pore of the Ca^{2+} channel, resulting in block of Ca^{2+} flux through the channel.

These results also provide direction for future investigations of the neurotoxic effects of MeHg. This includes but is not limited to: studies of effects of MeHg on neurotransmitter release, further examination of effects on channel function, effects of MeHg on Ca^{2+} channel functions other than neurotransmitter release, and how

effects on Ca^{2+} channels may relate to neurotoxicity observed during chronic exposure to MeHg.

Although effects of MeHg on neurotransmitter release have been demonstrated clearly at the neuromuscular junction, relatively little information exists concerning effects of MeHg on neurotransmitter release from the central nervous system (*See:* McKay *et al.*, 1986). Since effects of MeHg on Ca^{2+} channels in both central and peripheral terminals have been characterized, the ramifications of these effects on neurotransmitter release can be examined in more detail. Of particular importance is how alterations in Ca^{2+} channel function produced by MeHg impact MeHg-induced effects on neurotransmitter release. Release of neurotransmitters from the nerve terminal is thought to follow transient, localized elevations of $[Ca^{2+}]_i$ to near micromolar or micromolar levels (*see:* Smith and Augustine, 1988). Effects of MeHg on the rate and magnitude of this increase would be expected to result from block of Ca^{2+} channels. However, MeHg may have additional effects on neurotransmitter release independent of its effects on Ca^{2+} channel function (Atchison *et al.*, 1986; Traxinger and Atchison, 1987a). It has also been suggested that in addition to Ca^{2+} entry into the nerve terminal, voltage changes (beyond those which evoke Ca^{2+} entry) may be required for neurotransmitter release (Parnas *et al.*, 1986; 1989). Therefore alterations of membrane potential by MeHg (Hare and Atchison, 1990) may also contribute to block of the exocytotic process. Future studies of effects of MeHg on the release processes which occur after Ca^{2+} entry into the nerve terminal will provide additional information concerning the role of Ca^{2+}

channel block by MeHg, as well as possibly providing a link between effects of MeHg on Ca^{2+} channels and other neuronal systems.

Also important with respect to neurotransmitter release is the ability of MeHg to block both N- and L-type Ca^{2+} channels. Present interpretation of pharmacological and electrophysiological studies of Ca^{2+} channels involved in transmitter release suggest that N-type channels mediate release of small clear vesicles containing classical transmitters whereas L-type channels mediate release of dense-core vesicles containing peptide neurotransmitters (Hirning *et al.*, 1988; Smith and Augustine, 1988). Neuropeptides often are involved in modulation of neuronal activity, resulting in long term changes. Thus, the ability of MeHg to block L-type Ca^{2+} channels may be an effect that has important ramifications during chronic exposures to MeHg. Effects of MeHg on T-type Ca^{2+} channels have not been examined; this channel has been associated with dendritic action potentials and pacemaker activity in the CNS (Tsien, *et al.*, 1988). The ability of MeHg to affect the N- and L-type Ca^{2+} channels, as well as the ability of MeHg to affect Na^{+} channel function and neuronal excitability (Traxinger and Atchison, 1987b), suggest that possible effects of MeHg on T-type channels be considered in studies of MeHg effects in the CNS.

In addition to the role of Ca^{2+} channels in neurotransmitter release, effects of MeHg on channel function suggest topics for further study. The actions of MeHg on Ca^{2+} channels are unique in many ways from the actions of heavy metals such as Cd^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+} and Ni^{2+}; which also block Ca^{2+} channels (Nachshen, 1984). One subject of interest is how differences in charge and lipophilicity affect the ability

of mercurials to alter channel function. Recent studies suggest that only positively charged organic and inorganic mercurials are able to block Ca^{2+} channels, and only lipophilic mercurials block Ca^{2+} channels in a voltage-dependent manner (Hewett and Atchison, 1990). MeHg also alters Ca^{2+} channel ionic selectivity and inactivation (Shafer *et al.*, 1990; Shafer and Atchison, 1991), which suggests that interactions between MeHg and Ca^{2+} channels differ from those of inorganic Ca^{2+} channel blockers. Further characterization of effects of MeHg on Ca^{2+} channel function at the single channel and biochemical level will provide additional information regarding the mechanisms by which MeHg produces these effects.

In addition to effects on Ca^{2+} channels, this dissertation has presented evidence that MeHg produces effects on Na^+ channels in clonal cell lines and in motor nerve axons at similar concentrations to effects on Ca^{2+} channels. These results are consistent with previous studies of MeHg action (Quandt *et al.*, 1982; Traxinger and Atchison, 1987b). Alterations of Na^+ channel function by MeHg would certainly affect synaptic transmission in intact NMJ preparations (Traxinger and Atchison, 1987b; chapter 8), as block of impulse conduction by MeHg would prevent neurotransmitter release. However, due to the importance of Na^+ channels in neuronal excitability, these effects of MeHg may also have implications for other aspects of MeHg toxicity. Thus, future experiments on ionic channel function may include examinations of common mechanisms of interaction of MeHg with different channel types as well as how effects on different ionic channels in the membrane may contribute to disruption of cellular function by MeHg.

MeHg alters a number of cellular processes including Ca^{2+} channel function. It has been established that MeHg also alters mitochondrial function (Levesque and Atchison, 1987; 1988; 1991) as well as Na^{+} channel function (Quandt *et al.*, 1982; chapters 7 and 8) and nerve terminal membrane potential (Hare and Atchison, 1990). The reactivity of MeHg with sulfhydryl groups as well as amino and carboxyl groups makes it unlikely that block of synaptic transmission, or other neurotoxic effects of MeHg, are the result of disruption of a single process. Thus, it is important to characterize all of the effects of MeHg and try to synthesize sequences of events which contribute to toxicity. In addition to effects on neurotransmitter release, MeHg-induced block of Ca^{2+} channels in neuronal cells may have other effects on cellular function. For example, Ca^{2+} channels provide for transient, localized elevations in $[Ca^{2+}]_i$ involved in growth cone elongation (Streit and Lux, 1987; 1989; 1990) or activation of Ca^{2+}-dependent enzymes (Kennedy, 1989). Regardless, Ca^{2+} channels are one cellular component involved in regulation of $[Ca^{2+}]_i$; other components include mitochondria, endoplasmic reticulum and other proteins and organelles (Meldolesi *et al.*, 1988). Thus, effects of MeHg on Ca^{2+} channels (Atchison *et al.*, 1986; Shafer and Atchison, 1989; 1991; Shafer *et al.*, 1990), coupled with effects on mitochondrial function (Levesque and Atchison, 1987; 1988; 1990) and membrane depolarization (Hare and Atchison, 1990) suggest the possibility that alterations in Ca^{2+} regulation may occur in response to MeHg exposure. Disruption of Ca^{2+} regulation might produce a cascade of events leading ultimately to cell death (Choi, 1988).

In conclusion, interactions of MeHg with voltage-dependent Ca^{2+} channels have been examined by the experiments contained in this thesis. Disruption of Ca^{2+} channel function by MeHg has been observed in isolated and intact nerve terminals. In addition, MeHg alterats specific Ca^{2+} channel functions and disrupts funtion of two Ca^{2+} channel types which are presently associated with neurotransmitter release. Although further study of the effects of MeHg on neurotransmitter release are needed, evidence has been presented which supports the hypothesis that block of neurotransmitter release at the NMJ is, in part, the result of block of voltage-dependent Ca^{2+} channels which mediate Ca^{2+} entry into the nerve terminal. Furthermore, results of these experiments provide additional knowledge concerning interaction of MeHg with these critical membrane-associated molecules; which may contribute to our understanding of the mechanisms of neurotoxicity of this important environmental compound.

APPENDIX

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TRANSMITTER, ION CHANNEL AND RECEPTOR PROPERTIES OF PHEOCHROMOCYTOMA (PC12) CELLS: A MODEL FOR NEUROTOXICOLOGICAL STUDIES

A) INTRODUCTION

The use of isolated nerve terminals (synaptosomes) and intact nerve-muscle preparations to measure effects of MeHg on nerve terminal Ca^{2+} channels represent model systems in which have been extensively used to study neuronal Ca^{2+} channel channel function and therefore have been well characterized with respect to their strengths and limitations. By contrast, pheochromocytoma (PC12) cells are not of neuronal origin, and measurement of Ca^{2+} channel function in this cell line using patch voltage-clamp techniques assesses function of somatic or growth cone Ca^{2+} channels. However, because of the unique properties of PC12 cells, data collected under defined conditions may be applicable to neurons. PC12 cells have been a popular tool for neurobiological studies and represent a potentially useful tool for neurotoxicological studies as well. This chapter will focus on use of PC12 cells as model systems for studies of channel function as well as neurotransmitter release and receptor function.

PC12 is a clonal cell line derived from a catecholamine-secreting tumor (pheochromocytoma) of rat adrenal medulla (Greene and Tischler, 1976). When cultured under normal conditions, these cells resemble adrenal chromaffin cells in

morphology as well as many aspects of physiology and biochemistry. However, when cultured in the presence of nerve growth factor (NGF), PC12 cells differentiate to resemble sympathetic neurons morphologically. The process of differentiation is accompanied by physiological and biochemical changes which ultimately result in neuron-like function in PC12 cells. In the absence or presence of NGF, PC12 cells release dopamine (DA), norepinephrine (NE) and/or acetylcholine (ACh) in response to depolarization or activation of nicotinic or muscarinic ACh receptors. In addition, PC12 cells possess membrane-bound receptors, including receptors coupled to guanine nucleotide-binding proteins (G-proteins) and Na^+, K^+ and Ca^{2+} channels. As such, PC12 cells provide a useful model for studying: 1) processes associated with neuronal differentiation, 2) synthesis, storage and release of neurotransmitters, 3) function and regulation of ion channels and 4) interactions of compounds with membrane-bound receptors. The ability of PC12 cells to differentiate in response to NGF allows for selective expression of certain proteins and for comparisons of responses in undifferentiated, chromaffin-like and differentiated, neuron-like cells.

B) PC12 CELL DIFFERENTIATION. Inclusion of NGF in the culture medium elicits a characteristic response from PC12 cells. Within 24 hours after its addition, the cells stop dividing and begin forming neuronal processes (Greene and Tischler, 1976). After 4 to 14 days, the cell bodies have increased in size and grown long processes which form extensive networks. This process of morphological differentiation is accompanied by changes in the expression of a number of proteins including Na^+/K^+

ATPase (Inoue *et al.*, 1988), voltage-sensitive Na^+ channels (Dichter *et al.*, 1977; Reed and England, 1986; Rudy *et al.*, 1987) and increased choline acetyltransferase (CAT) activity resulting in increased ACh content (Greene and Rein, 1977b). Membrane electrical excitability and response of PC12 cells to ACh are increased by NGF-induced differentiation (Dichter *et al.*, 1977). In addition, changes in Ca^{2+} channel expression (Takahashi *et al.*, 1985) decrease the susceptibility of neurotransmitter release to blockade by dihydropyridine-(DHP) type Ca^{2+} channel antagonists (Kongsamut and Miller, 1986). Thus, the differential expression and/or function of proteins, including ion channels and receptors, can be induced by NGF-treatment of PC12 cells. Other substances such as fibroblast growth factor (FGF) also induce differentiation of PC12 cells (for review see Fujita *et al.*, 1989). However, the changes in enzyme and channel activity induced by FGF and other differentiating agents differ from those induced by NGF (Garber *et al.*, 1989; Matsuoka *et al.*, 1989; Damon *et al.*, 1990; Pollock *et al.*, 1990). Removal of NGF from the medium results in a rapid reversal of the process of differentiation (Greene and Tischler, 1976). The ability of PC12 cells to differentiate and de-differentiate in the presence or absence of NGF has made these cells a popular model for studying processes associated with NGF receptor activation and neuronal development and maturation. This use of PC12 cells has been reviewed elsewhere (Guroff, 1985; Fujita, *et al.*, 1989). Clearly, there is great potential for use of this cell line in examining how neurotoxic compounds affect critical processes during neuronal development.

One other aspect of PC12 cell differentiation deserves mention here. Both adrenal chromaffin cells and sympathetic neurons are derived from embryonic neural crest cells. By contrast to embryonic neural crest cells (Doupe *et al.*, 1985; Anderson and Axel, 1986; Naranjo *et al.*, 1986), Greene and Tischler (1976) reported that glucocorticoids such as dexamethasone (DEX) do not prevent the actions of NGF on PC12 cells. However, more recently, PC12 cells have been reported to respond to sodium butyrate (Byrd *et al.*, 1987) by differentiating to an adrenal chromaffin-like phenotype. Upon butyrate treatment, PC12 cells undergo slight morphological changes without neurite extension and produce greater amounts of opioid peptide neurotransmitters (Byrd *et al.*,1987). Co-culture of PC12 cells with adrenal medullary endothelial cells induces PC12 cells to differentiate into an adrenal chromaffin-like phenotype which is unresponsive to NGF (Mizrachi, *et al.*, 1990). Thus, PC12 cells respond to some stimuli by differentiating to an adrenal chromaffin-like phenotype, and other stimuli by differentiating to a neuron-like phenotype.

C) TRANSMITTERS, ION CHANNELS AND RECEPTORS IN PC12 CELLS.

1) Neurotransmitter Release from PC12 cells.

As previously mentioned, PC12 cells synthesize and release DA, NE (Greene and Tischler, 1976; Greene and Rein, 1977a) and ACh (Greene and Rein, 1977b), but not epinephrine (Greene and Tischler, 1976). These three transmitters are all released in response to depolarization (Table 1). In addition, undifferentiated and NGF-differentiated PC12 cells release catecholamines in a Ca^{2+}-dependent fashion

in response to nicotinic (Greene and Rein, 1977c) and muscarinic (Rabe *et al.*, 1987) cholinergic agonists.

The apparent decrease in activity of enzymes and amounts of neurotransmitters following treatment with NGF is most likely due to increased expression of new proteins induced by NGF, not to a decrease in the expression of enzymes associated with catecholamine synthesis (Greene and Tischler, 1976). The membranes of PC12 cells contain a saturable NE transporter which is sensitive to cocaine and the tricyclic antidepressant desmethylimipramine (Greene and Rein, 1977a). NE and DA are most likely stored in vesicles, as exposure to reserpine depletes cellular catecholamine levels (Greene and Rein, 1977a). Thus, the mechanisms of synthesis, storage and re-uptake of catecholamines in PC12 cells are similar to those of catecholaminergic neurons.

In undifferentiated PC12 cells, synthesis of NE, but not DA, changes with the cell cycle, being maximal during the G_2 phase. Release of catecholamines by depolarization is maximal during G_1, whereas carbamylcholine-induced release is maximal during the S and G_2 phases. Cell cycle-specific changes in $^{45}Ca^{2+}$ influx induced by K^+ depolarization and muscarinic receptor activation correlate with neurotransmitter release (Koike and Takashima, 1986). This characteristic of undifferentiated PC12 cells is clearly different from neurons, which do not divide.

Permeabilization of PC12 cells provides more direct access to release mechanisms. In staphylococcal α-toxin permeabilized cells, Mg-ATP, calmodulin or proteins containing -SH or -OH groups are not required for Ca^{2+}-dependent release

(Ahnert-Hilger and Gratzl, 1987). Furthermore, release of catecholamines from α-toxin or digitonin-permeabilized cells is enhanced by phorbol esters (Pozzan *et al.*, 1984; Ahnert-Hilger *et al.*, 1987; Carroll *et al.*, 1990) and reduced in a pertussis toxin-sensitive fashion by an activator of guanine binding proteins, GTPγS (Ahnert-Hilger *et al.*, 1987). These results indicate that protein kinase C (PKC) and G-proteins, respectively, may modulate catecholamine release from PC12 cells. However, the role of G-proteins in modifying catecholamine release from PC12 cells is not yet clearly defined. GTPγS stimulated only Ca^{2+}-independent NE release from digitonin-permeabilized PC12 cells, whereas an inhibitor of guanine binding proteins cyclase, GTPßS, reduced only the GTPγS component of release. Thus, the component of release affected by cyclic guanine nucleotides is not essential to NE release (Carroll *et al.*, 1990). Furthermore, the opposite actions of GTPγS in α-toxin and digitonin-permeabilized cells indicate that the agent used to permeabilize cells may influence G-protein-dependent pathways.

DA release evoked by depolarization and/or nicotinic ACh receptor activation is accompanied by increases in cyclic AMP (cAMP). Moreover, forskolin, an activator of adenylate-cyclase, enhances depolarization-(Rabe *et al.*, 1982; Baizer and Weiner, 1985) or carbachol-induced release (Baizer and Weiner, 1985). Thus, cAMP may also modulate catecholamine release in PC12 cells. However, the action of cAMP presumably is on a component of the release mechanism which functions after Ca^{2+} entry, as forskolin and/or its analogs either do not affect $^{45}Ca^{2+}$ entry (Rabe *et al.*,

1982), or block voltage-dependent Ca^{2+} channels in a cAMP-independent manner (Nishizawa *et al.*, 1990)

Effects of neurotoxic compounds on catecholamine synthesis and release have been studied in PC12 cells. Chronic exposure to ethanol (EtOH) decreased (muscarinic) ACh-induced release of [^{3}H]-NE from PC12 cells without affecting NE uptake or ACh binding. However, EtOH did decrease the rise in $[Ca^{2+}]_i$ induced by stimulation of muscarinic receptors on PC12 cells (Rabe and Weight, 1988). Concentrations of DA and NE in PC12 cells were decreased by a 6 h. treatment with 1 to 100 ppm Arochlor 1254 (Seegal *et al.*, 1989). Thus, PC12 cells may provide a useful *in vitro* system for examining mechanisms of chemical action on catecholamine levels and/or release processes in neurons.

In addition to catecholamine neurotransmitters, PC12 cells also synthesize and release ACh. This ability to synthesize and release two distinct classes of transmitters (ACh and catecholamines) permits PC12 cells to be used for differential studies of effects of neurotoxicants on the release of unique transmitters. In contrast to its effect on biosynthetic enzymes for catecholamines, NGF-differentiation increased CAT activity (Table 1). Treatment with retinoic acid instead of NGF resulted in an increase in CAT activity without morphological differentiation (Matsuoka *et al.*, 1989). Undifferentiated PC12 cells have a high-affinity (K_m = 12 μ), Na^+-dependent choline transporter which is relatively insensitive to hemicholinium-3 (Melega and Howard, 1981). This differs from normal cholinergic neurons for which the high-affinity choline transporter is sensitive to

Table A.1

Activity and Content of Noradrenergic and Cholinergic Synthetic Enzymes and Neurotransmitters in PC12 Cells

	Undifferentiated	NGF-Differentiated
Enzymes	Enzyme Activity (pmol/min/mg protein)	
Tyrosine Hydroxylase	39 ± 5	10 ± 1
Dopa decarboxylase	770 ± 99	130 ± 15
Dopamine β-hydroxylase	806 ± 84	161 ± 19
Choline Acetyltransferase*	440 ± 17	520 ± 20
Neurotransmitters	Neurotransmitter Content (pmol/mg protein)	
Dopamine	16.6 ± 1.7	4.4 ± 0.4
Norepinephrine	6.1 ± 6	1.5 ± 0.2
Epinephrine	<0.15	<0.15
Acetylcholine*	1335 ± 77	5594 ± 637

PC12 cells were treated with 50 mg/ml of NGF for 14 (noradrenergic properties) or 22 (cholinergic properties) days prior to measuring enzyme activity and neurotransmitter content. All values are expressed as mean ± SEM and protein content is total cellular protein content.

*Values from: Greene and Rein, *Nature (Lond.)* **268**: 349-352, 1977b.

All other values from: Greene and Tischler, *Proc. Natl. Acad. Sci. (U.S.A.)* **73**: 2424-2428, 1976.

hemicholinium-3. In undifferentiated PC12 cells, ACh and catecholamines are stored in dense-core vesicles, but based on reserpine sensitivity and electron microscopy, the two transmitters are not co-localized (Schubert and Kleir, 1977). Evidence that release of ACh from PC12 cells is quantal was demonstrated by co-culture of PC12 cells with a skeletal muscle clonal cell line, L-6. After as few as 3 days of co-culture in NGF-containing medium, α-bungarotoxin- and *d*-tubocurarine-sensitive miniature endplate-potentials could be recorded from L-6 myotubes (Schubert *et al.*, 1977). Howard and co-workers loaded PC12 cells with $^2H_4$-choline and used combined gas chromatography-mass spectrometry to measure levels of endogenous $^2H_0$-ACh and newly synthesized $^2H_4$-ACh in the presence and absence of vesamicol, which inhibits uptake of ACh into vesicles (Marshall, 1970). Their results clearly indicate that newly synthesized ACh in PC12 cells is stored in vesicles (Figure A.1A) and evoked-release of $^2H_4$-ACh (Figure A.1B)is predominantly vesicular in nature (Melega and Howard, 1984; Howard *et al.*, 1986). Thus, in these respects, release of ACh from PC12 cells mirrors normal release from native cholinergic terminals.

Effects of neurotoxins on cholinergic properties of PC12 cells have not been widely studied. The effects of tetanus toxin on ACh release from PC12 cells indicate that this neurotoxin inhibits ACh release at subnanomolar concentrations (Sandberg *et al.*, 1989a) and that tetanus toxin may inhibit ACh release by interfering with cyclic-GMP metabolism (Sandberg, *et al.*, 1989b).

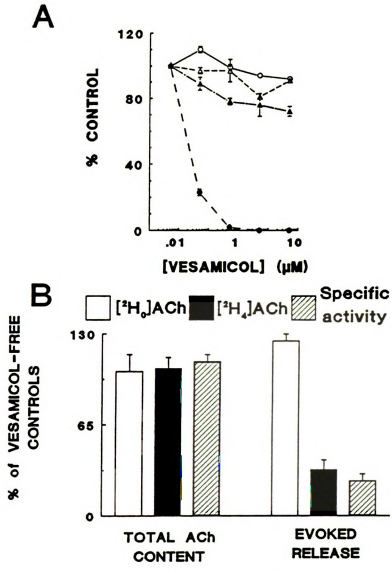

Figure A.1. Vesicular nature of ACh release from PC12 cells. Effects of vesamicol (AH5183) on the cellular and vesicular content of acetylcholine and on acetylcholine release in PC12 cells. A.) PC12 cells were incubated for 30 min with 10 μM [^{3}H]choline in the presence of vesamicol. Acetylcholine (ACh) was extracted from the cells (triangles) and from a vesicle fraction (circles) of a cell homogenate, and the levels of [$^2H_4$]ACh (solid symbols) and native [$^2H_0$]ACh (open symbols) were measured. The results are expressed as the percentage of control values. Values shown are the mean ± SD for triplicate incubations. B.) Release of ACh from vesamicol-treated cells during 5 min incubations. Total cellular ACh content is the sum of the [$^2H_0$]ACh or the [$^2H_4$]ACh released to the buffers during a 5 min incubation and the amount of ACh remaining in the cells after the incubations. Specific activity is the ratio of [$^2H_4$]-ACh/ total ACh. The results are expressed as a percentage of control values for cells that were incubated in the absence of vesamicol. The values are the mean ± SD for quadruplicate incubations.

Modified from: Howard *et al.*, In: *Calcium, Neuronal Function and Transmitter Release.* (R. Rahamimoff and B. Katz, eds). p. 282 (1986).

2) Ion channels in PC12 cells.

K^+ Channels. PC12 cells also express multiple K^+ conductances and channel subtypes. Several groups have reported the presence of a large Ca^{2+}-activated K^+ conductance in PC12 cells (Hoshi and Aldrich, 1988a; Auguste *et al.*, 1989; Pun and Behbehani, 1990). This current is blocked readily by agents which decrease Ca^{2+} influx into PC12 cells, such as inorganic divalent cations (Pun and Behbehani, 1990). More than one type of Ca^{2+}-activated K^+ channel may be present in PC12 cells, as $^{86}Rb^+$ efflux is only completely blocked by a combination of tetraethylammonium and apamin (Schmid-Antomarchi *et al.*, 1986), a polypeptide toxin from bee venom which blocks some types of Ca^{2+}-activated K^+ channels. PC12 cells express large amounts of the apamin receptor after as little as 4 days in culture. Interestingly, after 7 days in culture in the presence of NGF, the number of apamin receptors/mg protein was 10 fold less than in undifferentiated cells (Schmid-Antomarchi *et al.*, 1986). Thus, by contrast to Na^+ and Ca^{2+} channels, NGF treatment does not increase the expression of this channel type. Apamin was used in affinity labelling experiments in an attempt to identify components of the apamin receptor in PC12 cells. ^{125}I-labeled derivatives of apamin bound to a 30 kilodalton receptor in PC12 cell membranes (Auguste *et al.*, 1989). Since this cell line expresses large amounts of the apamin receptor, it may be useful in isolating the genetic sequences which code for this type of Ca^{2+}-activated K^+ channel.

Whole-cell currents similar to those mediated by rapidly inactivating (I_A) (Rogawski *et al.*, 1988; Pun and Behbehani, 1990) and delayed rectifier (I_K) K^+

channels (Rogawski, *et al.*, 1988; Hoshi and Aldrich, 1988a) have also been reported for PC12 cells. Phencyclidine (1-100 μM) and tetraethylammonium chloride (20 mM) block I_K (Rogawski, *et al.*, 1988), whereas I_A is inhibited by 2 mM 4-aminopyridine (Rogawski *et al.*, 1988). Hoshi and Aldrich (1988a,b) have characterized four voltage-dependent K^+ channels which may underlie whole-cell K^+ currents in PC12 cells. The kinetics of three of these channels are similar to those of delayed rectifier K^+ channels, whereas the kinetics of the fourth channel are similar to kinetics of the inactivating K^+ channel. However, the channels in PC12 cells also have characteristics which are unique from those of channels which mediate I_K and I_A (Hoshi and Aldrich, 1988a,b). Thus, it cannot be assumed that K^+ channels in PC12 cells behave in a fashion identical to those described elsewhere.

Finally, it has been demonstrated that the peptide galanin inhibits DA secretion from PC12 cells by activating a K^+ conductance (de Weille *et al.*, 1989). Galanin had no effect on L-type Ca^{2+} channels, but its action on K^+ conductances was inhibited by pretreatment with pertussis toxin. These results suggest that K^+ channels and the galanin receptor may be linked by a G-protein in PC12 cells (de Weille *et al.*, 1989). Whether or not the galanin response is mediated by a voltage-sensitive or receptor operated channel is presently unknown.

By contrast to Ca^{2+} channels, the effects of NGF-differentiation on voltage-sensitive K^+ channels have not yet been widely studied. Thus, it is unclear at this point whether or not changes in distribution or composition of K^+ channel types play

an important role in, or are affected by the process of differentiation of PC12 cells. Toxicological studies utilizing K^+ channels in PC12 cells are also lacking.

Na^+ channels. Like Ca^{2+} channels, expression of voltage-gated Na^+ channels is increased by differentiation of PC12 cells with NGF. TTX-sensitive, Ca^{2+}-independent action potentials in PC12 cells differentiated with NGF for 2 weeks were observed by Dichter *et al.* (1977). Undifferentiated PC12 cells express functional Na^+ channels, but differentiation with NGF increased TTX-sensitive influx of $^{22}Na^+$ by approximately 12 fold (Reed and England, 1986). Induction of a TTX-insensitive Na^+ flux by NGF has also been reported (Rudy *et al.*, 1987).

The mechanisms underlying NGF-induced increases in Na^+ channel function are presently unclear. Increases in Na^+ channel function by NGF were mimicked by increases in cAMP, and responses to NGF and cAMP were blocked by an inhibitor of cAMP-dependent protein kinase (PKA), suggesting that the expression of Na^+ channels in response to NGF is in part mediated by PKA (Kalman *et al.*, 1990). However, Pollock *et al.* (1990) reported that cAMP did not stimulate neurite outgrowth and decreased I_{Na} in PC12 cells, whereas FGF was as effective as NGF in increasing I_{Na}. In addition to NGF, treatment with DEX also increased the expression of Na^+ current in PC12 cells (Figure A.2, Garber *et al.*, 1989), although this response appears to be inconsistent. Treatment with DEX and NGF produced less of an increase in Na^+ current than treatment with NGF alone, and despite treatment with NGF and/or DEX, some PC12 cells failed to express measurable whole-cell Na^+ conductances (Figure A.2) (Garber *et al.*, 1989). Furthermore, Pollock

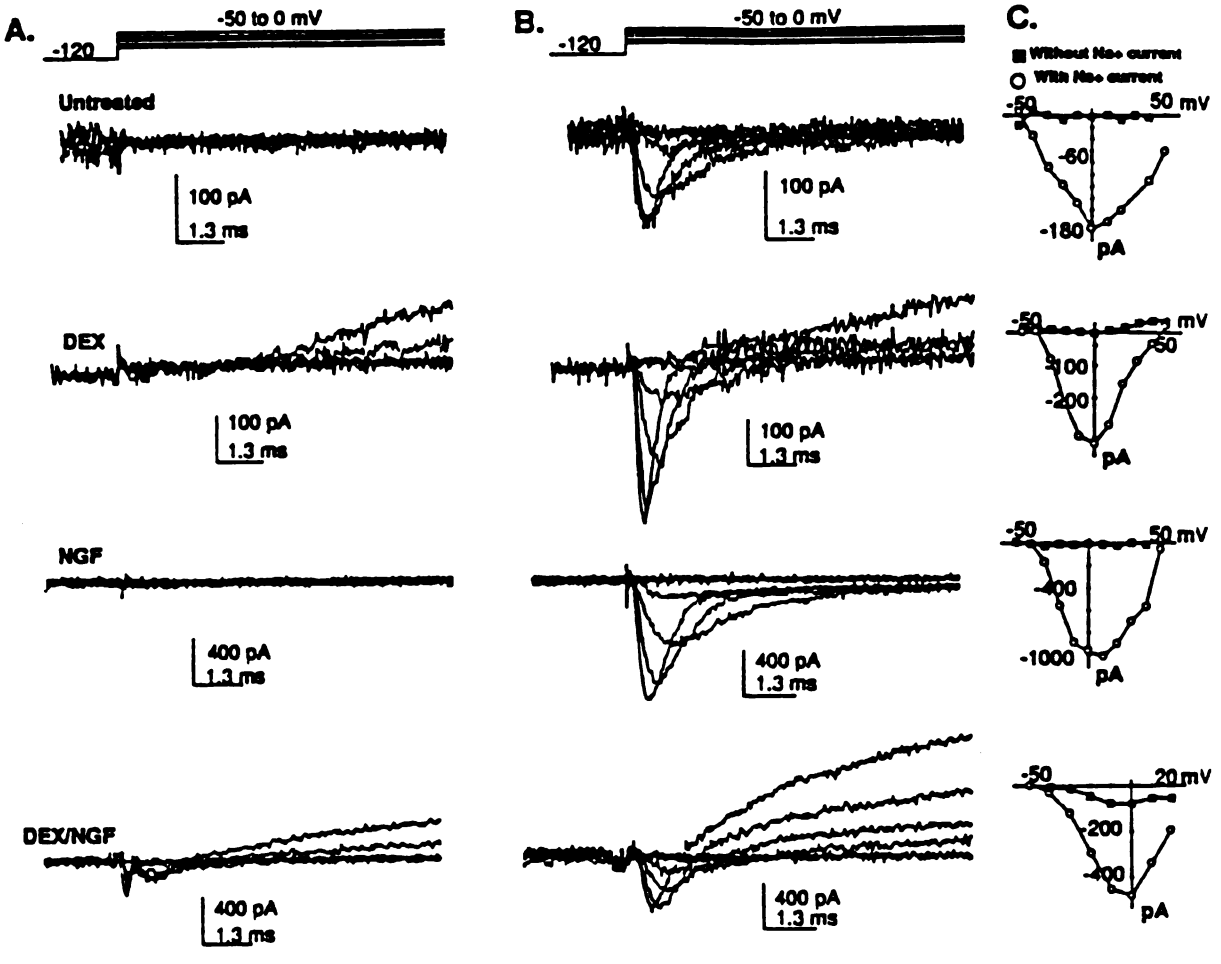

Figure A.2. Voltage-dependent Na^+ channels in PC12 cells. Representative families of the voltage-dependent Na^+ currents from untreated, dexamethasone-(DEX), nerve growth factor- (NGF), and DEX/NGF-treated cells recorded using the whole-cell patch voltage-clamp technique. In each treatment group, some cells (*A*) expressed no or little Na^+ current while others (*B*) expressed appreciable amounts of Na^+ current. *C*, Peak current-voltage (*I/V*) curves were obtained from cells which did not express the Na^+ current (*black squares*) and those which expressed the Na^+ current (*circles*). As K^+ was often used in internal solutions, outward K^+ currents are present in some families of current records.

From: Garber *et al.* *J. Neurosci.* 9:3976-3987 (1989).

et al., (1990) reported that DEX failed to increase I_{Na}, and inhibited NGF-induced increases in I_{Na} in PC12 cells. Finally, several other treatments including DEX, mitotic inhibitors and dimethyl sulfoxide, which induced physiological differentiation in neuroblastoma cell lines, were less effective at increasing Na^+ current than NGF. In addition, ACh responses induced by NGF in PC12 cells were not necessarily coupled to increases in Na^+ channel function (Ifune and Steinbach, 1990). Further study is needed to understand clearly the regulation of Na^+ channel expression in PC12 cells.

3) Receptors on PC12 cell membranes.

Another potential use of PC12 cells in neurotoxicology is for the study of interactions of compounds with neurotransmitter receptors or receptor/ligand-activated channels. Both nicotinic (Greene and Rein, 1977b) and muscarinic ACh receptors (Jumblatt and Tischler, 1982; Cross *et al.*, 1984) are expressed in PC12 cell membranes, as well as adenosine receptors (Guroff *et al.*, 1981). A recent report also suggests that PC12 cell membranes may also contain N-methyl-D-aspartate receptors which stimulate phosphoinositide hydrolysis, increased $[Ca^{2+}]_i$ and NE release (Kurozumi *et al.*, 1990).

Adenosine or adenosine receptor agonists stimulate adenylate cyclase resulting in increased tyrosine hydroxylase activity in PC12 cells (Rabe and M^{c}Gee, 1983; Noronha-Blob *et al.*, 1986). Radioligand binding studies have demonstrated that adenosine receptors in PC12 cells are of the A_2 subtype (Williams *et al.*, 1987). Short pretreatment of PC12 cells with adenosine increased depolarization-evoked release

of [^{3}H]-NE and [^{3}H]-ACh without altering $^{45}Ca^{2+}$ influx or depolarization as measured by $^{86}Rb^+$ efflux through nicotinic ACh receptor channels (Rabe and M^{c}Gee, 1983). It has been suggested that adenosine or adenosine nucleotides may act via A_2 receptors in a paracrine or autocrine fashion (Roskoski and Roskoski, 1989). ATP released during exocytosis of NE- or ACh-containing vesicles may be degraded to adenosine and stimulate A_2 receptors. In PC12 cells, the activity of adenosine is terminated by re-uptake (Roskoski and Roskoski, 1989).

Activation of muscarinic ACh receptors in undifferentiated PC12 cells results in elevations in inositol triphosphate (IP_3) and free $[Ca^{2+}]_i$ (Vincentini *et al.*, 1985), followed by neurotransmitter release (Rabe *et al.*, 1987). Inoue and Kenimer (1988) have suggested that a receptor-activated Ca^{2+} channel may mediate muscarinic receptor-induced neurotransmitter release. Muscarinic agonists increased $^{45}Ca^{2+}$ influx, and neurotransmitter release was not blocked by Ca^{2+} channel blockers. Moreover, the influx of $^{45}Ca^{2+}$ and neurotransmitter release were inhibited by pertussis toxin, indicating the possible involvement of G-proteins (Inoue and Kenimer, 1988). However, neurotransmitter release and phosphoinositide (PI) hydrolysis stimulated by muscarinic receptor activation appear to be separate events. In Ca^{2+}-free solutions, methacholine stimulated PI hydrolysis but not NE release. By contrast, activators of PKC inhibited PI turnover but not NE release (Takashima and Kenimer, 1989). Similarly, activation of muscarinic receptors in PC12 cells also results in an inhibition of cAMP which is a separate event from PI hydrolysis (Horwitz, 1989). However, there is no evidence that these actions are mediated by

different receptor subtypes (Horwitz, 1989). Thus, stimulation of the muscarinic ACh receptor in PC12 cells may also stimulate cellular processes in addition to neurotransmitter release.

Characterization of muscarinic receptor subtypes in undifferentiated PC12 cells by pharmacological sensitivity of [^{3}H]-NE release and [^{3}H]-telenzipine (a selective antagonist for the M_1-type muscarinic receptor) binding indicates that PC12 cells contain more than one type of muscarinic receptor (Bönish *et al.*, 1990). However, the predominant receptor subtype clearly is not of the M_2 or M_3 category, and also may not be of the M_1 subtype (Michel *et al.*, 1989; Bönish *et al.*, 1990). It has been proposed that the muscarinic ACh receptor in PC12 cells may be of the M_4 subtype (Michel *et al.*, 1989). Further study is needed to define better the type of muscarinic ACh receptor(s) present on PC12 cells.

Effects of organophosphate insecticides on muscarinic ACh receptors in PC12 cells indicate that 50 μM soman decreased the number of muscarinic receptors after 24 h. of exposure. However, the insecticides did not inhibit binding of radiolabeled muscarinic antagonists, nor did atropine prevent organophosphate-induced decreases in muscarinic receptor numbers. Furthermore, decreases in muscarinic receptors induced by organophosphates were qualitatively different from those produced by the ACh agonist carbamylcholine. Thus, organophosphate insecticides decrease the numbers of muscarinic ACh receptors by a mechanism other than agonist-induced receptor desensitization (Viana *et al.*, 1988).

Stimulation of nicotinic and muscarinic ACh receptors results in a rapid increase in PKC activity. However, this response appears to be largely the result of nicotinic receptor activation, as muscarinic antagonists only caused small reductions of carbachol-induced PKC activity (Messing *et al.*, 1989). Furthermore, the nicotinic response was dependent on Ca^{2+} influx via voltage-dependent Ca^{2+} channels, as the DHP antagonist nifedipine reduced PKC activity to the level observed during muscarinic receptor activation (Messing *et al.*, 1989). Recent binding studies of nicotinic ACh receptors in PC12 cells using [^{3}H]-ACh and α-bungarotoxin indicate that there may be heterogeneous expression of nicotinic receptor subtypes (Lukas, 1990).

M^{c}Gee and co-workers have used PC12 cells to examine agonist-induced down-regulation of the nicotinic ACh receptor (Robinson and M^{c}Gee, 1985). The nicotinic ACh receptor is not the target of cAMP-dependent phosphorylation in PC12 cells (M^{c}Gee and Liepe, 1984), but high concentrations of forskolin can impair receptor function by anesthetic-like effects (M^{c}Hugh and M^{c}Gee, 1986). Furthermore, nicotinic ACh receptors are not influenced by prolonged depolarization or elevations in $[Ca^{2+}]_i$ (DeLorme and M^{c}Gee, 1988). Adenylate cyclase does not appear to be involved in desensitization of nicotinic ACh receptors in PC12 cells, as forskolin and its analogs do not markedly alter agonist-induced desensitization of nicotinic ACh receptors in PC12 cells (Nishizawa *et al.*, 1990). Further study of agonist-induced desensitization of nicotinic ACh receptors is needed to elucidate the mechanisms responsible for desensitization in PC12 cells. However, enough information exists about the types

and functions of ACh receptors in PC12 cells to allow examination of interactions between neurotoxins and these receptor types.

D) CLONAL VARIABILITY AND SUBCLONES OF PC12

When using any model system, one must know the weaknesses and limitations of the model in order to maximize its usefulness while minimizing the likelihood of error or overinterpretation of results. One limitation of the PC12 cell line is that the number of passages or subcultures from the original isolation of the line is unknown. Second, PC12 cell cultures have a tendency to become heterogenous with respect to cell morphology after approximately 20 passages (Guroff, 1985). Thus, it is often difficult to compare directly results from different laboratories due to differences in passage number. This is most apparent for studies of the regulation of Na^+ channel expression, and undoubtedly limits the use of this line for screening and/or regulatory purposes. However, PC12 cells remain an excellent model for mechanistic studies of disruption of neuronal function. By routinely keeping track of the number of passages from receipt of the cell line, one can maintain internal consistency by using the same "passage number" for experiments and can begin a new culture from a frozen stock when the number of passages approaches 20.

A number of subclones of PC12 exist due to spontaneous and/or induced mutations. Heterogeneity in NE and DA synthesis and responses to glucocorticoids has been reported (Koike and Takashima, 1984). Other clones vary in their ability to respond to NGF or in the type of response induced by NGF (*see* Guroff, 1985).

Presently, the subclones of PC12 are used primarily in studies of the action of NGF. However, they may also represent a useful tool in neurotoxicology as well.

E) SUMMARY

PC12 cells have been widely used for studies of neuronal biochemistry and physiology. In addition to responses to NGF, the neurotransmitter, ion channel and receptor properties of this cell line have been well characterized. A well characterized and understood model system is a prerequisite for mechanistic studies of the action of toxic compounds. PC12 cells represent a potentially useful model for the study of mechanisms of action of neurotoxic compounds on neuronal differentiation and maturation, synthesis, storage and release of DA, NE and ACh, as well as the function of voltage-sensitive Na^+, K^+ and Ca^{2+} channels. With respect to Ca^{2+} channels, the subtypes of Ca^{2+} channels expressed in this cell line are extremely well characterized by ion flux, binding and electrophysiological techniques. In addition, the composition of Ca^{2+} channel subtypes can be altered predictably by NGF-differentiation, permitting accurate electrophysiological assessment of effects of putative toxicants on these important membrane macromolecules. Several receptor types, including nicotinic and muscarinic ACh receptors and adenosine receptors are also becoming well characterized in PC12 cells. Thus, this cell line, which has been used only sparingly in neurotoxicological studies to date, may in the future become an extremely useful model system.

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