This is to certify that the

dissertation entitled

CORRELATION OF EFFECTS OF METHYLMERCURY ON SPONTANEOUS QUANTAL RELEASE OF ACETYLCHOLINE FROM NERVE TERMINALS WITH DISRUPTION OF INTRATERMINAL CALCIUM REGULATION

presented by

Paul Charles Levesque

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology/Toxicology

William D. Atchison
Major professor

Date January 22nd, 1990

CORRELATION OF EFFECTS OF METHYLMERCURY ON SPONTANEOUS QUANTAL RELEASE OF ACETYLCHOLINE FROM NERVE TERMINALS WITH DISRUPTION OF INTRATERMINAL CALCIUM REGULATION

By

Paul Charles Levesque

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

1990

ABSTRACT

CORRELATION OF EFFECTS OF METHYLMERCURY ON SPONTANEOUS QUANTAL RELEASE OF ACETYLCHOLINE FROM NERVE TERMINALS WITH DISRUPTION OF INTRATERMINAL CALCIUM REGULATION

By

Paul C. Levesque

Methylmercury (MeHg) is a potent environmental neurotoxicant that increases spontaneous quantal release of acetylcholine (ACh) at both central and peripheral synapses. The overall objective of these studies was to investigate the cellular mechanisms underlying the stimulatory effects of MeHg on release of ACh. Changes in the frequency of spontaneous release of neurotransmitter are strongly related to the free Ca^{2+} concentration in the axon terminal. Since MeHg stimulates release of ACh in the absence of external Ca^{2+} or in Ca^{2+}-deficient preparations, the hypothesis proposed is that MeHg disrupts the action of intraterminal Ca^{2+} buffers to store Ca^{2+} leading to increased intraterminal Ca^{2+}. In turn, this would cause an increase in spontaneous quantal release of neurotransmitter.

Preliminary electrophysiological experiments utilizing conventional intracellular microelectrode recording techniques and the isolated hemidiaphragm preparation of rats were designed to ascertain whether mitochondria or smooth endoplasmic reticulum may be a source of the increased intraterminal Ca^{2+} for the increased spontaneous release of ACh produced by MeHg. Follow up neurochemical studies were designed to obtain more conclusive evidence in support of the proposed effects of MeHg on nerve terminal Ca^{2+} regulation and release of neurotransmitter. Radioflux studies were performed on synaptosomes and isolated mitochondria using $^{45}Ca^{2+}$, Me[^{203}Hg] and radiolabelled ACh. Results

of the electrophysiological and neurochemical studies indicate that MeHg enters the nerve terminal and induces release of bound Ca^{2+} from mitochondria and that release of this pool of Ca^{2+} by MeHg contributes to the increased spontaneous release of ACh induced by MeHg at both peripheral and central synapses. These studies provide useful information regarding the mechanisms underlying effects of MeHg on synaptic transmission at both physiological and biochemical levels. Perturbations of intracellular Ca^{2+} homeostasis by MeHg may underlie the effects of MeHg on other Ca^{2+}-dependent cellular functions in neuronal as well as in non-neuronal cells.

to my family, especially my parents, for their

encouragement and support

ACKNOWLEDGEMENTS

My sincere thanks go to my thesis advisor, Dr. William D. Atchison. His ideas, encouragement, support and advice have been essential in my scientific development and are greatly appreciated.

I would like to express my appreciation to Drs. Gerard Gebber, Jim Bennett, and John Wilson, who formed a helpful and constructive guidance committee.

I gratefully acknowledge all the individuals who provided technical support, including Julianna Caguiat, Ben Manning, Beth Bigler, Jacqueline Stout and Jennise Vincent. Special thanks go to Diane Hummel who has masterfully typed manuscripts, abstracts and posters.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

ACh	acetylcholine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular calcium concentration
CAF	caffeine
DAN	dantrolene
DC	dicoumarol
DNP	dinitrophenol
D-PEN	D-penicillamine
DTT	dithiothreitol
EPP	end-plate potential
GSH	glutathione
HKR	hepes-buffered krebs ringer
K^+	potassium
La^{3+}	lanthanum
MeHg	methylmercury
MEPP	miniature end-plate potential
mM	millimolar
μM	micromolar

LIST OF ABBREVIATIONS (continued)

Na^+	sodium
NMJ	neuromuscular junction
OUA	ouabain
RCR	respiratory control ratio
RR	ruthenium red
SAP	saponin
SER	smooth endoplasmic reticulum
SR	sarcoplasmic reticulum
TMB-8	N,N-dimethylamino-8-octyl-3,4,5-trimethoxybenzoate
VAL	valinomycin
YS035	N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine

CHAPTER ONE

INTRODUCTION

A. General Introduction

Methylmercury (MeHg) is a potent environmental neurotoxicant implicated in episodes of intoxication in Japan and Iraq. MeHg-induced neurotoxicity is characterized by sensory disturbances, cerebellar ataxia and generalized extremity weakness in exposed individuals. Synaptic transmission at both central and peripheral synapses is disrupted by MeHg. The cellular mechanisms underlying the neurotoxic effects of MeHg are unknown but are the subject of much research. The basic processes underlying information transfer within the nervous system, impulse conduction and chemical transmission across synapses, are dependent upon electrochemical flux of ions such as Na^+, K^+ and Ca^{2+}. Evidence in the literature suggests that abnormal mono- and polyvalent cations, such as Li^+, Mg^{2+}, Co^{2+} and La^{3+}, can alter these cationic conductances (Baker et al., 1971; Heuser and Miledi, 1971; Branisteanu and Volle, 1975). Thus, both inorganic and organic mercury may also affect these processes. Indeed, given the propensity of mercurials to interact with functional groups in biological membranes, it would be surprising if functional damage to the nervous system were not observed. Intracellular microelectrode recording studies of effects of acute bath administration of MeHg on synaptic transmission have revealed that nerve-evoked release of acetylcholine (ACh) is inhibited and spontaneous quantal release of ACh is first increased and then decreased (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982; Miyamoto, 1983; Atchison et al., 1984; Atchison, 1986; Atchison et al., 1986). Increased spontaneous release of ACh is measured electrophysiologically as increased miniature end-plate potential (MEPP) frequency and occurs presumably as a result of increased free intraterminal Ca^{2+}. The stimulatory effect

of MeHg on MEPP frequency occurs independently of extracellular Ca^{2+} since it occurs even in Ca^{2+}-deficient solutions (Atchison, 1986). This suggests that if MeHg-induced stimulation of spontaneous release of ACh is due to elevation of free intraterminal Ca^{2+}, the source of this Ca^{2+} may be an intracellular store. The objective of my dissertation research will be to determine whether the effects of MeHg on synaptic transmission are in part due to disruption of Ca^{2+} regulation within the axon terminal.

Buffering of Ca^{2+} within nerve terminals is thought to be controlled by mitochondria, smooth endoplasmic reticulum (SER) and perhaps by synaptic vesicles and Ca^{2+}-binding proteins (Blaustein et al., 1977; 1978a,b). If MeHg enters the nerve terminal and interacts with either of these Ca^{2+}-sequestering organelles to cause release of Ca^{2+}, then the resultant increase in intraterminal free Ca^{2+} might stimulate spontaneous release of ACh. Preliminary intracellular microelectrode recording studies were undertaken in hopes of ascertaining whether mitochondria or SER may be a source of the increased intraterminal Ca^{2+} for the increased MEPP frequency produced by MeHg (Levesque and Atchison, 1987a,b). In follow up neurochemical studies, the potential effects of MeHg on nerve terminal Ca^{2+} regulation and release of transmitter were characterized further by performing flux studies on synaptosomes and isolated mitochondria using $^{45}Ca^{2+}$, Me[^{203}Hg] and radiolabeled ACh.

One may question the relevance of functional changes following acute exposure to mercury to the pathological findings in patients poisoned by chronic exposure to MeHg. Although in some cases there may be no direct relationship, the functional changes may represent early cellular effects of MeHg which have not

progressed in the whole organism to the extent of observable pathology. Moreover, effects of MeHg on the processes underlying synaptic transmission, processes which are highly Ca^{2+}-dependent, may be representative of other effects of MeHg on the nervous system.

B. Significance

MeHg has become recognized as a potent environmental neurotoxicant following instances of mass intoxication in Minamata, Japan (Takeuchi et al., 1962) and in Iraq (Bakir et al., 1973). Exposure to MeHg is associated with sensory disturbances, cerebellar ataxia and generalized extremity weakness in exposed individuals (Hunter et al., 1940). The molecular and cellular mechanisms responsible for the neurotoxic effects of MeHg are not known. Pathological lesions of the central and peripheral nervous systems have been described for MeHg (Chang and Hartmann, 1972a,b) but these lesions are thought to occur in response to more subtle biochemical or physiological effects of MeHg on the nerve. The sensory and motor defects may be due to disruption of synaptic transmission.

The mammalian neuromuscular junction (NMJ) was chosen as a model cholinergic synapse for investigating effects of MeHg on synaptic transmission. Studies of the effects of MeHg on neuromuscular transmission are useful for a number of reasons. The NMJ serves as an excellent model synapse for studying the effects of MeHg on synaptic transmission since the biochemical and physiological processes involved in transmitter release at this cholinergic synapse are well characterized, and the basic processes which underlie Ca^{2+}-dependent

neurotransmitter release at the NMJ are in many ways similar to those at other peripheral and central synapses. This is important since the primary neurotoxic effect of MeHg and other organic mercurials is on central nervous system function. MeHg and other mercurials not only affect synaptic function at cholinergic synapses but cause analogous changes in release of non-cholinergic neurotransmitters from other peripheral and central synapses (Borowitz, 1974; Bondy et al., 1979; Nakazato et al., 1979; Kobayashi et al., 1980; Bartolome et al., 1982; and Tuomisto and Komulainen, 1983). Thus, it is possible that the mechanisms responsible for the effects of MeHg at the NMJ are similar to those mechanisms underlying MeHg's effects at other chemical synapses in the peripheral and central nervous system. Thus, studies of cellular effects of MeHg may serve a more predictive role for studying those effects on other neural cells. Moreover, studies of the effects of MeHg at the NMJ may provide useful information pertaining to the mechanisms of other neurotoxicants that are known to disrupt synaptic transmission. At the neuromuscular junction, spontaneous release of ACh occurs in two forms: quantal and non-quantal. Spontaneous quantal release, measured as the frequency of occurrence MEPPs, has a strong dependence on $[Ca^{2+}]_i$ and a slight dependence on $[Ca^{2+}]_e$. Non-quantal release does not give rise to MEPPs and is not dependent upon $[Ca^{2+}]_i$ or $[Ca^{2+}]_e$ (Vyskocil et al., 1989). MeHg-induced changes in MEPP frequency are studied for several reasons. The physiological relevance of the MEPP is not well understood but the MEPP is thought to represent the simplest form of quantal transmitter release and the mechanisms associated with vesicular exocytosis are thought to be identical for spontaneous and evoked release of neurotransmitter. Inasmuch as recent

findings indicate that MeHg may block axonal impulse conduction and prevent action potentials from reaching the nerve terminal (Traxinger and Atchison, 1987), measurements of direct actions of MeHg on transmitter release would be confounded by potential conduction failure for studies of evoked release. Spontaneous release of transmitter is not dependent on nerve terminal action potentials and so the effects of MeHg on MEPP frequency would occur even if impulse conduction were blocked. Thus, by studying spontaneous release we can determine whether MeHg affects the exocytotic release process and its components directly. This may lead to useful information pertaining to the processes underlying spontaneous quantal release of neurotransmitter. Also, information regarding the effects of MeHg on nerve terminal Ca^{2+} regulation can be gained by studying changes in MEPP frequency. Finally, measurement of MeHg-induced changes in frequency of spontaneous quantal release of transmitter gives a moment to moment bioassay of the effects of MeHg on free Ca^{2+} since the frequency of release is directly proportional to the level of intraterminal Ca^{2+}. Therefore, one can obtain a qualitative index of effects of MeHg on nerve terminal Ca^{2+} regulation by monitoring changes in MEPP frequency.

Neurochemical studies designed to characterize further the potential effects of MeHg on Ca^{2+} regulation by isolated nerve terminals (synaptosomes) and isolated mitochondria provide a logical conclusion to the initial electrophysiological studies. The molecular and cellular mechanisms underlying the pathological lesions that occur with MeHg intoxication are not yet clear, but undoubtedly occur in response to more subtle biochemical or physiological effects on nerve cell bodies or processes. Perhaps these effects are due at least in part to disruption

of Ca^{2+} regulation within the axon terminal by MeHg. Since synaptic transmission is dependent on precisely regulated changes in free intraterminal Ca^{2+} concentrations, disruption of intraterminal Ca^{2+} regulation would explain some of the known effects of MeHg on the transmitter release process. Synaptosomes, which are enriched nerve terminal preparations derived from central neurons, were used in neurochemical studies designed to determine the role of Ca^{2+} in the effects of MeHg on release of neurotransmitter from central synapses. Synaptosomes retain several functional properties of in situ nerve terminals including the ability to maintain a membrane potential, the existence of channel-mediated Na^+, K^+ and Ca^{2+} fluxes, and the ability to synthesize neurotransmitters and to release them in a Ca^{2+}-dependent manner in response to depolarization (Whittaker, 1984). Synaptosomes are an appropriate model since the effects of MeHg on transmitter release also occur in the central nervous system. Although synaptosomes obtained from whole brain homogenates are heterogeneous with respect to their neurotransmitter, the effects of MeHg on release of transmitter from CNS preparations are not unique to a particular transmitter type (Kobayashi et al., 1979; Komulainen and Tuomisto, 1981; 1982; Minnema et al., 1989).

Release of neurotransmitter is only one example of a Ca^{2+}-dependent process. If MeHg indeed alters cellular Ca^{2+} regulation by disrupting transmembrane Ca^{2+} fluxes or Ca^{2+} buffering by intracellular organelles, one could predict effects of MeHg on other Ca^{2+}-dependent cellular functions in neuronal as well as in non-neuronal cells.

Thus, the preliminary studies which utilized the NMJ as a model synapse in conjunction with the neurochemical studies of effects of MeHg on nerve terminal

Ca^{2+} regulation and spontaneous release of neurotransmitter provide a logical progression from an initial observation on neuronal function at the whole tissue in situ or in vitro level to neurochemical analyses of isolated nerve terminals or components of nerve terminals as a means of examining putative mechanisms in greater detail. Moreover, this combined approach also provides useful information regarding the mechanisms underlying effects of MeHg on synaptic transmission at both the physiological and biochemical levels.

C. Methylmercury Intoxication

Mercury-containing compounds are utilized extensively for a variety of industrial and agricultural uses worldwide. Elemental and inorganic mercury are used in the electrical apparatus, industrial control, instrumentation, chloralkali and paint industries. MeHg, an organomercurial, is used mainly for its fungicidal properties and can be found in seed grain dressings, orchard sprays and preservative solutions for wood, paper pulp and leather. Mercury is released into the environment from these sources through waste-water discharges or atmospheric venting. Contamination also occurs through burning of fossil fuels and through surface run-off and release of wastes into rivers. Mercury poisoning most commonly occurs via ingestion of mercury contaminated food. Elemental and inorganic mercury are very poorly absorbed from the gastrointestinal tract but up to 95% of MeHg is absorbed. Industrial waste products containing inorganic mercury had been discharged into the atmosphere and waterways for years but were of little concern because they were not considered to be hazardous since inorganic mercury is only minimally absorbed by plants and animals. However, it

is now known that inorganic mercury can be converted readily to MeHg primarily by microorganisms present in the sediment of river and lake beds. Organic mercury can then enter the food chain after being taken up by algae and fish. Upon ingestion and subsequent absorption, mercury is transported in the plasma and red blood cells. Inorganic mercury is not distributed uniformly and becomes highly concentrated in the kidneys. MeHg is distributed somewhat evenly to the various tissues, with the highest concentrations in the brain and blood. Histochemical analysis shows that, intracellularly, MeHg is bound to membranous organelles such as mitochondria, endoplasmic reticulum, golgi complex, nuclear envelopes and lysosomes. Mercury penetrates and damages the blood-brain barrier, leading to a dysfunction of this protective system. Degenerative changes in nerve fibers occur with MeHg intoxication. In nerve fibers, MeHg is localized primarily on myelin sheaths and mitochondria. Excretion of inorganic mercury occurs via both the urine and feces, while MeHg is primarily excreted in the feces (Chang, 1977).

Exposure to MeHg in the food chain has led to large-scale incidents of intoxication in Minimata and Niigata, Japan (Takeuchi et al., 1962; 1968) and Iraq (Bakir et al., 1973). The most publicized incident in Japan occurred in the 195O's when industrial waste containing organic and inorganic mercury was discharged into Minimata Bay. Inorganic mercury was converted by microorganisms into MeHg, a much more toxic form of mercury, which then passed through the food chain ultimately reaching humans who consumed contaminated fish. Approximately 1500 people were affected and 46 deaths were reported. In addition, many infants were born with severe nervous system damage from

prenatal mercury intoxication. The Iraqi incident occurred in 1972, after seed grain contaminated with a MeHg-containing fungicide was used for baking bread. This acute episode of MeHg intoxication resulted in 450 deaths. In both instances, exposure to MeHg caused prominent neurotoxic signs characterized by cerebellar ataxia, generalized extremity weakness and sensory disturbances including impairments of speech, vision and hearing. A myasthenia gravis-like weakness was reported in the Iraqi outbreak. This condition was treated successfully with the acetylcholinesterase inhibitor, neostigmine (Rustam et al., 1975). Pathological examinations of tissue from affected patients have revealed lesions of both the central and peripheral nervous systems (Chang, 1977). The cellular mechanisms underlying the pathological lesions and the neurotoxic effects of MeHg are unknown.

D. Specific Background for Research Objectives

1. Mechanisms of Synaptic Transmission

Chemical synaptic transmission at peripheral and central synapses involves several steps (Figure 1). Presynaptically, an electrical signal is converted into a chemical signal and postsynaptically, the chemical signal invokes either an electrical or biochemical signal. Synaptic transmission at the NMJ is chemical in nature; acetylcholine (ACh) is the neurotransmitter. Synthesis of ACh occurs in the presynaptic nerve terminal. The precursors are choline and acetyl CoA and the catalytic enzyme is choline acetyltransferase. Newly-synthesized neurotransmitter is packaged into vesicles for storage and for protection against enzymatic

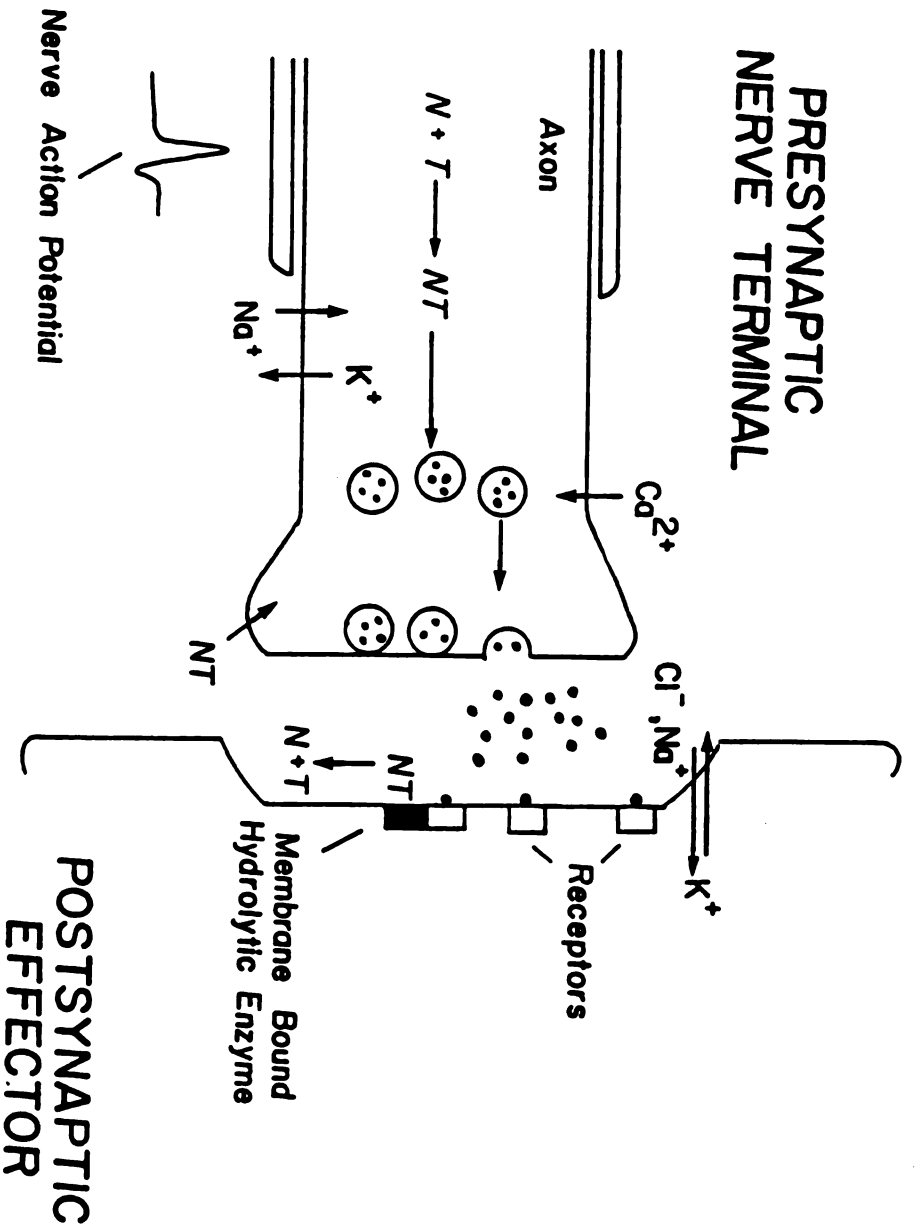

Figure 1. Steps underlying chemical synaptic transmission. Acetylcholine was used in the text as specific example of a chemical neurotransmitter in describing these processes. (From Atchison, 1988).

breakdown. Under normal physiological conditions, intraterminal Ca^{2+} becomes elevated in response to depolarization of the nerve terminal by an action potential. Action potentials are propagated along the axon towards the nerve terminal by inward and outward movement of Na^{+} and K^{+} ions through their respective channels. Nerve terminal depolarization causes opening of voltage-sensitive Ca^{2+} channels, and Ca^{2+} moves down its electrochemical gradient into the nerve terminal. ACh vesicles are discharged synchronously from the presynaptic nerve terminal subsequent to an elevation of free intraterminal Ca^{2+}. Release of ACh vesicles occurs at specialized regions of the nerve terminal known as active release zones. The precise mechanism underlying Ca^{2+}-induced transmitter release is unknown. An interaction between Ca^{2+} and Ca^{2+}-binding proteins is thought to cause fusion of synaptic vesicles with the axon terminal plasma membrane, resulting in release of ACh. ACh released into the synaptic cleft can interact with specific receptor proteins on the postsynaptic membrane. Binding of ACh to its receptor induces opening of a cationic-nonspecific channel associated with the receptor. Both Na^{+} and K^{+} move through this channel along their respective concentration gradients. The movement of these ions along their gradients causes a graded depolarization of the endplate membrane known as the end-plate potential (EPP). Action potentials are generated postsynaptically if the EPP reaches a threshold voltage. The action of ACh is terminated by a very efficient cholinesterase enzyme which is associated with the ACh receptor on the post-synaptic membrane (for reviews, see Silinsky, 1985; Atchison, 1987; Augustine, 1987).

Two well-characterized forms of quantal neurotransmitter release at the

neuromuscular junction are spontaneous release and synchronous evoked release. Spontaneous quantal release involves the random release of single packets of ACh from the nerve terminal (Fatt and Katz, 1952), the frequency of which varies directly with the intraterminal Ca^{2+} concentration. Under normal conditions, spontaneous release occurs randomly at a frequency of O.2-3.O/sec (Hz) (Fatt and Katz, 1952; Llinas and Nicholson, 1975). Spontaneous release of a single quantum of ACh can be measured electrophysiologically as a small, short-lived depolarization of the postsynaptic membrane, known as a miniature end-plate potential (MEPP). The MEPP is considered to represent the fundamental quantum of secretion. The other type of quantal neurotransmitter release, synchronous evoked release, involves the simultaneous release of many quanta of ACh from the axon terminal. This occurs subsequent to depolarization of the nerve terminal by an action potential and requires Ca^{2+} entry into the nerve terminal through specific voltage-sensitive Ca^{2+} channels (Katz and Miledi, 1967; Llinas et al., 1981). This form of release can be measured electrophysiologically as large, graded depolarizations of the postsynaptic membrane at the skeletal muscle end-plate region. The graded depolarization is known as an endplate potential (EPP) (Fatt and Katz, 1951).

Release of ACh from the neuromuscular junction is quantal in nature. This has been shown experimentally by reducing the extracellular ratio of Ca^{2+} to Mg^{2+} in increments. Manipulation of these cations in this way will cause the evoked EPP to fluctuate in discrete steps which correspond in size to multiples of the spontaneously-occurring MEPP (del Castillo and Katz, 1954). In addition to quantal release, non-quantal release of neurotransmitter from neuromuscular preparations also occurs (Mitchell and Silver, 1963; Fletcher and Forrester, 1975; Vyskocil et al.,

1989). This form of release of neurotransmitter occurs under normal conditions and accounts for much of the ACh released. The specific mechanisms underlying this form of transmitter release are not clear but are thought to involve more complicated processes than leakage or passive diffusion of transmitter from the nerve terminal (Polak et al., 1981; Vyskocil et al., 1989).

2. Effects of MeHg on Synaptic Transmission

Intracellular microelectrode recording studies of effects of acute bath administration of MeHg on synaptic transmission have revealed that nerve-evoked release of ACh is inhibited (Figure 2) and spontaneous quantal release of ACh is first increased and then decreased in a biphasic manner (Figure 3) (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982; Miyamoto, 1983; Atchison et al., 1984; Atchison, 1986; Atchison et al., 1986; Levesque and Atchison, 1987). Block of nerve-evoked release is observed as a complete cessation of the EPP. Block of EPPs by MeHg occurs rapidly and is complete by 20 min with 100 μM MeHg (Atchison and Narahashi, 1982; Atchison et al., 1984; Traxinger and Atchison, 1986). Changes in spontaneous quantal release of transmitter at the NMJ are measured electrophysiologically as changes in MEPP frequency. The MeHg-induced increase in MEPP frequency occurs after an initial latent period (Atchison and Narahashi, 1982; Atchison et al., 1984; Atchison, 1986). Increasing the concentration of MeHg does not increase mean peak frequency of MEPPs, however, it does shorten the latency to onset (Atchison and Narahashi, 1982). This latent period may reflect the time required for MeHg to enter the presynaptic nerve terminal and stimulate transmitter release. Thus, increasing the concentration of MeHg would be expected to hasten its entrance into the cell by increasing the

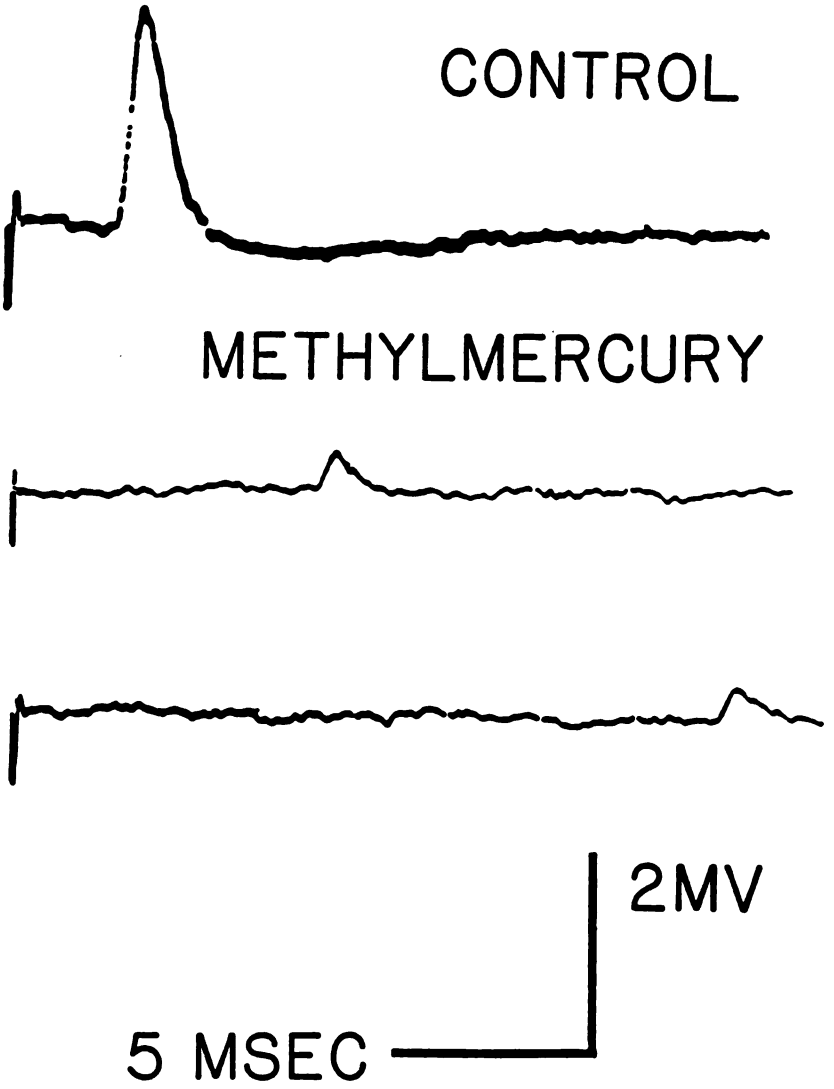

Figure 2. MeHg-induced block of synchronous evoked release of acetylcholine. EPP before (top) and 10 min after bath application of 100 μM MeHg (bottom). As seen on the bottom traces, MEPPs are present even after the EPP is blocked completely (From, Atchison and Narahashi, 1982).

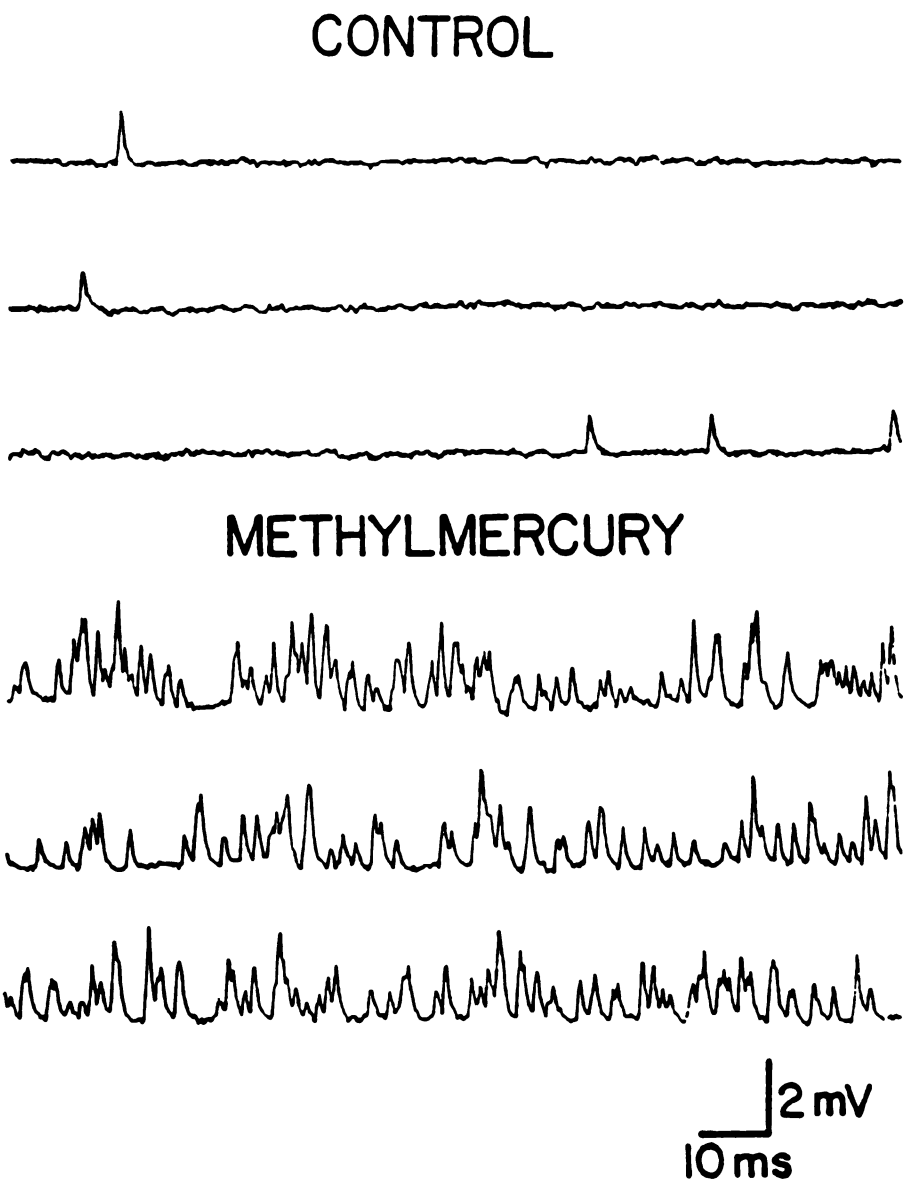

Figure 3. Stimulation of MEPP frequency by MeHg. MEPPs were recorded before (top) and 30 min after exposure to 40 μM MeHg (bottom). (from Atchison et al., 1984).

chemical driving force. Neither suppression of the EPP nor block of MEPPs by MeHg can be attributed to postjunctional block of the receptor-ionic channel complex because the endplate depolarization produced by iontophoretically applied ACh is not suppressed even after 60 min exposure to 100 μM MeHg, by which time evoked and spontaneous release of ACh are abolished completely (Atchison and Narahashi, 1982). Also, MEPP amplitude is not affected at the time that the nerve-evoked EPP is blocked by MeHg (Juang, 1976; Atchison and Narahashi, 1982). Changes in MEPP frequency, such as those induced by MeHg, are known to occur as a result of presynaptic and not postsynaptic events. Taken together, the above findings indicate that the effects of MeHg on neuromuscular transmission appear to be directed primarily towards presynaptic elements. However, depletion of releasable stores of ACh cannot be considered a possible presynaptic mechanism responsible for the ultimate block of transmitter release since treatment with La^{3+} after initial suppression of spontaneous quantal release by MeHg results in restoration of MEPPs (Atchison, 1986) albeit at a frequency lower than that observed following La^{3+} treatment in the absence of MeHg.

Effects of MeHg on synchronous evoked release and spontaneous quantal release of ACh occur with different time courses, suggesting that they may occur by different mechanisms. EPP amplitude is decreased within 5 min of initiating MeHg treatment (Atchison and Narahashi, 1982; Atchison et al., 1984) and increased MEPP frequency does not occur until at least 30-60 min after beginning treatment, depending on the concentration of MeHg applied (Juang, 1976; Atchison and Narahashi, 1982). Thus, the effects of MeHg on spontaneous release occur well after nerve-evoked release is blocked. MeHg-induced block of EPPs

occurs suddenly and without significant decrement of EPP amplitude (Traxinger and Atchison, 1987). This is consistent with block of impulse conduction into the nerve terminal or perhaps block of divalent cation influx. During K^+-induced depolarization, MeHg irreversibly blocks entry of $^{45}Ca^{2+}$ into isolated nerve terminals (synaptosomes) derived from rat forebrain (Atchison et al., 1986; Shafer and Atchison, 1989). MeHg may interact with voltage-dependent Ca^{2+} channels to inhibit Ca^{2+} influx into nerve terminals (Shafer and Atchison, 1989). This could explain, at least in part, the rapid, irreversible block of synchronous evoked release caused by MeHg (Juang, 1976; Atchison and Narahashi, 1982).

The mechanism by which MeHg ultimately increases spontaneous release of ACh is unknown. MeHg may stimulate spontaneous discharge of ACh quanta by elevating intracellular Ca^{2+}. Techniques which result in elevated free-Ca^{2+} concentrations in the presynaptic nerve terminal have been shown to increase MEPP frequency (Liley, 1956; Miledi, 1973; Kita and Van der Kloot, 1976). Agents suspected of increasing intracellular Ca^{2+} concentrations such as ruthenium red (Alnaes and Rahamimofff, 1975), dinitrophenol (Kraatz and Trautwein, 1957), warfarin (Rahamimoff and Alnaes, 1973), tetraphenylboron (Marshall and Parsons, 1975), and cardiac glycosides (Elmqvist and Feldman, 1965; Baker and Crawford, 1975) also increase MEPP frequency markedly. MeHg increases MEPP frequency in the absence of extracellular Ca^{2+} or in Ca^{2+}-deficient preparations although not to as great an extent as in the presence of Ca^{2+} (Atchison, 1986). MeHg also increases Ca^{2+} in isolated nerve terminals in the absence of external Ca^{2+} (Komulainen and Bondy, 1987a). Thus, if MeHg-induced stimulation of transmitter release is due to elevation of intracellular Ca^{2+}, the source of this Ca^{2+} may be an

intracellular store. MeHg may interact with one or more of these Ca^{2+} storage sites to induce release of bound Ca^{2+} into the nerve terminal cytoplasm, resulting ultimately in stimulated release of neurotransmitter.

In order for MeHg to stimulate MEPP frequency subsequent to an interaction with an intraterminal Ca^{2+} store, MeHg would have to penetrate the plasma membrane and enter the nerve terminal cytoplasm. Whether MeHg enters the nerve terminal has not been shown directly; however, a growing body of evidence indirectly suggests that MeHg may indeed enter the nerve terminal. It is possible that the aliphatic side chain may confer sufficient lipophilicity upon the molecule to permit its entry through the cell membrane via passive diffusion (Lakowicz and Anderson, 1980). There is also evidence which suggests that MeHg may enter through ionic channels in the axon terminal membrane (Atchison, 1986; Atchison, 1987). The latent period prior to the MeHg-induced increase in MEPP frequency can be shortened considerably by depolarizing the nerve terminal with high extracellular K^+. This effect is not due solely to depolarization-induced entry of Ca^{2+} through voltage-dependent Ca^{2+} channels since the latency is also shortened by K^+ depolarization in Ca^{2+}-deficient solutions. The MeHg-induced increase in MEPP frequency is also shortened significantly by direct activation of Ca^{2+} channels with Bay K 8644, even in Ca^{2+}-deficient solutions (Atchison, 1987). This suggests that MeHg may enter the nerve terminal through existing transmembrane Ca^{2+} channels.

3. Nerve Terminal Ca^{2+} Regulation

Neurotransmitter release from the nerve terminal occurs following a transient increase in intracellular Ca^{2+} (Llinas and Nicholson, 1975; Llinas et al., 1981). A recently reported value for the concentration of free Ca^{2+} in polarized brain

synaptosomes of 370 nM was obtained using the fluorescent Ca^{2+} indicator fura-2 (Komulainen and Bondy, 1987b). This level of free Ca^{2+} is far lower than extracellular levels, which generally exceed 1 mM. Most of the Ca^{2+} responsible for triggering release of transmitter enters the presynaptic nerve terminal by moving down this steep concentration gradient through voltage-dependent plasmalemmal Ca^{2+} channels opened by nerve terminal depolarization (Baker et al., 1971). The elevated intraterminal Ca^{2+} concentration gives rise to transmitter release which decays rapidly, within about 2 msec after membrane repolarization (Katz and Miledi, 1968). The Ca^{2+} that enters during periods of neuronal activity must subsequently be extruded against the electrochemical gradient, in order for the terminal to return to the resting steady state condition. Extrusion of Ca^{2+} from the axon terminal occurs relatively slowly, with a time constant on the order of seconds or minutes (Blaustein and Ector, 1976; Blaustein et al., 1978). Thus, there are intraterminal buffering mechanisms that play a crucial role in rapidly lowering cytosolic Ca^{2+}, immediately following a period of neuronal activity (Figure 4) (Blaustein et al., 1978). As plasma membrane extrusion mechanisms gradually lower the level of intraterminal Ca^{2+}, bound Ca^{2+} is slowly released from intraterminal storage sites. This Ca^{2+} is also extruded until the normal resting Ca^{2+} concentration is reestablished (Blaustein et al., 1978). So, the excess Ca^{2+} is only transiently redistributed or buffered within the nerve terminal until it is finally extruded from the nerve terminal cytoplasm. The ultimate extrusion of Ca^{2+} from the nerve terminal probably involves a Na^{+}-Ca^{2+} exchange mechanism in the plasmalemma (Blaustein and Ector, 1976; Blaustein et al., 1980; Nicholls, 1986; Carafoli, 1988) and an

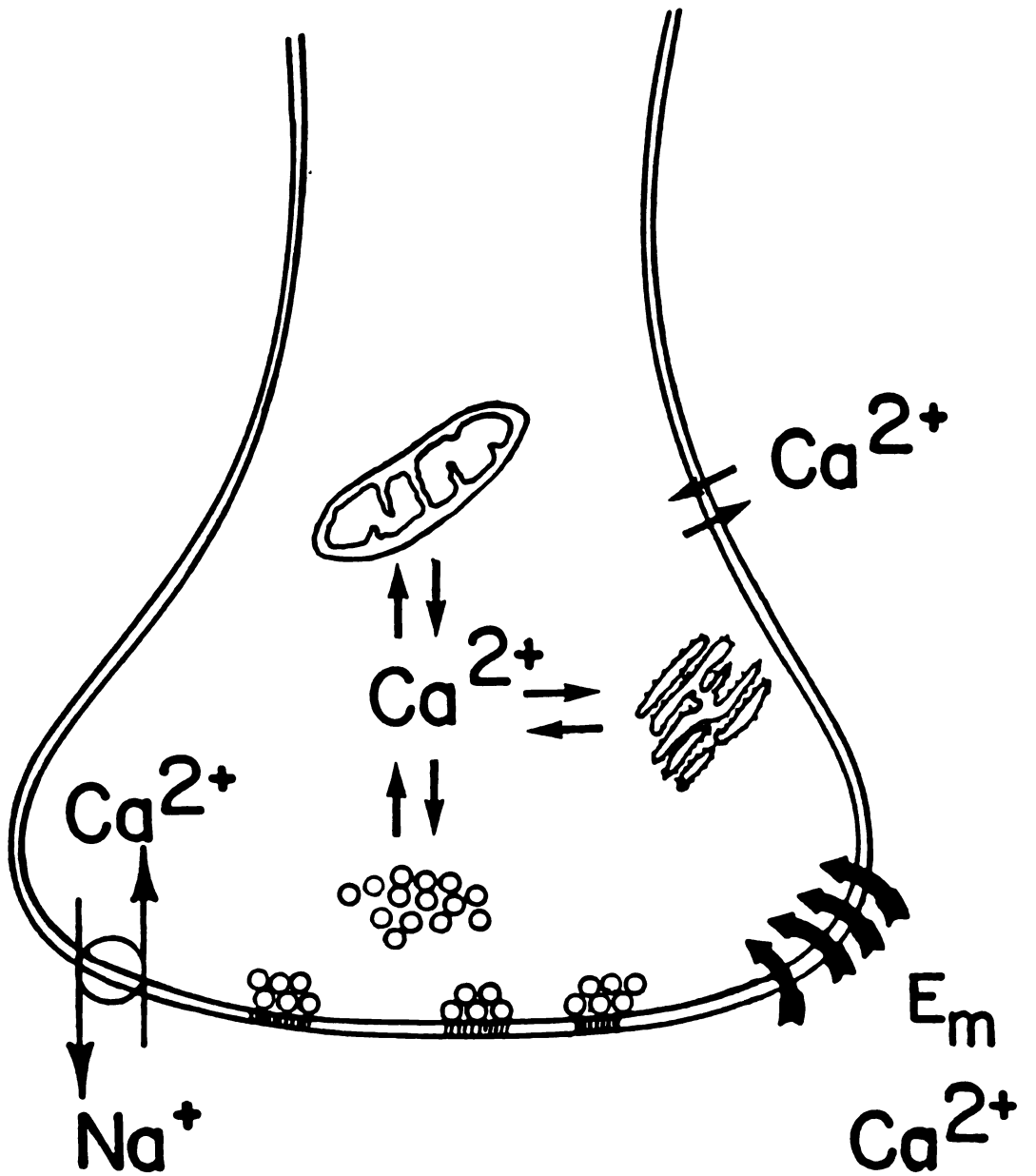

Figure 4. Regulation of free intracellular Ca^{2+} concentrations at the presynaptic nerve terminal. Both influx and efflux mechanisms and residual Ca^{2+} concentrations contribute to the intraterminal Ca^{2+} concentration. Following an action potential, influx occurs through voltage-sensitive (E_m) membrane ionic channels. Resting intraterminal Ca^{2+} is maintained by mitochondria, smooth endoplasmic reticulum and perhaps by synaptic vesicles and calcium binding proteins which temporarily store excess Ca^{2+}. Excess Ca^{2+} is eventually extruded from the nerve terminal by a Na^{+}/Ca^{2+} exchange system and a Ca^{2+} pump. (from Atchison, 1988).

energy-dependent Ca^{2+} pump. The primary source of energy for the exchange process is thought to be the Na^{+} gradient across the plasma membrane, while activity of the Ca^{2+} pump is thought to depend on ATP.

One major site of Ca^{2+} buffering within nerve terminals whose importance is widely recognized is the mitochondrion (Blaustein et al., 1978; 1980). Presynaptic nerve terminals contain many mitochondria; these organelles occupy 6-7% of the volume of motor nerve terminals (Alnaes and Rahamimoff, 1975). The importance of mitochondria

in buffering intracellular Ca^{2+} is widely recognized. Mitochondria accumulate Ca^{2+} at the expense of energy derived from either electron transport or ATP hydrolysis (Lehninger, 1970). Mitochondria have a lower affinity for accumulating Ca^{2+} than other nonmitochondrial Ca^{2+} storage sites but the capacity of mitochondria for storing Ca^{2+} is 10 times greater than other storage sites. (Blaustein et al., 1978). The conclusion that mitochondria play an important role in nerve terminal Ca^{2+} sequestration resulted from studies which showed that mitochondrial poisons markedly enhanced spontaneous release of transmitter (Kraatz and Trautwein, 1957; Glagoleva et al., 1970; Rahamimoff and Alnaes, 1973 ; Alnaes and Rahamimoff, 1975). This effect is due presumably to block of uptake or evoked release of sequestered Ca^{2+} from mitochondria and a subsequent increase in free intraterminal Ca^{2+}.

The specific mechanisms underlying transport of Ca^{2+} by the mitochondrion have been studied in detail (for review, see Carafoli, 1982). Uptake of Ca^{2+} by mitochondria is energy dependent (Vasington and Murphy, 1962) and is driven by a transmembrane electrical gradient (Rottenberg and Scarpa, 1974; Heaton and

Nicholls, 1976). Electron carriers of the respiratory chain actively transport or pump H^+ ions from the mitochondrial matrix into the cell cytosol. The inner membrane of the mitochondrion is otherwise impermeable to H^+ ions and this creates an electrical gradient across the inner membrane of approximately 150 mV with a net negative charge on the inside (Mitchell and Moyle, 1969; Nicholls, 1974; Rottenberg, 1975). The Ca^{2+} uptake kinetics suggest that the uptake reaction is mediated by a specific carrier (Lehninger and Carafoli, 1969). Ca^{2+} influx is now believed to be mediated by an uptake uniport protein driven by the electrical gradient (Carafoli, 1982). Several groups have isolated what they propose to be the uptake uniport protein from the inner mitochondrial membrane (Sottocasa et al., 1972; Carafoli and

Sottocasa, 1974; Jeng and Shamoo, 1980). Inhibitors of the uniporter include ruthenium red (Moore, 1971; Vasington et al., 1972), which is a hexavalent polysaccharide stain (Luft, 1971), and lanthanum and other cations of the lanthanide series (Mela, 1968, 1969; Reed and Bygrave, 1974). In order to maintain the transmembrane potential, extensive Ca^{2+} uptake can only occur in the presence of anions which can permeate the inner membrane. Such anions are bicarbonate, hydroxybutyrate, glutamate and phosphate which can be transported along with Ca^{2+} into the mitochondrion (Lehninger et al., 1963; Brand et al., 1976; Debise et al., 1978; Elder and Lehninger, 1973; Harris, 1978). When phosphate accompanies Ca^{2+} into the mitochondrial matrix, very large quantities of Ca^{2+} can be sequestered (Rossi and Lehninger, 1963). During "massive" loading of mitochondria with Ca^{2+}, Ca^{2+} and phosphate precipitate inside mitochondria and can be visualized with the electron microscope as electron-dense masses within

the matrix space (Greenawalt et al., 1964).

Release of Ca^{2+} by the mitochondria may occur by several pathways. First, extensive Ca^{2+} extrusion occurs when the mitochondrial membrane potential is disrupted (for discussion, see Carafoli, 1982a; Nicholls and Akerman, 1982; Akerman and Nicholls,

1983). Ca^{2+} efflux induced by lowering of the membrane potential can be attributed, in part, to reversal of the uptake uniporter (Akerman, 1978; Scarpa and Azzone, 1970; Wikstrom and Saari, 1976; Gunter et al., 1978). After disruption of the membrane potential, the net Ca^{2+} efflux observed is the sum of the activities of the reversed uniporter and of an independent Ca^{2+} efflux pathway (Rossi et al., 1973; Carafoli and Crompton, 1976; Puskin et al., 1976). The latter pathway functions continuously, even in the presence of a maintained membrane potential (Nicholls, 1978). The ongoing efflux that is independent of the transmembrane potential, is believed to counterbalance the energy-driven uptake of Ca^{2+} into mitochondria (Carafoli, 1979). This separate Ca^{2+} efflux pathway is thought to involve a specific Na^{+}-Ca^{2+} exchange mechanism (Carafoli et al., 1974; Crompton et al., l976a,b; Nicholls, 1978). Exposure of energized mitochondria to Na^{+}-containing solutions induces a rapid efflux of Ca^{2+} which is directly proportional to the concentration of Na^{+} added (Carafoli, l974).

It has been proposed that the influx and efflux mechanisms function in unison to maintain intracellular Ca^{2+} homeostasis. The efflux rate is essentially constant while the activity of the influx uniporter depends on the free Ca^{2+} concentration in the cytosol (Carafoli, 1982a). Thus, when free Ca^{2+} rises above a set point, increased activity of the uptake uniporter lowers cytosolic free Ca^{2+}.

When intracellular Ca^{2+} falls below the set point, activity of the uniporter will decline and net efflux via Na^{+}-Ca^{2+} exchange will result in elevated intracellular free Ca^{2+}. Mitochondrial uptake and release of Ca^{2+} may also serve to regulate Ca^{2+} levels within the mitochondrial matrix (Carafoli, 1988). Several enzymes within the mitochondrion require precise regulation of intramitochondrial Ca^{2+} to function normally. Moreover, too much Ca^{2+} within the mitochondrion is toxic to the organelle.

The smooth endoplasmic reticulum (SER) is the other intraterminal organelle that is believed to play an important role in Ca^{2+} sequestration. SER occupy about 1.8% of the total volume of synaptosomes isolated from mammalian brain (McGraw et al., 1980). SER have a much higher affinity for Ca^{2+} but a far lower capacity for storing or binding Ca^{2+} than do mitochondria. Ca^{2+} uptake depends on ATP (Blaustein et al., 1978; 1980; McGraw et al., 1980). Unlike mitochondrial ATP-dependent Ca^{2+} uptake, Ca^{2+} buffering by SER is not affected by uncouplers of oxidative phosphorylation (McGraw et al., 1980). Little is known about the specific mechanisms underlying Ca^{2+} transport by the SER; however, cellular mechanisms underlying Ca^{2+} transport by muscle sarcoplasmic reticulum (SR), have been well characterized (for review, see Carafoli, 1982a). Nerve terminal SER is morphologically very similar to muscle SR (Henkart et al., 1976; McGraw et al., 1980) and the kinetic properties of Ca^{2+} transport by the two organelles are also very similar (Blaustein et al., 1978). Perhaps the cellular mechanisms underlying Ca^{2+} transport by SER and SR are analogous. Uptake of Ca^{2+} by SR is driven by an ATP-dependent Ca^{2+} pump and release occurs via membrane ionic channels (Carafoli, 1988).

At Ca^{2+} concentrations approaching 1 μM, most free intraterminal Ca^{2+} is taken up by mitochondria; or, at lower Ca^{2+} levels of about 0.3 μM, mitochondria show little uptake activity, and most of the Ca^{2+} is taken up by the SER (McGraw et al., 1980). It has been suggested therefore, that SER plays the predominant role in buffering intraterminal Ca^{2+} under normal physiological conditions. Mitochondria probably sequester Ca^{2+} when nerve terminal depolarization is maintained or during pathological conditions in which large amounts of Ca^{2+} may enter the axon terminal (Blaustein, 1980). Perhaps the most important function of the mitochondrion is to phosphorylate ADP using energy derived from electron transport (Rossi and Lehninger, 1964; Drahota et al., 1965). When intraterminal Ca^{2+} becomes sufficiently elevated, mitochondria utilize their energy to sequester Ca^{2+} rather than to phosphorylate ADP. Thus, intraterminal Ca^{2+} may normally be buffered at a level sufficiently low so that mitochondria can utilize most of the energy available from electron transport to phosphorylate ADP. Since SER have a higher affinity for Ca^{2+} and are located in close proximity to mitochondria (McGraw et al., 1980), perhaps the SER acts as a "screen" for the mitochondria, blunting fluctuations in Ca^{2+} before mitochondria are affected. In this way, the SER would protect mitochondria from Ca^{2+} overload.

4. Possible Mechanisms of Interaction of MeHg with Mitochondria and Smooth Endoplasmic Reticulum

MeHg-induced increases in MEPP frequency occur in preparations treated with Ca^{2+}-deficient solutions (Atchison, 1986), implying that MeHg acts on intracellular stores of bound Ca^{2+}, perhaps to evoke its release and/or block its uptake. The subsequent increase in free ionized cytosolic Ca^{2+}, presumably

underlies the increased discharge of ACh, observed electrophysiologically as increased MEPP frequency (Llinas and Nicholson, 1975). Biochemical and electron-microscopic localization studies have revealed that mercury binds intracellularly to membranous structures including the two major organelles involved in Ca^{2+} regulation: mitochondria and SER (Chang and Hartmann, 1972a; Chang, 1977). This primary association of mercury with mitochondria and SER suggests that mercury may alter normal functioning of either of these organelles including disruption of their ability to sequester Ca^{2+}.

Mitochondria are considered a likely site for MeHg-induced disruption of nerve terminal Ca^{2+} regulation for several reasons. First, they are the most abundant Ca^{2+} sequestering organelle in the presynaptic nerve terminal; they have the largest capacity for storing Ca^{2+} and are involved in buffering Ca^{2+} in a variety of cell types (Carafoli, 1982). Second, mitochondrial inhibitors, such as dinitrophenol, ruthenium red and dicoumarol have effects similar to those of MeHg on spontaneous transmitter release (Blioch et al., 1968; Rahamimoff and Alnaes, 1973). Third, inorganic and organomercurials inhibit respiration (Norseth and Brendeford, 1971; Magnaval et al., 1975 ; Sone et al., 1977), ATP production (Sone et al., 1977), and Ca^{2+} uptake in isolated mitochondria (Binah et al., 1978). Inhibition of these processes would disrupt the normal mitochondrial membrane potential and could result in efflux of Ca^{2+} from mitochondria. If this were to occur in nerve terminal mitochondria, the elevated intraterminal Ca^{2+} could stimulate spontaneous release of ACh.

Evidence suggesting that MeHg can act on nerve terminal mitochondria arose from experiments in which neonatal rats were injected subcutaneously with

MeHg (O'Kusky, 1983). Ultrastructural changes were observed in mitochondria from presynaptic terminals of cortical neurons in MeHg-treated rats. The pathological changes seen in the neonates were attributed to adverse effects of MeHg on mitochondria.

There is little doubt that MeHg deleteriously affects normal mitochondrial functioning. MeHg induces ultrastructural changes in mitochondria (Yoshino et al., 1966 a,b; Norseth, 1969; Desnoyers and Chang, 1975) which are consistent with inhibition of respiration (Trump and Arstilla, 1971). Several other groups have obtained evidence suggesting that MeHg inhibits mitochondrial respiration (Norseth and Brendeford, 1971, 1975; Magnaval et al., 1975; Verity et al., 1975; Fowler and Woods, 1977; Von Burg et al., 1979; O'Kusky, 1983; Ally et al., 1984). Swelling is one frequently observed pathological change which occurs in organomercurial-intoxicated mitochondria. This effect is not due to inhibited respiration (Bogucka and Wojtczak, 1979) but more likely to mercurial-induced potassium accumulation (Brierley et al., 1978; Scott et al., 1970; Southard et al., 1973, 1974; Verity et al., 1975; Sone et al., 1977; Bogucka and Wojtczak, 1979). Increased permeability of the inner mitochondrial membrane to K^+ induced by MeHg may occur secondarily to release of membrane-bound Mg^{2+} from mitochondria (Duszynski and Wojtczak, 1977; Bogucka and Wojtczak, 1979). Removal of endogenous Mg^{2+} from the inner mitochondrial membrane is believed to increase greatly transmembrane, electrophoretic permeability to monovalent cations (Settlemire et al., 1968; Wherle et al., 1976). This effect of mercurials has been postulated (Southard et al., 1974a,b) to lead to unmasking of an endogenous mitochondrial ionophore which renders the inner membrane permeable to cations such as K^+. Other groups (Scott

et al., 1970; Brierley et al., 1978; Harris and Baum, 1980) have suggested that membrane permeability changes are the result of interactions between the organomercurial and membrane-bound thiol groups which may be involved in keeping Mg^{2+} bound to the membrane, in which case its permeability is low (Harris et al., 1979). Inhibition of mitochondria with thiol-specific reagents has been reported to stimulate mitochondrial Ca^{2+} efflux (Harris et al., 1979; Harris and Baum, 1980). Modification of membrane sulfhydryl groups by MeHg may lead to an extensive perturbation of membrane integrity. Regardless of the mechanism, mercurial-induced K^{+} accumulation is thought to collapse the mitochondrial membrane potential, ultimately resulting in inhibited phosphorylation of ADP and decreased ATP production (Paterson and Usher, 1971; Southard et al., 1973; 1974a; Sone et al., 1977; O'Kusky, 1983) in intoxicated mitochondria. Collapse of the electrical potential would result in Ca^{2+} leakage from mitochondria via reversal of the uptake uniporter. Thus, MeHg-induced collapse of the mitochondrial membrane potential would result in increased nerve terminal Ca^{2+}.

The nerve terminal SER is the other major Ca^{2+} sequestering organelle that could be considered a potential source for the putative increase in intraterminal Ca^{2+} induced by MeHg. The SER has a high affinity for Ca^{2+} and sequesters Ca^{2+} at the expense of ATP hydrolysis (Kendrick et al., 1977; Eroglu and Keen, 1977; Blaustein et al., 1978). MeHg could inhibit SER Ca^{2+} uptake by decreasing intraterminal ATP. MeHg has been shown to uncouple oxidative phosphorylation and inhibit ATP synthesis in isolated mitochondria, ultimately resulting in decreased intracellular ATP (Paterson and Usher, 1971; Sone et al., 1977). Another possible mechanism whereby MeHg could evoke release of Ca^{2+} from the

SER could be to increase the permeability of the SER membrane to Ca^{2+}. Inorganic and organomercurials cause rapid release of Ca^{2+} from SR vesicles derived from skeletal muscle (Martinosi et al., 1964; Chiesi and Inesi, 1979; Abramson et al., 1983; Bindoli et al., 1983). Binding of sulfhydryl groups by mercurials causes a dramatic increase in the Ca^{2+} permeability of the SR (Bindoli et al., 1983; Abramson, 1983). Given the close similarity between nerve terminal SER and muscle SR it is possible that MeHg also increases the Ca^{2+} permeabilty of the SER membrane.

E. Research Objectives

The primary goal of this thesis project was to investigate the cellular mechanisms underlying the stimulatory effects of MeHg on spontaneous release of ACh. Since changes in the frequency of spontaneous release of neurotransmitter are strongly related to the free Ca^{2+} concentration in the axon terminal and MeHg stimulates release of ACh in the absence of external Ca^{2+}, the hypothesis proposed is that MeHg disrupts the action of intraterminal Ca^{2+} buffers to store Ca^{2+} leading to increased intracellular Ca^{2+}. In turn, this leads to an increase in spontaneous quantal release of neurotransmitter. Preliminary intracellular microelectrode recording experiments were designed to delineate potential sources of bound intraterminal Ca^{2+} which could be mobilized by MeHg. The specific objective was to test whether inhibitors of Ca^{2+} buffering by mitochondria or SER could alter or block the stimulatory effects of MeHg on spontaneous quantal release of ACh.

Subsequent neurochemical studies which utilized isolated brain mitochondria

were performed to follow up on results of the electrophysiological experiments which provided preliminary evidence supporting the proposal that MeHg interacts with Ca^{2+} sequestration by nerve terminal mitochondria to induce spontaneous release of ACh. The goals of this study were to determine 1) whether MeHg blocks Ca^{2+} uptake into mitochondria; 2) whether MeHg causes release of Ca^{2+} from preloaded mitochondria; 3) whether the effects of MeHg on Ca^{2+} regulation are blocked by treatment with inhibitors of mitochondrial Ca^{2+} transport, and 4) whether MeHg impairs the functional integrity of mitochondria.

Since MeHg has been shown to affect cholinergic neurotransmission at both central and peripheral synapses, experiments utilizing synaptosomes were used to tie together neurochemically the effects of MeHg on spontaneous release of ACh from central nerve terminals and effects on Ca^{2+} buffering. The primary goal was to assess the relative contributions of extracellular Ca^{2+} and nerve terminal mitochondria to the effects of MeHg on spontaneous release of ACh.

In a final study, the binding characteristics of MeHg to synaptosomes and mitochondria were examined using radiolabeled MeHg. The specific objectives were to examine the possible modes of entry of MeHg into nerve endings and to assess the effects of inhibitors of Ca^{2+} transport by mitochondria on binding of MeHg to mitochondria and synaptosomes.

CHAPTER TWO

INTERACTIONS OF MITOCHONDRIAL INHIBITORS WITH METHYLMERCURY ON SPONTANEOUS QUANTAL RELEASE OF ACETYLCHOLINE

ABSTRACT

The interaction of methylmercury (MeHg) with various inhibitors of mitochondrial function (dinitrophenol, 50 μM; dicoumarol, 100 μM; valinomycin, 20 μM; and ruthenium red, 20 μM) on spontaneous quantal release of acetylcholine was tested at the neuromuscular junction of the rat. The objective was to determine whether these mitochondrial inhibitors blocked the MeHg-induced increase of spontaneous release of acetylcholine, an effect measured electrophysiologically as increased miniature endplate potential (MEPP) frequency. MEPPs were recorded from myofibers of the rat hemidiaphragm using conventional, intracellular microelectrode recording techniques. When given alone, all four inhibitors increased MEPP frequency from resting levels of 1-2/sec (Hz) to approximately 10-60 Hz after a latency which ranged from 5-30 min. MEPP frequency subsequently returned to control levels. Subsequent concomitant application of MeHg (100 μM) with dinitrophenol, dicoumarol or valinomycin increased MEPP frequency sharply to peak values of 40-60 Hz after 15-20 min. MEPP frequency subsided to pre-MeHg levels 10 min later. The time course and peak MEPP frequency elicited by MeHg after pretreatment with these uncouplers was similar to results obtained in preparations treated with MeHg alone. Ruthenium red, a putative, specific inhibitor of the Ca^{2+} uptake uniporter in mitochondria, increased MEPP frequency to 12 Hz after 8.5 min when given alone. MEPP frequency returned to control levels approximately 10 min later. Subsequent application of MeHg and ruthenium red for up to 80 min failed to increase MEPP frequency. The inability of MeHg to increase MEPP frequency in ruthenium red-

treated preparations was not due to depletion of acetylcholine nor to block of postjunctional receptors by ruthenium red since subsequent treatment with La^{3+} (2 mM) increased MEPP frequency to 12.5 Hz within 10 min. Thus, ruthenium red blocked the stimulatory effect of MeHg on MEPP frequency while uncouplers of oxidative phosphorylation and a K^+ ionophore did not. The results with ruthenium red are consistent with the proposal that MeHg may block mitochondrial uptake of Ca^{2+} or promote its release leading to an increased free cytosolic Ca^{2+} concentration which in turn stimulates spontaneous release of acetylcholine.

INTRODUCTION

Exposure to methylmercury (MeHg) causes prominent neurotoxic signs, characterized by sensory disturbances, cerebellar ataxia and generalized extremity weakness in exposed individuals (Hunter et al., 1940). Pathological lesions of the central and peripheral nervous systems have been described for MeHg (Chang and Hartmann, 1972a,b) but the cellular mechanisms underlying these lesions are unclear.

Intracellular microelectrode studies of effects of acute bath administration of MeHg on synaptic transmission have revealed that nerve-evoked release of acetylcholine (ACh) is inhibited and spontaneous quantal release of ACh is first increased and then decreased (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982; Miyamoto, 1983; Atchison et al., 1984; Atchison, 1986; Atchison et al., 1986). Changes in spontaneous release of transmitter are measured electrophysiologically as alterations of miniature endplate potential (MEPP) frequency. The MeHg-induced increase in MEPP frequency occurs after an initial latent period (Atchison and Narahashi, 1982; Atchison et al., 1984; Atchison, 1986). Increasing the concentration of MeHg does not increase mean peak frequency of MEPPs, however, it does shorten the latency to onset (Atchison and Narahashi, 1982). This latent period may reflect the time required for MeHg to enter the presynaptic nerve terminal and stimulate transmitter release. Thus, increasing the concentration of MeHg would be expected to hasten its entrance into the cell by increasing the chemical driving force. Once inside the cell MeHg may stimulate spontaneous discharge of ACh quanta by elevating intracellular Ca^{2+}. Techniques

which result in elevated free-Ca^{2+} concentrations in the presynaptic nerve terminal have been shown to increase MEPP frequency (Liley, 1956b; Miledi, 1973; Kita and Van der Kloot, 1976). Agents suspected of increasing intracellular Ca^{2+} concentrations such as ruthenium red (Alnaes and Rahamimoff, 1975), dinitrophenol (Kraatz and Trautwein, 1957), warfarin (Rahamimoff and Alnaes, 1973), tetraphenylboron (Marshall and Parsons, 1975), and cardiac glycosides (Elmqvist and Feldman, 1965; Baker and Crawford, 1975) also increase MEPP frequency markedly.

MeHg increases MEPP frequency in the absence of extracellular Ca^{2+} or in Ca^{2+}-deficient preparations (Atchison, 1986). Thus, if MeHg-induced stimulation of transmitter release is due to elevation of intracellular Ca^{2+}, the source of this Ca^{2+} may be an intracellular store. Buffering of calcium within the axon terminal is thought to be under control of mitochondria, smooth endoplasmic reticulum and perhaps synaptic vesicles (Blaustein et al., 1977, 1978a,b). It is known that mitochondria in particular can store large quantities of Ca^{2+} (Lehninger et al., 1963; Lehninger, 1970). Perhaps MeHg enters the presynaptic nerve terminal where it disrupts normal mitochondrial function resulting in leakage of Ca^{2+} into the extramitochondrial space. To test this possibility, we performed experiments to determine whether dinitrophenol (DNP), dicoumarol (DC), valinomycin (VAL) and ruthenium red (RR), all of which inhibit normal buffering of Ca^{2+} by mitochondria, could block the effects of MeHg on spontaneous transmitter release. Like MeHg, each of these inhibitors causes a biphasic, time-dependent increase and concomitant decrease in MEPP frequency (Glagoleva et al., 1970; Rahamimoff and Alnaes, 1973; Alnaes and Rahamimoff, 1975). Stimulation of MEPP frequency is

presumed to result from inhibitor-induced release of Ca^{2+} from mitochondria into the terminal cytosol. DC and DNP, which uncouple oxidative phosphorylation, inhibit mitochondrial Ca^{2+} uptake by acting as proton ionophores. As a result, Ca^{2+} is released from this organelle following collapse of the mitochondrial membrane potential (Carafoli, 1967; Lehninger et al., 1967). RR specifically inhibits the so-called Ca^{2+} influx "uniporter" protein in the mitochondrial membrane (Moore, 1971). Specific Ca^{2+} efflux pathways are insensitive to RR thus there is a net efflux of Ca^{2+} from mitochondria (Moore, 1971; Vasington et al., 1972; Carafoli, 1982a,b). The resulting increase in cytosolic Ca^{2+} is presumed to cause transmitter release. VAL is a K^+-selective ionophore which causes influx of K^+ into mitochondria, uncoupling of oxidative phosphorylation and eventual cell depolarization (Moore and Pressman, 1964; Chappell and Crofts, 1965; Scarpa and Azzone, 1970; Pressman, 1973).

METHODS

Preparation and solutions. All experiments utilized the isolated hemidiaphragm (Bulbring, 1946) of male rats (175-225 g, Harlan Sprague-Dawley) and conventional microelectrode recording techniques. Preparations were pinned out in a sylgard-coated, plexiglas chamber and superfused continuously with a physiological saline solution modified from that described by Liley (1956a). This solution consisted of (mM): NaCl, 135; $CaCl_2$, 2; KCl, 5; MgCl, 1; glucose, 11; and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 14. Solutions were aerated continuously with 100% O_2 throughout all experiments. All solutions were adjusted to pH 7.4. Experiments were performed at room temperature of 23-26°C. Separate hemidiaphragm preparations were used for each individual experiment. All experiments were replicated in a minimum of four animals with each animal serving as its own control.

Intracellular recording. Intracellular recordings of miniature endplate potentials (MEPPs) were made using conventional techniques with borosilicate glass microelectrodes (10-20 M resistance) filled with 3 M KCl. MEPPs were amplified (M707, WP Instruments, Hartford, CT) and displayed on an oscilloscope (2090, Nicolet Instruments, Verona, WI) and recorded simultaneously on magnetic tape using an FM instrumentation tape recorder (Model B, Vetter Instruments, Rebersburg, PA) for later analysis. MEPP frequency and amplitude were determined by manual measurements from chart records of the taped data made with a Gould 2200 chart recorder.

The appropriate concentrations of each of the mitochondrial inhibitors for

this preparation had to be determined in separate experiments. Effects of several concentrations of each inhibitor on MEPP frequency were tested in the diaphragm. Different hemidiaphragms were used for each concentration of inhibitor. The final concentration selected for an inhibitor was one which did not cause rapid depolarization of the postsynaptic membrane potential or cessation of MEPPs. After selecting inhibitor concentrations, an identical protocol was followed for all four inhibitors tested. Experiments in which the membrane potential depolarized to below -50 mV in the presence of a mitochondrial inhibitor were terminated and deemed invalid. This minimized the possibility of stimulation of MEPP frequency due to large changes in nerve terminal membrane potential (Liley, 1956b) due to inhibitor-induced depolarization. While maintaining impalement of the same cell, the perfusion medium was switched to one containing the inhibitor being tested. Superfusion with this solution was continued either until the effects of the inhibitor on MEPP frequency declined and became constant or until MEPPs disappeared altogether. While maintaining impalement of the same cell, the perfusion solution was then switched to one which contained both the inhibitor and MeHg.

Materials. Methylmercuric acetate was obtained from Pfaltz-Bauer Chemical Co. (Stamford, CN). Stock solutions of MeHg (2 mM) were made by dissolving the salt in 4% (v/v) glacial acetic acid. Test solutions were made from dilutions of the stock solution. The concentration of MeHg used in all of the experiments was 100 μM. This concentration has been found to increase MEPP frequency to identical peak levels as lesser concentrations (20 μM) that are closer to blood levels attained during intoxication episodes but the higher concentration shortens an otherwise lengthy latent period to onset of MEPP stimulation (Atchison and Narahashi, 1982).

HEPES was obtained from the United States Biochemical Co. (Cleveland, Ohio). 2,4-dinitrophenol, dicoumarol, ruthenium red and lanthanum chloride were obtained from Sigma Chemical Co. (St. Louis, Mo.). Valinomycin was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Animals. Prior to experimentation, animals were housed in plastic cages in a room which received 12 hr of light per day. Room temperature was maintained at 22 to 24°C and relative humidity at 40 to 60%. Food (Purina Rat Chow) and water were provided ad libitum.

Statistical Analysis. Statistical analysis of the effects of MeHg in conjunction with DC, DNP and VAL data was performed by a one-way analysis of variance (ANOVA) (Steel and Torrie, 1960). Significance was set at $p \leq 0.05$.

RESULTS

All of the inhibitors tested (DC, DNP, VAL, RR) produced significant increases in MEPP frequency when added alone to the hemidiaphragm perfusion solution (Table 1). Control MEPP frequency in all experiments was between 1-2 Hz. The time course and degree of stimulation of ACh release varied among the different types of inhibitors. Because both DC and DNP inhibit mitochondrial Ca^{2+} buffering by uncoupling oxidative phosphorylation, they were expected to produce similar effects on transmitter release. Indeed, the time of onset, time to peak increase in MEPP frequency and peak MEPP frequencies were similar for these inhibitors.

The third inhibitor tested was VAL. This antibiotic selectively increases the K^+ permeability of biological membranes including mitochondrial membranes (Moore and Pressman, 1964) and causes mitochondria to take up K^+ from the cell cytosol where the concentration of this ion is higher. As a result of the K^+ accumulation, oxidative phosphorylation is uncoupled and depolarization of the mitochondrial membrane occurs. Although the inhibitory mechanism of VAL differs from that of DNP and DC, mitochondrial Ca^{2+} buffering is disrupted by the uncoupling effect of all three of these inhibitors. Therefore, it was not surprising to find that the time course of the stimulatory effects on MEPP frequency for VAL were quite similar to those of DC and DNP.

The final inhibitor tested was the mucopolysaccharide dye RR. RR specifically inhibits the Ca^{2+} uptake uniporter in the mitochondrial membrane (Moore, 1971; Vasington et al., 1972). When the uptake uniporter is blocked by

RR, net Ca^{2+} efflux occurs (Rossi et al., 1973) via a distinct efflux pathway which functions in the presence of a maintained membrane potential (Puskin et al., 1976; Nicholls, 1978). Thus, the mechanism by which RR blocks Ca^{2+} influx into the mitochondria differs from that of the uncouplers and VAL. The data show that the stimulation of MEPP frequency by RR differs from that of the uncouplers. There is a very short latent period (3.5 min) which precedes the onset of RR-induced stimulation of MEPP frequency. Peak MEPP frequencies were obtained only 7-10 min after treatment with RR, and the entire biphasic effect was completed within about 20 min.

Pretreatment with DC or DNP had little effect on the stimulation of MEPP frequency by MeHg (Figure 1). In both cases, MeHg increased MEPP frequency approximately 15-20 min (16.3 ± 6 min for DC and 18.6 ± 4.2 min for DNP) after its introduction into the perfusion medium. MEPP frequency reached a peak shortly after the onset and then declined back towards control levels. Peak MEPP frequencies with MeHg after DNP and DC were 53.5 ± 9.8 Hz (mean ± SEM, n=4) and 61 ± 8.9 Hz (n=4), respectively. Peak MEPP frequency with MeHg in combination with DNP and DC occurred after 25 ± 5.3 min (n=4) and 27.4 ± 6.7 min (n=4), respectively. The time course of MeHg-stimulated transmitter release, following pretreatment with either DC or DNP, was very similar to the time course observed in experiments with MeHg in which there was no pretreatment with inhibitor (Atchison and Narahashi, 1982).

As with the uncouplers, pretreatment with VAL did not block MeHg-induced stimulation of MEPP frequency (Figure 2). There was a much shorter latent period for the onset of action of MeHg following pretreatment of the preparation with VAL

(9.1 ± 1.4 min). MEPP frequency rose more gradually after VAL pretreatment than after pretreatment with DC or DNP. Peak MEPP frequency after treatment with MeHg and VAL occurred after 23.3 ± 3.9 min, and was 42.2 ± 6.9 Hz (n=5) as compared to 54 and 61 Hz with MeHg in combination with DNP and DC, respectively. It was nearly impossible to maintain impalement of the same cells long enough in VAL plus MeHg to observe the usual decline in MEPP frequency which follows initial stimulation, however, in several experiments, MEPP frequency had begun to decline while impalements were maintained.

Statistical analysis of the comparative effects of MeHg on MEPP frequency after pretreatment with DNP, DC and VAL revealed no significant differences ($p<0.05$) in time of onset, time to peak or peak MEPP frequency after pretreatment with these inhibitors. This may indicate that these inhibitors affect mitochondrial Ca^{2+} buffering in a manner which has no bearing on the mechanism whereby MeHg stimulates MEPP frequency. It is also possible that all of these inhibitors may have similar effects on the mitochondria which alter MeHg stimulation of MEPP frequency to the same extent.

Unlike any of the above inhibitors, pretreatment with RR completely blocked MeHg-induced stimulation of MEPP frequency (Figure 3). Impalement of the same cells was maintained for as long as 80 min after initiating superfusion with MeHg and RR and yet no increase in MEPP frequency was observed. It was possible that RR may have been blocking junctional transmission at the postsynaptic membrane by blocking the ACh receptor/channel complex or at the presynaptic nerve ending by blocking ACh release. It this were the case, a potential subsequent increase in transmitter release by MeHg would be blocked by

functional antagonism. In attempts to rule out either postsynaptic block or transmitter depletion by RR, we tested the effects of lanthanum on transmitter release following pretreatment with RR (Figure 4). La^{3+} causes a massive release of any available transmitter stores from the presynaptic terminal (Heuser and Miledi, 1971). In every case, La^{3+} induced a rapid increase in MEPP frequency in preparations in which MEPP frequency was suppressed by RR. Thus, RR was not blocking postsynaptic receptors for ACh nor depleting transmitter stores.

Our results indicate that MeHg-induced Ca^{2+} release is RR-sensitive. Uncoupler-induced Ca^{2+} release from mitochondria has been found by some investigators to be insensitive to RR (Vasington et al., 1972; Puskin et al., 1976; Pozzan et al., 1977). The differences in RR sensitivity may be due to the existence of different Ca^{2+} pools in the mitochondria or different release pathways. The inability of MeHg to induce release of ACh in RR pretreated preparations prompted us to wonder whether RR-sensitive and insensitive pathways might also be found in nerve terminal mitochondria. To test this we tested the effects of RR on DNP-induced release. As shown in Figure 5, pretreatment of the hemidiaphragm with RR also prevented the increase in MEPP frequency that would otherwise occur upon subsequent perfusion of the hemidiaphragm with DNP. The typical biphasic increase and decrease in MEPP frequency produced by RR was observed but there were no significant increases in frequency attributable to the addition of DNP to the perfusion medium for up to 30 min after the addition of this uncoupler. Again, RR was not blocking junctional transmission since subsequent treatment with La^{3+} in the presence of RR resulted in increased MEPP frequency after about 3 min (results not shown). The absence of any detectable stimulation of MEPP

frequency by DNP after treatment with RR is not due to a presynaptic exhaustion of releasable transmitter stores nor to postsynaptic block by RR, as demonstrated by the effect of La^{3+} after RR.

TABLE 1

Effect of Mitochondrial Inhibitors on Spontaneous MEPP Frequency

Inhibitor[a]	Time of Onset of[b] Increased MEPP Frequency (min)	Time to Peak Increase in MEPP Frequency (min)	Peak Frequency (Hz)
dicoumarol	23.8±2.0[c]	33.8±6.0	63.3±6.7
dinitrophenol	18.8±3.2	30.6±3.7	58.0±9.5
valinomycin	15.0±1.8	33.8±3.2	52.0±8.5
ruthenium red	3.5±0.6	8.5±1.1	11.9±1.4

[a]Concentrations of inhibitors were as follows: dicoumarol, 100 μM; dinitrophenol, 50 μM; valinomycin, 20 μM; ruthenium red, 20 μM.

[b]This refers to the interval from starting superfusion with the inhibitor to increased MEPP frequency.

[c]Values for dicoumarol, dinitrophenol, valinomycin and ruthenium red are the mean ± SEM of 4, 4, 6 and 13 determinations, respectively.

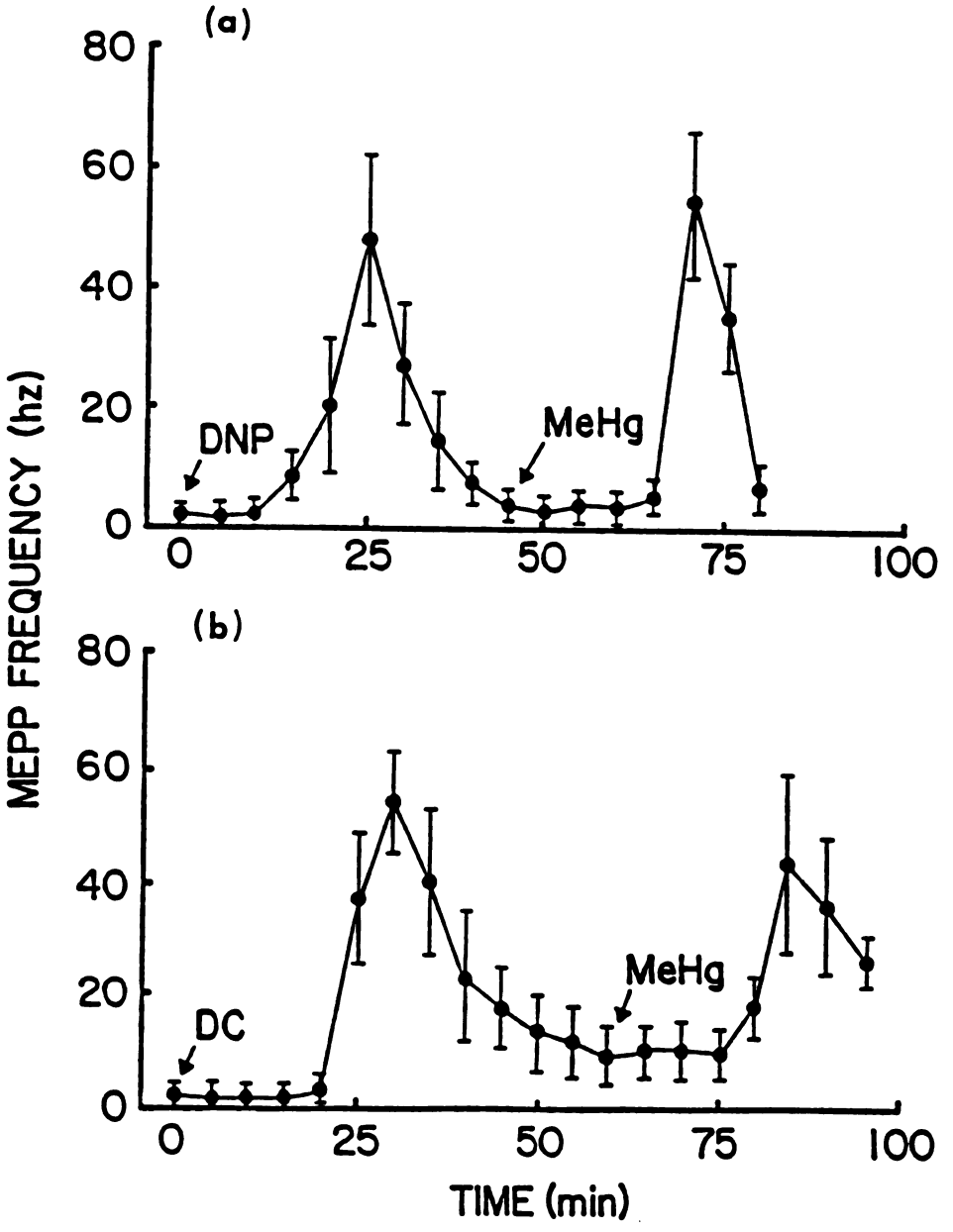

Figure 1. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with (a) dinitrophenol (DNP, 50 μM) and (b) dicoumarol (DC, 100 μM). DC or DNP was applied at time zero. MEPP frequency was allowed to return towards prestimulation levels before the preparation was superfused with MeHg in conjunction with DC or DNP. MeHg was added at times indicated by arrows. MEPP frequency was determined in 5-min increments. Values for DNP and DC are the mean ± SEM of 4 preparations.

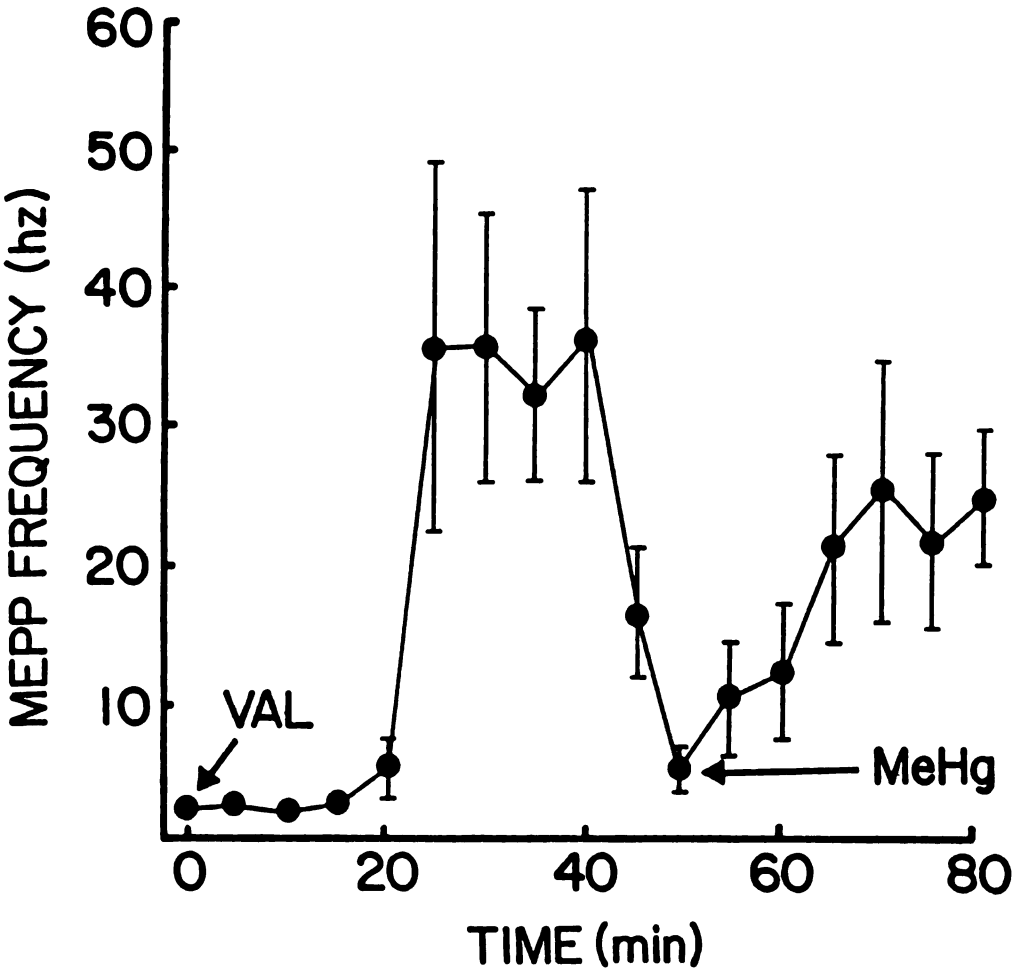

Figure 2. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with valinomycin (VAL, 20 μM). VAL was applied at time zero. As MEPP frequency returned towards pre-stimulation levels, the preparation was superfused with MeHg in conjunction with VAL at the time indicated by the arrow. MEPP frequency was determined in 5 min increments. Values are the mean ± SEM of 6 preparations.

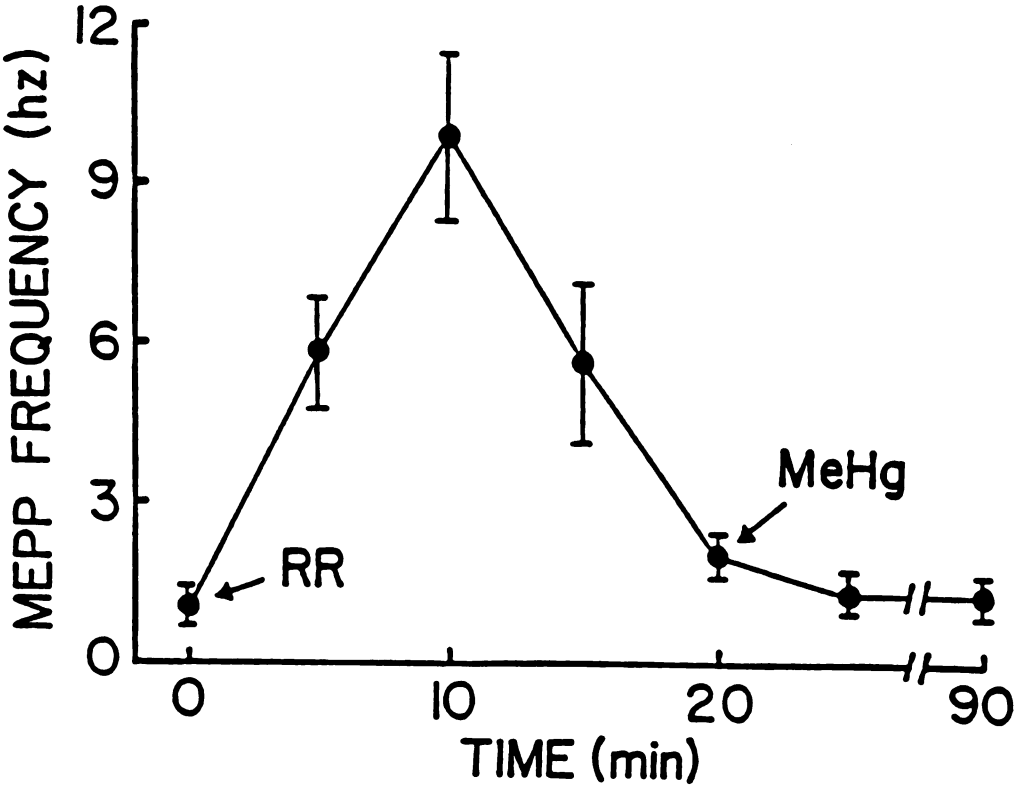

Figure 3. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with ruthenium red (RR, 20 μM). RR was applied at time zero. As MEPP frequency returned towards pre-stimulation levels, the preparation was superfused with MeHg in conjunction with RR at the time indicated by the arrow. MEPP frequency was determined in increments of 2.5 min. Values are the mean ± SEM of 8 preparations.

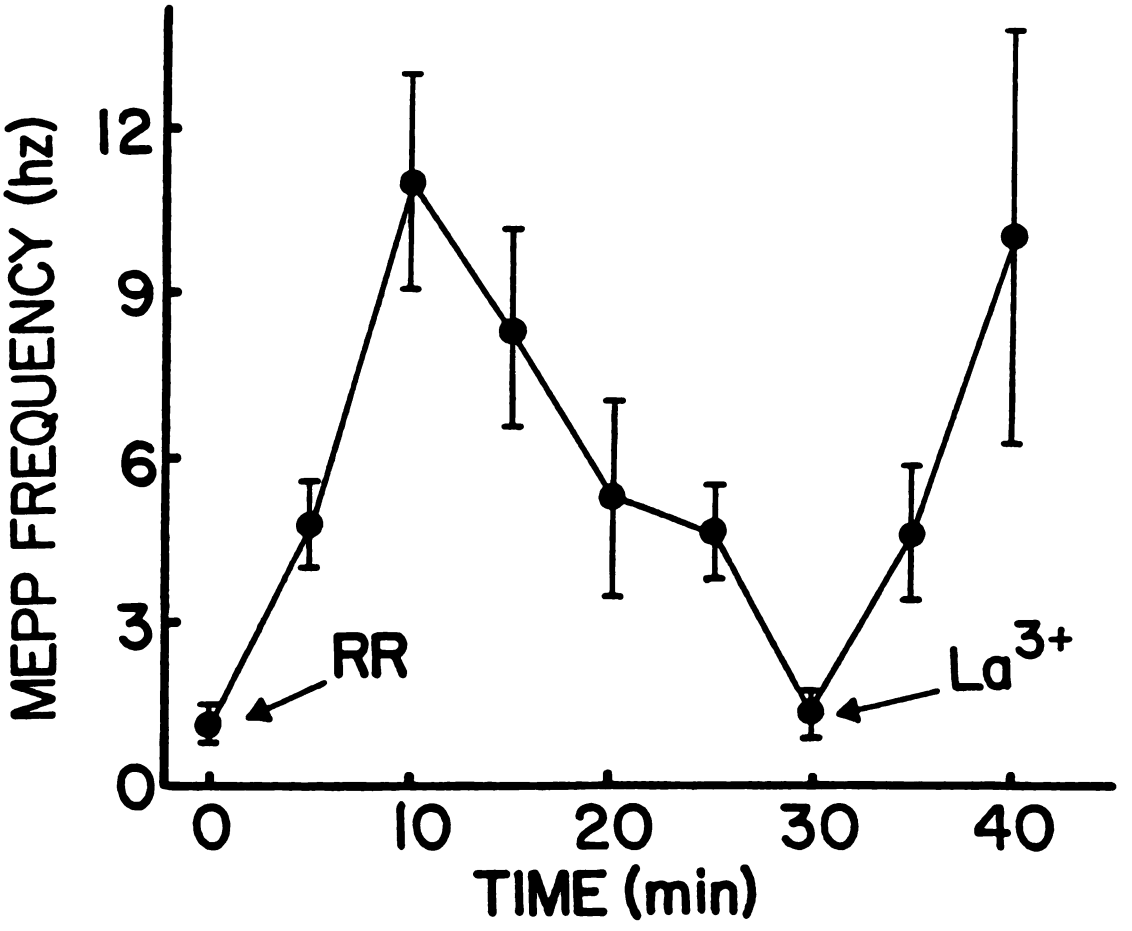

Figure 4. La^{3+}-induced (2 mM) stimulation of MEPP frequency after suppression of MEPPs by ruthenium red (RR, 20 μM). MEPP frequency was determined in increments of 2.5 min. Values are the mean ± SEM of 5 determinations.

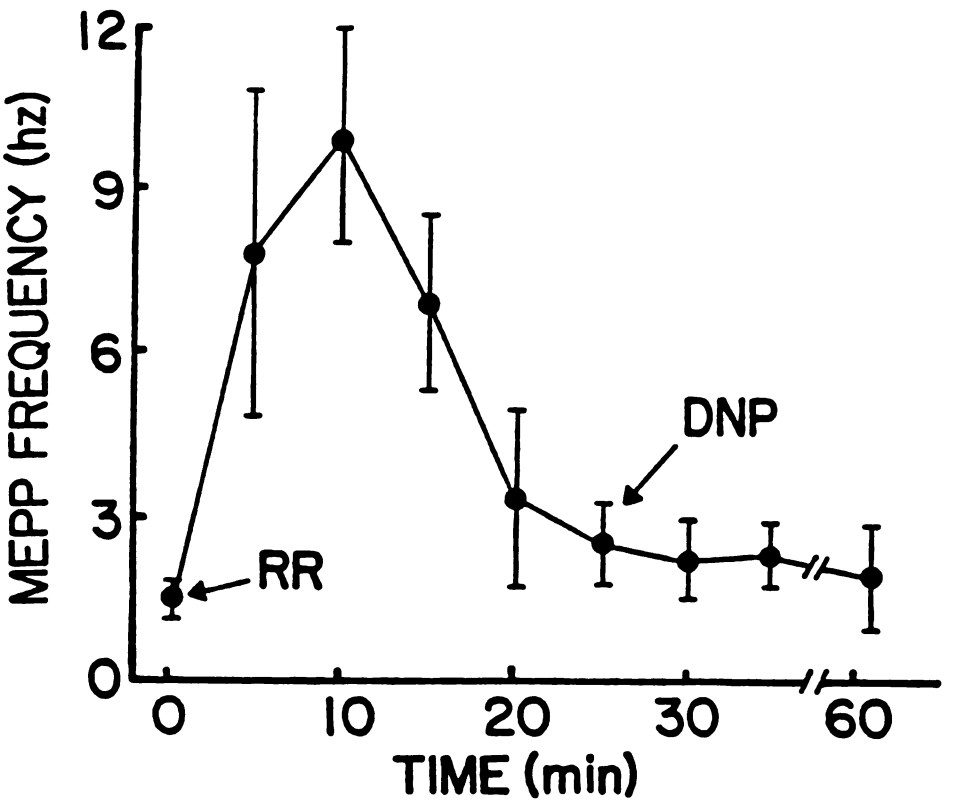

Figure 5. Time course of effects of (a) dinitrophenol (DNP, 50 μM), on MEPP frequency after pretreatment with ruthenium red (RR, 20 μM). RR was applied at time zero. MEPP frequency was allowed to return towards pre-stimulation levels before the preparation was superfused with DNP in conjunction with RR. Administration of DNP occurred at the times indicated by the arrows. MEPP frequency was determined in 5 min increments. Values are the mean ± SEM of 5 preparations.

DISCUSSION

The MeHg-induced stimulation of spontaneous, quantal release of ACh at the neuromuscular junction is not blocked by agents which depolarize the mitochondrial membrane such as DNP, DC or VAL but it is blocked by an agent which blocks Ca^{2+} influx specifically via the so-called "uniporter". Thus, simple inhibition of mitochondrial function does not prevent MeHg from eliciting an increase in MEPP frequency.

MeHg-induced increases in MEPP frequency occur in preparations treated with Ca^{2+}-deficient solutions as well as in preparations pretreated with EGTA/Ca^{2+}-free solutions to lower intracellular Ca^{2+} (Atchison, 1986). In these latter experiments the time required for MeHg to increase spontaneous, quantal secretion is prolonged markedly compared to preparations treated with normal $[Ca^{2+}]_o$. Taken together, these results imply that MeHg acts on intracellular stores of bound Ca^{2+} perhaps to provoke its release. The increase in free ionized cytosolic Ca^{2+} in turn is thought to promote the increased quantal discharge of ACh observed as increased frequency of MEPPs.

The present experiments were undertaken to ascertain whether mitochondria were a possible source of this increased $[Ca^{2+}]_i$. Mitochondria were targeted in this study for several reasons. First, mitochondria are the most important Ca^{2+} buffering organelle in the presynaptic nerve terminal; they store more Ca^{2+} under normal physiologic conditions than any other intracellular Ca^{2+} buffer and are the primary intracellular organelle responsible for maintaining Ca^{2+} homeostasis in a variety of cell types (Carafoli, 1982a). Second, mitochondrial inhibitors, such as DNP, RR

and DC have effects similar to MeHg on spontaneous transmitter release (Blioch et al., 1973; Rahamimoff and Alnaes, 1973). Third, inorganic and organomercurials inhibit respiration (Norseth and Brendeford, 1971; Magnaval et al., 1975; Sone et al., 1977) ATP production (Sone et al., 1977) and Ca^{2+} uptake in isolated mitochondria (Binah et al., 1978). Inhibition of these processes would disrupt normal mitochondrial membrane potential and could result in efflux of Ca^{2+} from mitochondria. If this were to occur in nerve terminal mitochondria, the elevated intraterminal Ca^{2+} could stimulate spontaneous release of ACh.

There is little doubt that MeHg deleteriously affects normal mitochondrial functioning. MeHg induces ultrastructural changes in the mitochondria (Yoshino et al., 1966a,b; Norseth, 1969; Desnoyers and Chang, 1975; O'Kusky, 1983) which are consistent with inhibition of respiration (Trump and Arstila, 1971) in that organelle. Swelling is one frequently observed pathological change which occurs in organomercurial-intoxicated mitochondria. The swelling is not due to inhibited respiration (Bogucka and Wojtczak, 1979), but is more likely due to mercurial-invoked K^+ accumulation (Brierley et al., 1968; Scott et al., 1970; Southard et al., 1973, 1974a,b; Verity et al., 1975; Sone et al., 1977; Bogucka and Wojtzak, 1979) which is thought to collapse the mitochondrial membrane potential, ultimately resulting in inhibited phosphorylation of ADP and decreased ATP production (Paterson and Usher, 1971; Southard et al., 1973; Southard et al., 1974a; Sone et al., 1977; OKusky, 1983). Collapse of the mitochondrial membrane potential would result in Ca^{2+} leakage into the nerve terminal cytosol.

A common mechanism for increasing intraterminal Ca^{2+}, collapse of the mitochondrial electrical gradient and subsequent Ca^{2+} leakage from this organelle,

would explain the close similarities between the effects of MeHg and DC, DNP and VAL on spontaneous release of transmitter. Yet none of these agents blocked the effects of MeHg on MEPP frequency. The Ca^{2+}-releasing ability of MeHg may be more complex than that of the other inhibitors since the stimulatory effects of MeHg on MEPP frequency still occur after pretreatment with the other inhibitors. Assuming that there are different mitochondrial Ca^{2+} pools (Carafoli, 1967), it may be that MeHg releases an additional pool of Ca^{2+} from mitochondria which is not accessible to DNP, DC or VAL. Unlike the other mitochondrial inhibitors used in our experiments, MeHg can interact directly with membrane thiol groups. Binding of these groups by MeHg might result in disruption of membrane integrity, uptake of otherwise impermeable cations, and collapse of the membrane potential. Inhibition of mitochondria with thiol-specific reagents stimulates mitochondrial Ca^{2+} efflux (Harris et al., 1979; Harris and Baum, 1980). Thus, modification of membrane sulfhydryl groups by MeHg may lead to a more extensive perturbation of membrane integrity and a more complete inhibition of mitochondrial Ca^{2+} buffering than is achievable with the other inhibitors. Alternatively, MeHg may act on other intraterminal Ca^{2+} buffers in addition to mitochondria such as the SER, cytosolic Ca^{2+} binding proteins and perhaps synaptic vesicles (Blaustein et al., 1977; 1978a,b).

Pretreatment with RR blocked the stimulatory effects of MeHg on MEPP frequency. Net Ca^{2+} leakage is induced by agents which lower the membrane potential and thereby allow efflux via reversal of the uniporter. Since RR is an effective inhibitor of Ca^{2+} uptake via the uniporter, Ca^{2+} efflux by uniporter reversal is believed to be blocked by this agent although other efflux pathways are not

affected. RR could be predicted to block the MeHg-induced efflux of Ca^{2+} effectively if this efflux occurred solely through the reversed uniporter. Alternatively, RR may prevent access of MeHg to the mitochondria. RR binds to proteins and lipids on membrane surfaces and could block the stimulatory effects of MeHg by preventing entry of MeHg into the nerve terminal or by blocking a site on the mitochondrial membrane at which MeHg may act.

In conclusion, block of mitochondrial function at different steps produces a different pattern of interaction with MeHg on spontaneous release of ACh. The results with RR suggest an interaction between MeHg and mitochondria to induce release of bound Ca^{2+} stores into the nerve terminal cytoplasm, resulting ultimately in stimulated release of neurotransmitters.

CHAPTER THREE

EFFECT OF ALTERATION OF NERVE TERMINAL Ca^{2+} REGULATION ON INCREASED SPONTANEOUS QUANTAL RELEASE OF ACETYLCHOLINE BY METHYLMERCURY

ABSTRACT

Agents known to disrupt Ca^{2+} buffering, N,N-dimethylamino-8-octyl-3,4,5,-trimethoxybenzoate (TMB-8), 25 μM; caffeine, 7.5 mM; N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035), 180 μM; ouabain 200 μM; and dantrolene, 50 μM, were tested for the ability to alter effects of methylmercury (MeHg) on spontaneous quantal release of acetylcholine (ACh) at the rat neuromuscular junction. In particular, we sought to determine whether any of the above agents could prevent the MeHg-induced increase of spontaneous release of ACh, an effect measured electrophysiologically as increased frequency of miniature end-plate potentials (MEPPs). MEPPs were recorded continuously from myofibers of the rat hemidiaphragm using conventional, intracellular recording techniques during pretreatment with an inhibitor of Ca^{2+} regulation and subsequently with the inhibitor plus MeHg (100 μM). When given alone, caffeine and ouabain, which release Ca^{2+} from the smooth endoplasmic reticulum and mitochondria, respectively, increased MEPP frequency in a biphasic manner. Following pretreatment, concomitant application of MeHg with caffeine or ouabain increased MEPP frequency after a brief latent period to peak values of 53 and 92 Hz, respectively. TMB-8 and dantrolene, putative inhibitors of Ca^{2+} release from smooth endoplasmic reticulum, differed in their effects on MEPP frequency; TMB-8 alone decreased MEPP frequency to approximately 10% of drug-free control, whereas dantrolene did not significantly alter control MEPP frequency. Subsequent concomitant application of MeHg with TMB-8 or dantrolene increased MEPP frequency to peak values of 40 and 100 Hz after 17 and 30 min, respectively.

YS035, a putative inhibitor of mitochondrial uptake and release of Ca^{2+}, decreased MEPP frequency to less than 10% of control after 15 min when given alone. Application of MeHg following YS035 pretreatment failed to increase MEPP frequency for up to 90 min. YS035 did not mask a MeHg effect by blocking postsynaptic sensitivity to ACh or preventing its release since subsequent treatment with La^{3+} (2 mM) after YS035 had abolished spontaneous release, increased MEPP frequency within 5 min. Thus, of the five inhibitors of nerve terminal Ca^{2+} regulation tested, only YS035 prevented the stimulatory action of MeHg on MEPP frequency. Results of the present study suggest that release of Ca^{2+} from nerve terminal mitochondria contributes to the increased MEPP frequency caused by MeHg while release of Ca^{2+} from smooth endoplasmic reticulum may not.

INTRODUCTION

The cellular mechanisms by which MeHg causes neurotoxicity have been the focus of several recent studies. Two effects of MeHg on synaptic transmission are observed during intracellular microelectrode recording studies and acute bath administration of MeHg: first, nerve-evoked, synchronous, quantal release (Silinsky, 1985) of acetylcholine (ACh) is inhibited (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982: Atchison et al., 1984; Atchison et al., 1986; Traxinger and Atchison, 1987a,b) and second, following a latent period spontaneous, asynchronous, quantal release of ACh is first increased and then decreased (Barrett et al., 1974; Juang 1976; Atchison and Narahashi, 1982; Atchison, 1986, 1987; Levesque and Atchison, 1987). Increased spontaneous release of ACh is measured electrophysiologically as increased miniature end-plate potential (MEPP) frequency and occurs as a result of changes in free intraterminal Ca^{2+} concentration ($[Ca^{2+}]$).

The stimulatory effect of MeHg on MEPP frequency occurs independently of extracellular Ca^{2+} since it occurs even in Ca^{2+}-deficient solutions (Atchison, 1986). This suggests that if MeHg-induced stimulation of spontaneous release of ACh is due to elevation of $[Ca^{2+}]$, the source of this Ca^{2+} may be an intracellular store. The two most important buffers of intraterminal Ca^{2+} are thought to be mitochondria and smooth endoplasmic reticulum (SER) (Blaustein et al., 1977, 1978). If MeHg enters the nerve terminal and interacts with either of these Ca^{2+}-sequestering organelles to cause release of Ca^{2+}, then the resultant increase in $[Ca^{2+}]$ might stimulate spontaneous release of ACh. This speculation prompted previous

complementary experiments which were undertaken to assess whether mitochondria may be a source of increased [Ca^{2+}] for the increased MEPP frequency produced by MeHg (Levesque and Atchison, 1987). Block of mitochondrial function at different steps caused a different pattern of interaction with MeHg on spontaneous quantal release of ACh. Pretreatment of the preparation with dinitrophenol, dicoumarol or valinomycin, all of which release Ca^{2+} subsequent to depolarization of the mitochondrial membrane, did not block the MeHg-induced increase of MEPP frequency. However, ruthenium red (RR), which is thought to be a specific inhibitor of the Ca^{2+} influx uniporter in the mitochondrial membrane (Moore, 1971), blocked the stimulatory effect of MeHg on MEPP frequency. The complete block of MeHg-induced stimulation of MEPP frequency after pretreatment with RR may indicate that both RR and MeHg act on a similar Ca^{2+} store and may suggest an action of MeHg on mitochondria to induce release of bound Ca^{2+} stores into the nerve terminal cytoplasm, ultimately resulting in stimulated release of neurotransmitter.

The present experiments were designed to gain further information on effects of MeHg on nerve terminal Ca^{2+} regulation. Agents which disrupt Ca^{2+} buffering by the SER including caffeine, dantrolene and 8-(dimethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) were tested for potential interaction with MeHg on MEPP frequency. Caffeine is thought to induce release of Ca^{2+} (Elmqvist and Feldman, 1965b; Hofmann, 1969; Roed, 1982) while TMB-8 and dantrolene are thought to inhibit release of sequestered Ca^{2+} (Stefani and Chiarandini, 1973; Malagodi and Chiou, 1974; Putney and Bianchi, 1974; Chiou and Malagodi, 1975; Van Winkle, 1976; Francis, 1978; Danko et al., 1985) from the

SER. Also, ouabain and N,N-bis-(3,4-dimethoxyphenylethyl)-N-methylamine (YS035), which are presumed to cause Ca^{2+} mobilization from mitochondria (Govier and Holland, 1964; Elmqvist and Feldman, 1965a; Baker and Crawford, 1975) and inhibit mitochondrial uptake and release of Ca^{2+} (Deana et al., 1984), respectively, were tested for interaction with MeHg. Since these agents inhibit mitochondria and SER by different mechanisms, it was of interest to compare the interaction of each inhibitor and MeHg on spontaneous release of transmitter. These experiments, together with those performed previously (Levesque and Atchison, 1987), were carried out with the hopes of delineating potential sources for the putative release of bound intracellular Ca^{2+} by MeHg which is presumed to be the cause of the MeHg-induced increase in MEPP frequency.

METHODS

Preparation and solutions. All experiments utilized the isolated hemidiaphragm (Bulbring, 1946) of male rats (175-225 g, Harlan Sprague-Dawley) and conventional intracellular microelectrode recording techniques. The hemidiaphragm was pinned out in a sylgard-coated (Dow-Corning Co., Midland, MI) plexiglas chamber and superfused continuously with a physiological saline solution modified from that described by Liley (1956). The composition of this fluid was (mM): NaCl, 135; $CaCl_2$, 2; KCl, 5; $MgCl_2$, 1; glucose, 11; and HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid), 14. When the concentration of KCl was lowered in experiments with "cut muscles" (see below), equimolar increases were made in NaCl to maintain [Cl^-] and osmolarity constant. It was necessary to substitute HEPES for the usual phosphate-bicarbonate buffer (Liley, 1956) to prevent precipitation of MeHg. Solutions were oxygenated continuously with 100% O_2 during all experiments and pH was adjusted to 7.4. All experiments were carried out at room temperature of 23-26°C. Each individual experiment required using a separate hemidiaphragm preparation. All experiments were replicated in a minimum of four rats and each preparation served as its own control.

Intracellular recording. Intracellular recordings of MEPPs were made using borosilicate glass microelectrodes filled with 3 M KCl and having impedances of between 10-30 megaohms. MEPPs were amplified (M707, WP Instruments, Hartford, CT), displayed on an oscilloscope (2090, Nicolet Instruments, Verona, WI) and recorded on magnetic tape using an FM instrumentation tape recorder (Model B, A. R. Vetter Instruments, Rebersburg, PA). MEPP frequency and amplitude

were determined by manual measurements from chart records made from taped data with a Gould 2200 (Gould Inc., Cleveland, OH) chart recorder.

To determine concentrations of each inhibitor of Ca^{2+} regulation suitable for use in this preparation, separate preliminary experiments were performed employing different concentrations of the test agent. The final concentration selected for an agent was one that did not interfere with measurements of MEPP frequency either by suppressing MEPPs altogether or by causing rapid depolarization of the membrane of the impaled postsynaptic cell. The same protocol was followed for each agent once final concentrations were selected. After a short equilibration period with control physiological saline solution, a cell was impaled and control MEPP frequency was measured. After impalement, some cells failed to maintain near normal resting potentials and depolarized rapidly. This necessitated impalement of a different cell. While maintaining impalement of the same cell, the perfusion solution was switched to one containing the inhibitor being tested. MEPP frequency was recorded continuously in this solution until MEPP frequency declined and became constant or until MEPPs were no longer observed. While still maintaining impalement of the same cell, the perfusion solution was switched to a final one containing both the agent and MeHg. Again, MEPPs were measured until any changes induced by MeHg subsided or until MEPPs disappeared altogether.

In all experiments with caffeine, a "cut-fiber" preparation (Barstad and Lilleheil, 1968; Hubbard and Wilson, 1972; Traxinger and Atchison, 1987a,b) was used to prevent contraction of the diaphragm subsequent to caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum (SR) of the muscle. Cut muscles

were bathed in cold physiological saline containing a lowered concentration of KCl (2.5 mM) for about 1 hr and then were returned to room temperature prior to being used in an experiment. There were no differences in control MEPP frequencies between cut and uncut diaphragms. Also, there was no significant difference in time to peak frequency or peak MEPP frequency induced by MeHg in cut and uncut preparations (Atchison, 1987).

To ensure that the concentration of dantrolene utilized in the experiments could affect release of Ca^{2+} from SR, we performed a bioassay in which we observed the effects of dantrolene on the muscle twitch evoked by electrical stimulation of the phrenic nerve. The nerve-evoked muscle twitch was almost completely inhibited by dantrolene sodium (50 μM) within 30 min (data not shown).

Materials. Methylmercuric acetate was obtained from Pfaltz-Bauer Chemical Co. (Stamford, CN) and was dissolved in 4% (v/v) glacial acetic acid to yield a 2 mM stock solution. Dilutions of this stock solution were made to yield a final MeHg concentration of 100 μM. This final dilution was adjusted to pH 7.4 prior to experimentation. At 100 μM, MeHg produced identical peak MEPP frequencies as do lower concentrations (20, 40 μM) but the latent period preceding the onset of increased MEPP frequency is shorter for the higher concentration (Atchison and Narahashi, 1982; Atchison et al., 1984). HEPES was obtained from the U.S. Biochemical Co. (Cleveland, OH). Lanthanum chloride and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO.). Caffeine and TMB-8 [8-(dietylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride] were obtained from Aldrich Chemical Co. (Milwaukee, WI). Dantrolene sodium was obtained from Norwich Eaton Pharmaceutical Co. (Manati, Puerto Rico) and was dissolved in

physiological saline by stirring vigorously for 1 hr and then filtering. Dantrolene solutions were made just prior to experimentation due to rapid precipitation of this agent. As indicated above, dantrolene solutions were bioassayed for efficacy prior to use. YS035 (N,N-bis(3,4-dimethoxyphenethyl)-N-methylamine) was synthesized by the Organic Synthesis Lab of the Michigan State University Department of Chemistry according to the methods of Deana et al. (1984). YS035 was dissolved in ethanol and then added to the physiological saline solution so that the final concentration of ethanol was 0.1% (v/v). Control solutions contained an identical concentration of the respective vehicle. Caffeine, TMB-8 and ouabain were dissolved in the physiological saline solution.

Statistical analysis. Statistical analysis of the effects of MeHg in conjunction with dantrolene, TMB-8, caffeine, ouabain and YS035 was performed by a one-way analysis of variance (ANOVA) (Steel and Torrie, 1960). Significance was set at $p \leq 0.05$.

RESULTS

When tested alone, each of the five agents which disrupt intraterminal Ca^{2+} regulation (dantrolene, TMB-8, caffeine, ouabain and YS035) had different effects on the time course and degree of stimulation of MEPP frequency (Figure 1). These findings may reflect differences in mechanism of inhibition, site of action and concentration between the different agents used or simply differences in efficacy in stimulating release. Control MEPP frequency prior to treatment with an inhibitor was between 1-2 Hz in all experiments.

Dantrolene and TMB-8 are both thought to block the release of bound Ca^{2+} from SER (Stefani and Chiarandini, 1973; Malagodi and Chiou, 1974; Putney and Bianchi, 1974; Chiou and Malagodi, 1975; Danko et al., 1985). Dantrolene (50 μM) was the only agent tested that had no significant effect on MEPP frequency when given alone. Even after an hour of perfusing the diaphragm with a solution containing dantrolene, MEPP frequency was maintained at about 1-2 Hz. At this concentration the muscle twitch evoked by electrical stimulation of the phrenic nerve was virtually completely blocked (results not shown), so dantrolene clearly was reducing or preventing release of Ca^{2+} from muscle SR. Durant et al. (1980) have also reported finding no effect of dantrolene on spontaneous release of ACh at the mammalian neuromuscular junction. However, these data are at variance with results of Statham and Duncan (1976) who reported that dantrolene markedly depressed MEPP frequency at the amphibian neuromuscular junction. Perhaps the contrasting results may be due to a species difference in regulation of $[Ca^{2+}]_i$ in motor nerve terminals or in the role of Ca^{2+} in spontaneous release of transmitter

at the neuromuscular junction. Alternatively, the differences may merely reflect differences in diffusional barriers such as connective tissue which may have precluded access of dantrolene to the nerve terminal interior. Administration of TMB-8 caused a marked reduction of MEPP frequency within 30 min to values of 0.1 ± 0.1 Hz or 10% of control.

Caffeine was the third agent tested. Unlike dantrolene and TMB-8, caffeine is thought to evoke Ca^{2+} release from internal stores thereby stimulating spontaneous release of transmitter at the neuromuscular junction (Elmqvist and Feldman, 1965a; Hofmann, 1969; Onodera, 1973; Roed, 1982). In our experiments, caffeine caused a rapid increase in MEPP frequency which peaked at values of 62.1 ± 4.7 Hz (n=7) after 14.4 ± 1.4 min.

The fourth agent tested was the cardiac glycoside ouabain. When given alone, ouabain increased MEPP frequency to peak values of 97.6 ± 4.1 Hz after 91.6 ± 7.7 min. There was a lengthy latent period of 48.3 ± 6.0 min prior to the onset of the increase in MEPP frequency. These results are consistent with previous reports (Elmqvist and Feldman, 1965b; Birks and Cohen, 1968; Baker and Crawford, 1975; Branisteanu et al., 1979) that ouabain increases spontaneous release of ACh at the neuromuscular junction. A major action of ouabain is inhibition of Na^+/K^+-ATPase, an enzyme found in high concentration in nerve terminals.

The final agent tested was the putative Ca^{2+} antagonist YS035. Deana et al. (1984) synthesized this compound and extensively investigated its activity on Ca^{2+} transport. YS035 was effective in inhibiting Ca^{2+} uptake by brain synaptosomes and by isolated cells of excitable tissues. YS035 also inhibited ruthenium red-

induced and Na^+-dependent, Ca^{2+} efflux from isolated liver and brain mitochondria. Because of its apparent ability to inhibit cellular uptake and mitochondrial efflux of Ca^{2+}, we wanted to investigate effects that YS035 alone may have on synaptic transmission. Treatment with YS035 (180 μM) significantly decreased control MEPP frequency to values of 0.12 ± 0.1 (n=6) Hz within 20 min. Lower concentrations of YS035 (30, 90, 120 μM) did not appear to affect MEPP frequency. Perhaps slow, steady leakage of Ca^{2+} from the mitochondria contributes significantly to the normal spontaneous release of ACh. YS035 could decrease control MEPP frequency by inhibiting the role of mitochondria in spontaneous release. These results are consistent with the presumed ability of YS035 to prevent increases in or cycling of intracellular Ca^{2+}.

Pretreatment with dantrolene or TMB-8 did not block the MeHg-induced stimulation of MEPP frequency (Figure 2). MeHg increased MEPP frequency to 102.5 ± 3.1 (n=5) Hz after 37.3 ± 6.6 min following pretreatment with dantrolene. The latency to onset of increased MEPP frequency with MeHg was 30.0 ± 5.4 min. Subsequent to pretreatment with TMB-8, MeHg increased MEPP frequency to peak values of 25.5 ± 9.7 (n=5) Hz after 40.0 ± 6.2 min. The stimulatory effect of MeHg on MEPP frequency first began 17.0 ± 2.6 min after its addition to the perfusion medium. Thus, inhibitors of Ca^{2+} release from the SER were ineffective in blocking the MeHg-induced increase in spontaneous release of ACh. Although TMB-8 did not block the effect of MeHg on MEPP frequency, the peak frequency with MeHg and TMB-8 was less pronounced than in experiments in which there was no pretreatment with TMB-8 (Atchison, 1986, 1987).

Similar results were obtained after pretreatment with caffeine and ouabain

(Figure 3). However, the time course of the effect of MeHg on MEPP frequency was hastened considerably with both agents. Following caffeine pretreatment, MeHg rapidly increased MEPP frequency after only 5.4 ± 1.2 (n=7) min (Figure 3A). MEPP frequency rose to peak values of 53.2 ± 9.0 Hz after 9.1 ± 2.7 min before declining towards control levels. When ouabain was used to disrupt Ca^{2+} regulation prior to MeHg (Figure 3B), MEPP frequency began to increase just 2.5 ± 1.6 (n=7) min after addition of MeHg to the perfusion medium. A peak MEPP frequency of 91.7 ± 7.1 Hz was reached after 8.1 ± 2.1 min. The frequency declined towards control values shortly after peaking. The time of onset and time to peak MEPP frequency induced by MeHg were considerably shortened by pretreatment with caffeine or ouabain in comparison to control experiments with MeHg alone (Atchison, 1986, 1987).

In contrast to the results with the above agents, pretreatment with YS035 completely blocked stimulation of MEPP frequency by MeHg (Figure 4). There was no detectable increase in MEPP frequency for up to 90 min (N=6) after initiating superfusion with MeHg and YS035.

It was possible that YS035 may have been masking a potential MeHg-evoked increase in transmitter release by blocking junctional transmission either by inhibiting the process by which ACh is released from the presynaptic nerve terminal or blocking the ACh receptor/channel complex at the postsynaptic membrane. To test for a possible block of junctional transmission by YS035, we examined the effects of lanthanum on transmitter release following pretreatment with YS035 (Figure 5). La^{3+} was chosen because it is known to facilitate the release of available transmitter stores from the nerve terminal (Heuser and Miledi, 1971).

Following suppression of MEPPs by YS035, concomitant administration of La^{3+} rapidly resulted in a 35-fold (n=4) increase in MEPP frequency. This ruled out the possibility that YS035 may have been blocking presynaptic release of ACh or causing postsynaptic block of the action of ACh. These results indicate that the stimulatory effect of MeHg on MEPP frequency is YS035-sensitive.

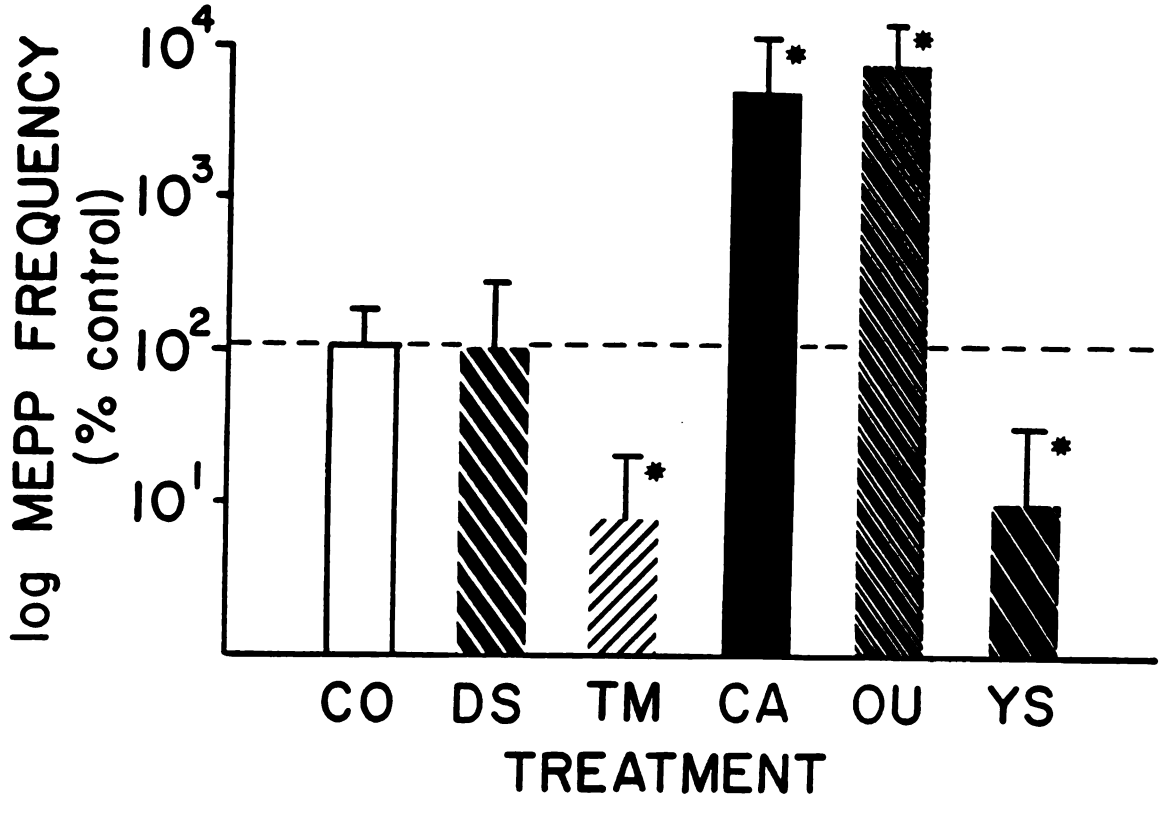

Figure 1. Effects of inhibitors of Ca^{2+} regulation by SER or mitochondria on MEPP frequency. The first bar (CO) signifies control MEPP frequency prior to addition of an inhibitor. The dashed horizontal line represents values equal to 100% of control. The concentration of inhibitors were as follows: Dantrolene sodium (DS), 50 μM; N,N-dimethylamino-8-octyl-3,4,5-trimethoxybenzoate (TM), 25 μM; caffeine (CA), 7.5 mM; ouabain (OU), 200 μM; and N,N-bis-(3,4-dimethoxyphenethyl)-N-methylamine or YS035 (YS), 180 μM. Values for CO, DS, TM, CA, OU and YS are the means ± SEM of 12, 4, 4, 7, 4 and 6 determinations, respectively. The asterisk (*) indicates a value significantly different from that of control ($p \leq 0.05$).

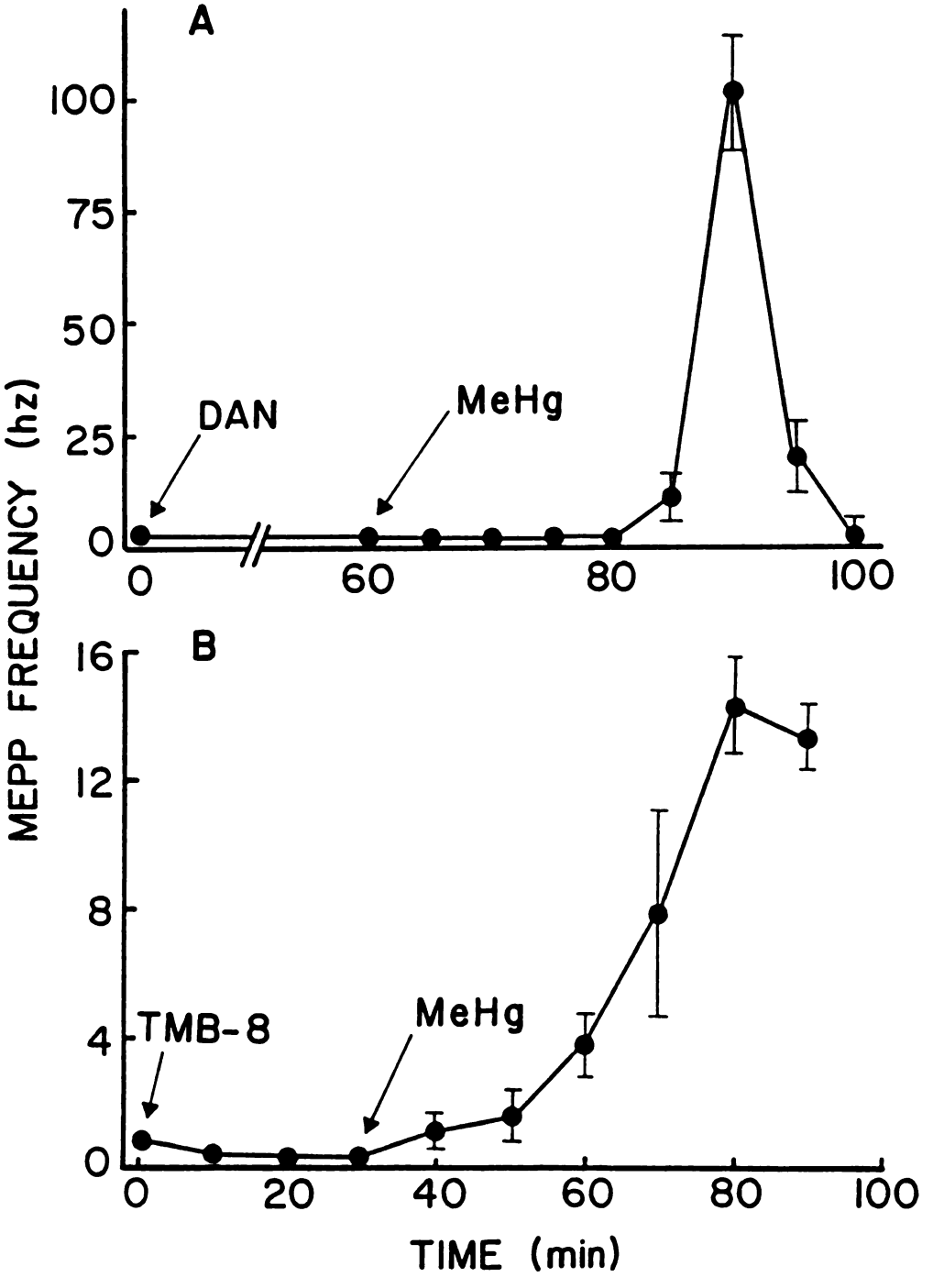

Figure 2. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with (A) dantrolene sodium (DS, 50 μM) or (B) N,N-dimethylamino-8-octyl-3,4,5-trimethoxybenzoate (TMB-8, 25 μM). Dantrolene or TMB-8 were applied at time zero. The preparation was superfused with MeHg in conjunction with dantrolene or TMB-8 at the times indicated by arrows. MEPP frequency was determined in increments of 5 min for dantrolene and 10 min for TMB-8. Values for dantrolene and TMB-8 are the means ± SEM of 5 determinations.

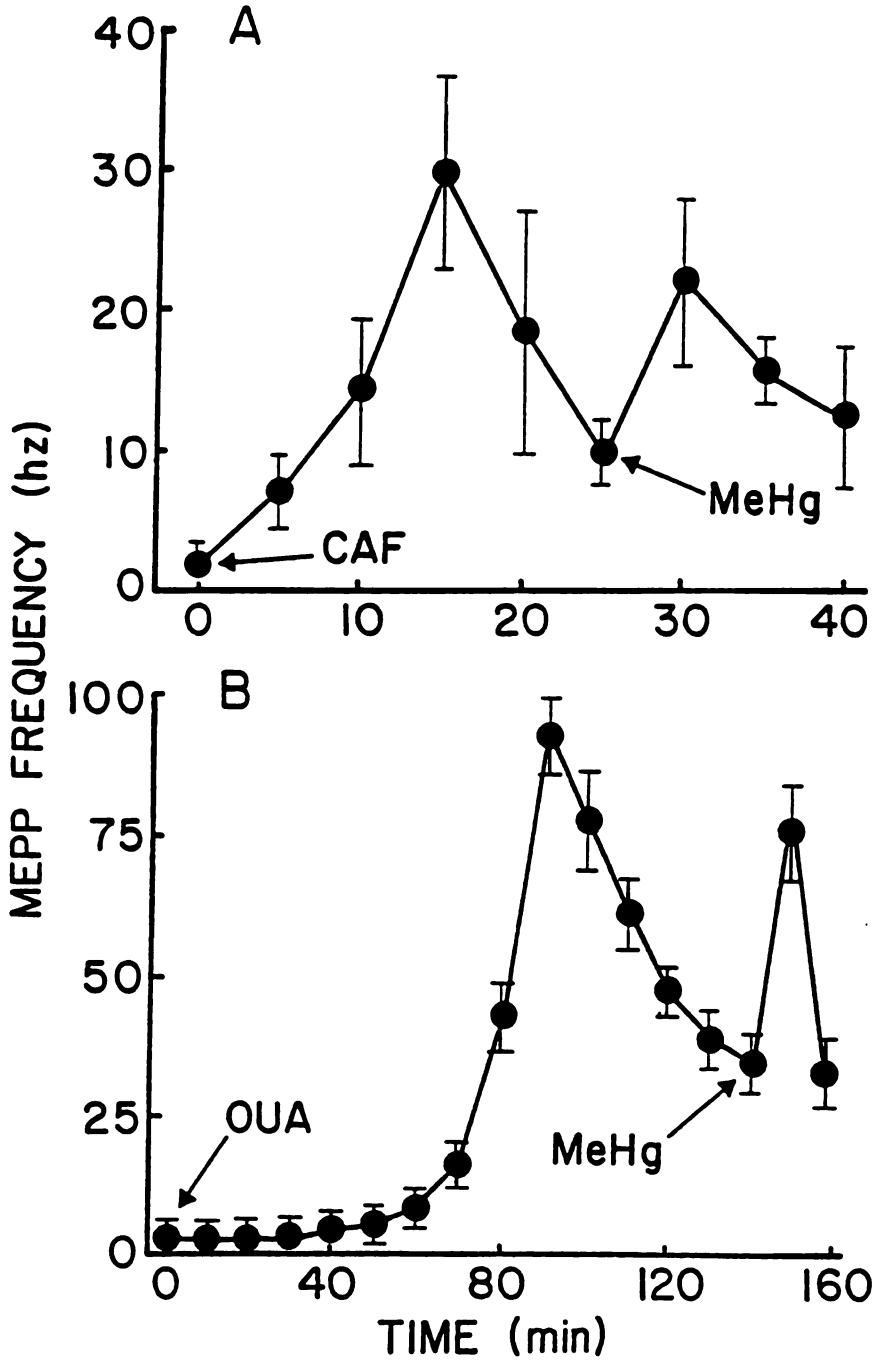

Figure 3. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with (A) caffeine (CAF, 7.5 mM) or (B) ouabain (OUA, 200 μM). CAF or OUA were applied at time zero. As MEPP frequency declined towards prestimulation levels, the preparation was superfused with MeHg in conjunction with CAF or OUA. MEPP frequency was determined in increments of 5 min in (A) and 10 min in (B). Values are the mean ± SEM of 7 determinations.

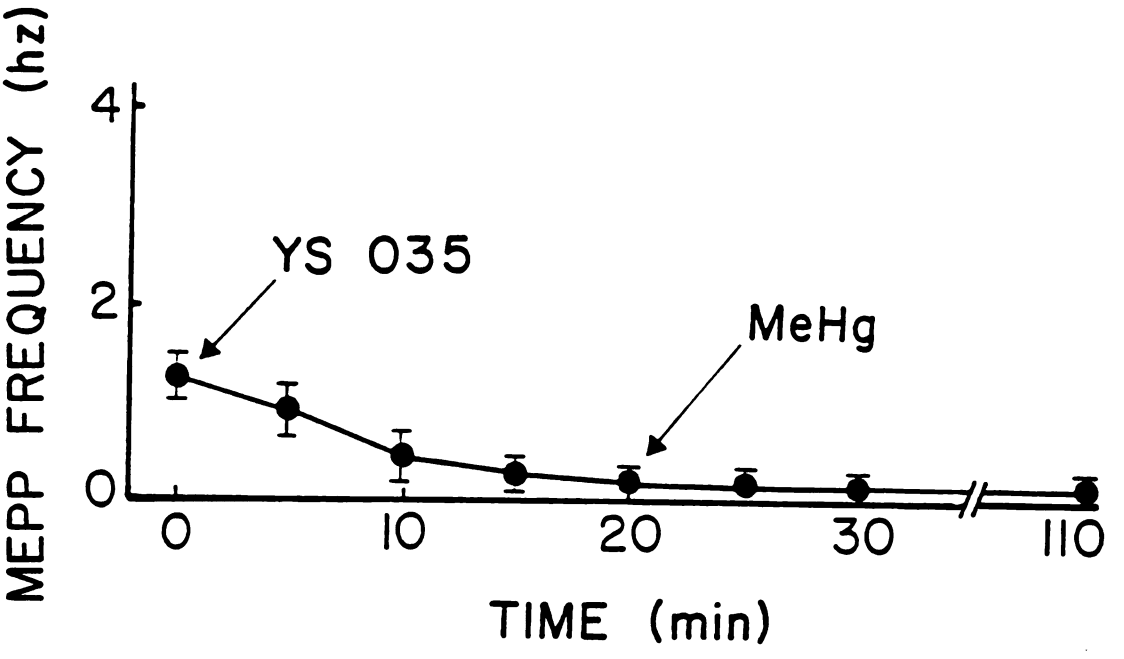

Figure 4. Time course of effects of MeHg (100 μM) on MEPP frequency after pretreatment with N,N-bis-(3,4-dimethoxyphenethyl)-N-methylamine (YS035, 180 μM). YS035 was applied at time zero. The preparation was superfused in conjunction with MeHg in conjunction with YS035 at the time indicated by the arrow. MEPP frequency was determined in increments of 5 min. Values are the mean ± SEM of 6 determinations.

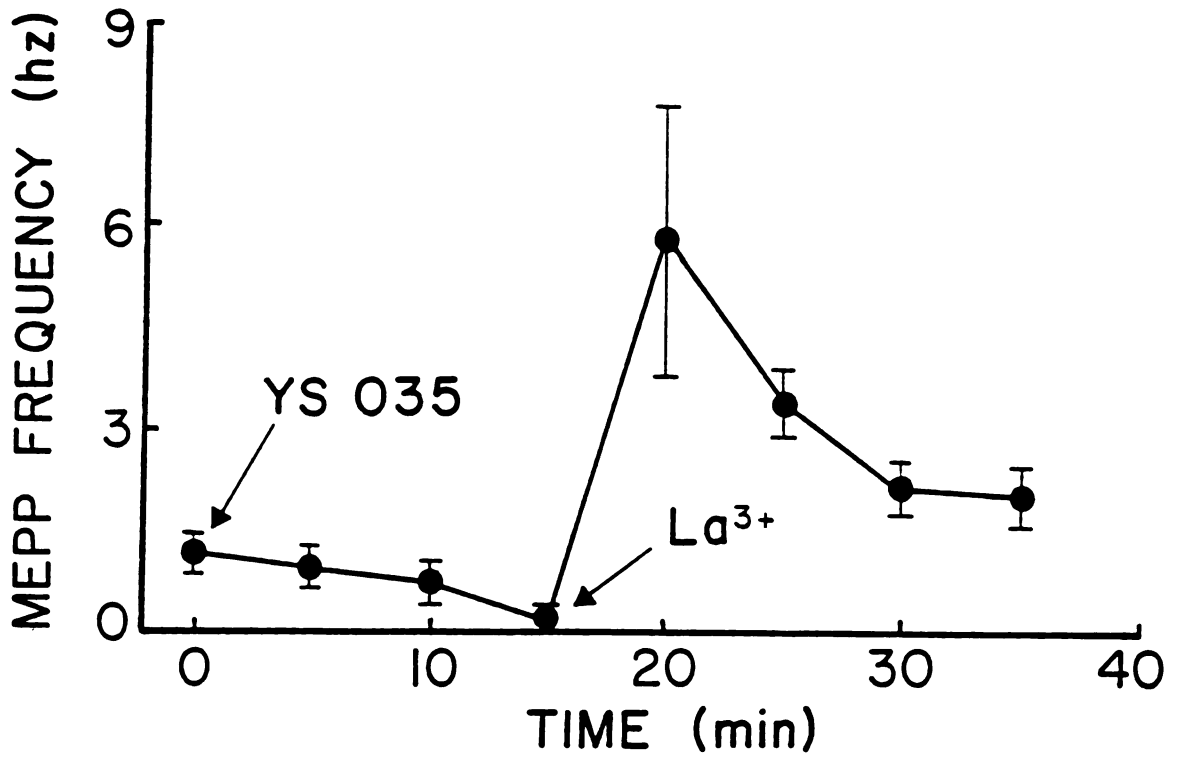

Figure 5. La^{3+}-induced (2 mM) stimulation of MEPP frequency after suppression of MEPP frequency by YS035 (180 μM). The preparation was superfused with La^{3+} in conjunction with YS035 at the time indicated by the arrow. MEPP frequency was determined in increments of 5 min. Values are the mean ± SEM of 3 determinations.

DISCUSSION

Previously we have demonstrated that agents that block release of Ca^{2+} from mitochondria by disruption of the mitochondrial membrane potential (2,4-dinitrophenol, valinomycin, warfarin) would not prevent the increased MEPP frequency elicited by MeHg, while block of Ca^{2+} release via reversal of the mitochondrial uniport protein by ruthenium red would (Levesque and Atchison, 1987). These results suggested to us that disruption of the Ca^{2+} transport mechanism in mitochondria could preclude MeHg from inducing Ca^{2+} release and subsequently increasing ACh release. These findings prompted the present study which was based on two goals: namely, testing another source of Ca^{2+} storage within the terminal for possible interaction with MeHg, and examining further the tentative link established in the previous studies regarding effects of MeHg on mitochondrial Ca^{2+} buffering.

Agents which disrupt Ca^{2+} buffering by the SER either by evoking Ca^{2+} release (caffeine), or preventing its release (TMB-8, dantrolene) were ineffective at preventing the MeHg-induced increase in MEPP frequency. Ouabain, which causes Na^{+}-dependent Ca^{2+} mobilization from nerve terminal mitochondria also did not prohibit MeHg from evoking a large increase in MEPP frequency. YS035, which is proposed to prevent uptake and release of Ca^{2+} from mitochondria did block the enhanced spontaneous release of ACh evoked by MeHg. Taken together these results would seem to reinforce our previous conclusion, and suggest tentatively that disruption of Ca^{2+} handling by SER may not play a critical role in the enhancement of ACh release by MeHg.

MEPP frequency is related directly to the free $[Ca^{2+}]_i$ concentration in the axon terminal. As MeHg induces an increase in MEPP frequency in solutions deficient in $[Ca^{2+}]_o$ (Atchison, 1986; 1987), it is assumed that intracellular stores of Ca^{2+} are a target of action of MeHg. Recently, the fluorescent Ca^{2+} indicator fura-2 was used to demonstrate directly a large increase in $[Ca^{2+}]_i$ in rat brain synaptosomes after treatment with MeHg (Komulainen and Bondy, 1987).

Intraterminal Ca^{2+} buffering systems are thought to play a crucial role in maintaining free $[Ca^{2+}]_i$ within a narrow, low range at rest and during activity. The two major sites of intraterminal Ca^{2+} buffering are considered to be mitochondria and SER (Blaustein et al., 1977, 1980). Of the two, mitochondria appear to have a large buffering capacity, but a low affinity for Ca^{2+} while the SER has a much lower capacity but higher affinity for Ca^{2+} (Blaustein et al., 1978). In principle then, MeHg could interact with either, or both systems to prevent uptake and/or induce release of bound Ca^{2+} eventually leading to a rise in $[Ca^{2+}]_i$.

Disruption of SER buffering of Ca^{2+} in the presence of caffeine, dantrolene or TMB-8 was unable to prevent the increase in MEPP frequency upon exposure to MeHg. Perhaps Ca^{2+} stored in the SER does not contribute to the effects of MeHg on MEPP frequency or is only responsible for a small fraction of the increased MEPP frequency evoked by MeHg, and thus its absence did not dramatically alter the response to MeHg in the presence of TMB-8 or dantrolene.

Mitochondria are the major storage source of Ca^{2+} in the presynaptic nerve terminal. An abundance of evidence implicates an interaction of MeHg with nerve terminal mitochondria to disrupt Ca^{2+} storage. Organomercurials inhibit mitochondrial respiration (Norseth and Brendeford, 1971; Fox et al., 1975;

Magnaval et al., 1975; Verity et al., 1975; Sone et al., 1977) and induce K^+ accumulation (Brierly et al., 1968; Scott et al., 1970; Southard et al., 1973; 1974a,b; Verity et al., 1975; Sone et al., 1977; Bogucka and wojtzak, 1979). This in turn would depolarize the mitochondrial membrane resulting in decreased ATP production (Paterson and Usher, 1971; Southard et al., 1973; 1974a,b; Sone et al., 1977; O'Kusky, 1983) and Ca^{2+} leakage from the organelle (Carafoli, 1982a). As we have shown previously that ruthenium red, which blocks release of mitochondrial Ca^{2+} via reversal of the uniport protein, prevents the increase in MEPP frequency elicited by MeHg, we sought to study the apparent interaction of MeHg and mitochondria further in the present study by use of YS035 to prevent Ca^{2+} release from mitochondria.

YS035 completely blocked the stimulatory effects of MeHg on spontaneous quantal release of ACh. Thus YS035 may have prevented an increase in MEPP frequency by blocking the elevation of free $[Ca^{2+}]$ which may normally occur subsequent to MeHg treatment. One would expect YS035 to be effective in preventing an increase in free $[Ca^{2+}]$ since this compound inhibits cellular uptake and mitochondrial release of Ca^{2+} (Deana et al., 1984). Thus, this result supports the hypothesis that MeHg may stimulate spontaneous quantal release of ACh subsequent to an interaction with mitochondria, to release bound Ca^{2+} and elevate free $[Ca^{2+}]$.

Pretreatment with ouabain, another agent that acts on mitochondria, did not block the stimulatory effect of MeHg on MEPP frequency. Quite to the contrary, the times of onset and peak effect of MeHg were hastened considerably. Ouabain inhibits Na^+/K^+-ATPase and in so doing causes accumulation of Na^+ in the nerve

terminal cytosol. The decreased onset and time to peak with MeHg after ouabain may be due to depolarization of the nerve terminal by ouabain leading to enhanced entry of Ca^{2+} and/or MeHg into the terminal. Treatments that facilitate Ca^{2+} entry into the nerve terminal such as depolarizing with high $[K^+]_o$ or veratridine, a Na^+ channel activator, and also via direct activation of Ca^{2+} channels by Bay K 8644, hasten the increased MEPP frequency with MeHg (Atchison, 1986; 1987). Alternatively, ouabain may "prime" the release process by elevating $[Ca^{2+}]_i$ in a Na^+-dependent manner (Carafoli and Crompton, 1978; Carafoli, 1982b) or prevent Na^+/Ca^{2+} exchange by elevating $[Na^+]_i$.

The failure of ouabain pretreatment to inhibit the stimulatory action of MeHg on MEPP frequency parallels that of other agents known to release Ca^{2+} from mitochondria such as dinitrophenol, warfarin and valinomycin. Ca^{2+} is stored in mitochondria in a nonhomogeneous manner and various agents which promote Ca^{2+} release may act on only a portion of the stored Ca^{2+} (Carafoli, 1967). Perhaps MeHg releases a pool of mitochondrial Ca^{2+} not accessible to ouabain.

In conclusion, agents which disrupt Ca^{2+} buffering by the SER did not block the stimulatory effects of MeHg on MEPP frequency but they did alter the normal time course and magnitude of the MeHg effect. Ouabain hastened the onset of the stimulatory effect of MeHg, possibly by depolarizing the nerve terminal membrane. The results with YS035 suggest that MeHg may stimulate MEPP frequency as a result of releasing Ca^{2+} from mitochondria leading to a subsequent increased MEPP frequency. Taken together, the results obtained in a previous study with ruthenium red and in the present study with YS035 implicate the mitochondrion as the site upon which MeHg acts to release Ca^{2+} and elevate MEPP frequency. The experi-

ments with ruthenium red and YS035 utilized a preparation with intact cells and block of MeHg-induced stimulation of MEPP frequency may have occurred by some other mechanism than the one proposed such as preventing entry of MeHg into the nerve terminal cytoplasm or by blocking the access of MeHg to a site on the mitochondrial membrane on which it may act. Thus, future experiments will entail use of simpler isolated systems to test this hypothesis more directly.

We cannot be certain of the relationship between the results of our experiments dealing with acute bath application of MeHg to a model synapse and the histopathological findings in patients poisoned by chronic exposure to MeHg. The molecular and cellular mechanisms underlying pathological lesions that occur with MeHg intoxication are not yet clear, but undoubtedly occur in response to more subtle biochemical or physiological effects on nerve cell bodies or processes. Perhaps these effects are due at least in part to disruption of Ca^{2+} regulation within the axon terminal by MeHg. Since synaptic transmission is dependent on precisely regulated changes in free [Ca^{2+}], disruption of intraterminal Ca^{2+} regulation would explain some of the known effects of MeHg on the transmitter release process. Moreover, release of neurotransmitters is only one example of a Ca^{2+}-dependent process. If MeHg does indeed alter cellular Ca^{2+} regulation by interfering with transmembrane Ca^{2+} fluxes or Ca^{2+} buffering by intracellular organelles, one could predict effects of MeHg on other Ca^{2+}-dependent cellular functions in neuronal as well as in non-neuronal cells.

CHAPTER FOUR

DISRUPTION OF BRAIN MITOCHONDRIAL CALCIUM SEQUESTRATION BY METHYLMERCURY

ABSTRACT

Effects of methylmercury (MeHg) on Ca^{2+} transport and respiratory control of mitochondria isolated from rat forebrain were examined to determine whether MeHg disrupted sequestration of Ca^{2+} by mitochondria. Uptake of $^{45}Ca^{2+}$ by mitochondria and release of $^{45}Ca^{2+}$ from preloaded mitochondria were measured as a function of time and MeHg concentration in the presence and absence of ATP. Release of $^{45}Ca^{2+}$ from preloaded mitochondria by MeHg was measured in the presence and absence of ruthenium red (RR), a putative inhibitor of the mitochondrial Ca^{2+} uptake uniporter. During incubation intervals ranging from 10 sec to 5 min, 10 μM MeHg reduced mitochondrial uptake of $^{45}Ca^{2+}$ by about 50% and 100 μM MeHg completely prevented $^{45}Ca^{2+}$ uptake. The inhibitory effect of MeHg on $^{45}Ca^{2+}$ uptake occurred in both the presence and absence of ATP. Exposure of mitochondria preloaded with $^{45}Ca^{2+}$ to MeHg for 10 sec resulted in efflux of $^{45}Ca^{2+}$; 10% and greater than 65% of bound $^{45}Ca^{2+}$ were released by 10 μM and 100 μM MeHg respectively in both the absence and presence of ATP. Loading mitochondria with $^{45}Ca^{2+}$ in the presence of 20 μM RR reduced total uptake of $^{45}Ca^{2+}$ and greatly attenuated MeHg-induced release of $^{45}Ca^{2+}$ from mitochondria. RR did not inhibit the effects of MeHg on Ca^{2+} release by merely preventing the binding of MeHg to mitochondria since RR did not alter binding of labeled Me[^{203}Hg] to the organelle. The ratio of state 3 to state 4 respiration (respiratory control ratio) was measured as a means of assessing functional integrity of isolated mitochondria in the absence and presence of MeHg. Control ratios of from 3 to 5 were only marginally reduced by 2 μM MeHg but were greatly reduced by 10 and 20 μM

MeHg. The results of this study indicate that concentrations of MeHg which stimulate spontaneous transmitter release impair mitochondrial respiration, thus, impairing the functional integrity of the organelle. As a consequence, the ability of mitochondria to sequester Ca^{2+} is disrupted, inducing efflux and inhibiting uptake of Ca^{2+}. The MeHg-induced efflux of Ca^{2+} from mitochondria was prevented by block of the mitochondrial Ca^{2+} uniporter. Disruption of mitochondrial Ca^{2+} regulation by MeHg may increase cytoplasmic [Ca^{2+}] resulting in the well-described increase of spontaneous quantal release of transmitter by MeHg.

INTRODUCTION

Intracellular microelectrode recording studies have shown that the neurotoxic organomercurial methylmercury (MeHg) increases spontaneous quantal release of acetylcholine (ACh) at the neuromuscular junction (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982; Miyamoto, 1983; Atchison et al., 1984; Atchison, 1986). Increased spontaneous release of ACh is measured electrophysiologically as increased miniature end-plate potential (MEPP) frequency and occurs presumably as a result of increased free intraterminal Ca^{2+} ($[Ca^{2+}]_i$) (Llinas and Nicholson, 1975). The stimulatory effect of MeHg on MEPP frequency does not require extracellular Ca^{2+} since it occurs even in Ca^{2+}-deficient solutions (Atchison, 1986). Thus, a portion of the Ca^{2+} responsible for the increased MEPP frequency induced by MeHg may be released from bound intracellular stores. Komulainen and Bondy (1987) used a fluorescent probe for Ca^{2+} to demonstrate directly a significant increase in free Ca^{2+} in isolated nerve terminals after treatment with MeHg in Ca^{2+}-free solutions however the source of this apparent increase in cytoplasmic free Ca^{2+} is not known with certainty.

Major sites of Ca^{2+} buffering within nerve terminals are the mitochondrion and smooth endoplasmic reticulum (SER) (Blaustein et al., 1977; Blaustein et al., 1978; 1980; Scott et al., 1980; Akerman and Nicholls, 1981; Nicholls, 1986). Mitochondria are a likely source of Ca^{2+} for the increased MEPP frequency induced by MeHg. Mitochondrial inhibitors, such as dinitrophenol, dicoumarol or ruthenium red affect MEPP frequency in a manner similar to that of MeHg (Blioch et al., 1968; Glagoleva et al., 1970; Rahamimoff and Alnaes, 1973). Moreover, inorganic and

organomercurials inhibit mitochondrial respiration (Norseth and Brendeford, 1971; Magnaval et al., 1975; Verity et al., 1975; Sone et al., 1977; O'Kusky, 1983; Kauppinen et al., 1989) and ATP production (Sone et al., 1977; Kauppinen et al., 1989). In nerve terminals, this sequence of events results in increased $[Ca^{2+}]$ (Ashley et al., 1982; Heinonen et al., 1984).

Electrophysiological experiments at the rat neuromuscular junction provided preliminary evidence that nerve terminal mitochondria may be a source of Ca^{2+} for the increased spontaneous release of ACh produced by MeHg (Levesque and Atchison, 1987; 1988). Several different inhibitors of Ca^{2+} buffering by SER or mitochondria were tested for their ability to alter or perhaps block the stimulatory effects of MeHg on MEPP frequency. Only ruthenium red (RR), which blocks mitochondrial transport of Ca^{2+} via the uptake uniport protein (Moore, 1971), and N,N-bis (3,4-dimethoxyphenylethyl)-N-methylamine (YS035), which inhibits mitochondrial release of Ca^{2+} (Deana et al., 1984), completely prevented the stimulatory effects of MeHg on MEPP frequency. The complete block of MeHg-induced stimulation of MEPP frequency after pretreatment with RR or YS035 may indicate that these agents and MeHg act on a similar Ca^{2+} store or that they prevent access of MeHg to its site of action.

Ca^{2+} regulation in intact cells is a complex process and it is difficult to obtain a clear picture of potential interactions between MeHg and specific intracellular Ca^{2+} storage sites from observations made on intact tissue. Thus, to follow up on the electrophysiological data implicating mitochondria as a source of Ca^{2+} for the increased MEPP frequency produced by MeHg, the present experiments were designed to obtain detailed information regarding direct effects of MeHg on Ca^{2+}

transport by mitochondria isolated from rat brain. Separate pathways for uptake and efflux of Ca^{2+} operate unidirectionally to permit continuous cycling of Ca^{2+} across the inner mitochondrial membrane (Nicholls and Crompton, 1980; Carafoli, 1982). This enables mitochondria to regulate precisely the distribution of Ca^{2+} between the cytosol and the mitochondrial matrix. Changes in net flux of Ca^{2+} could result from perturbations of either the uptake or efflux pathways. Extra-mitochondrial free Ca^{2+} could become elevated following an interaction between MeHg and mitochondria to prevent uptake or induce release of Ca^{2+}. Therefore, effects of MeHg on Ca^{2+} sequestration by mitochondria were assessed by measuring uptake and release of $^{45}Ca^{2+}$ from mitochondria as a function of time and MeHg concentration. The specific goals of these experiments were to determine 1) whether MeHg blocks Ca^{2+} uptake into mitochondria; 2) whether MeHg causes release of Ca^{2+} from preloaded mitochondria; 3) whether the effects of MeHg on Ca^{2+} regulation are blocked by treatment with RR; 4) whether ruthenium red inhibits passive uptake of Me[^{203}Hg] by mitochondria, and; 5) whether MeHg disrupts mitochondrial respiratory control.

METHODS

Preparation of mitochondria. Mitochondria were isolated from forebrains of male Sprague-Dawley rats (Harlan, 175-225g) using a modification of the method of Booth and Clark (1978). This isolation procedure, which utilizes Ficoll/sucrose discontinuous gradients, was used because the free mitochondria obtained by the method are less contaminated by synaptosomes and have greater biochemical integrity than those prepared by sucrose-gradient techniques (Rafalowska et al., 1980; Dagani et al., 1985). The Ficoll/sucrose gradient method eliminates hyperosmotic gradients and lengthy preparation times which are known to compromise the functional integrity of mitochondrial preparations (Biesold, 1974; Booth and Clark, 1978; Dagani et al., 1985). Rats were sacrificed by decapitation and the forebrains were rapidly removed and dropped into ice-cold isolation medium (0.32 M-sucrose/ 1 mM-potassium EDTA/10 mM-Tris/HCl, pH 7.4). All isolation procedures were carried out at 0-4°C. The tissue was minced and then homogenized in a Dounce-type homogenizer by 8 up-and-down strokes at 550 rpm (pestle clearance 0.1 mm). The homogenate was diluted to 60 ml with isolation medium and spun at 1300g for 3 min in a Sorvall RC2-B high-speed centrifuge. The supernatant from this spin was centrifuged at 17,000g for 15 min, producing a crude mitochondrial/synaptosomal pellet. This pellet was resuspended in a total of 6 ml isolation medium and then 34 ml of 15% Ficoll/sucrose medium (15% (w/w) Ficoll, 0.32 M-sucrose, 50 μM-potassium EDTA, pH 7.4) was added. The crude suspension was then divided equally, introduced into separate 40 ml centrifuge tubes and above each, 12 ml of 7.5% Ficoll/sucrose medium (7.5%

(w/w) Ficoll, 0.32 M-sucrose, 50 μM-potassium EDTA, pH 7.4) was carefully layered. Finally, 5 ml of isolation medium was slowly layered to top off each tube. The tubes were centrifuged at 99,000g for 45 min in an SW27 swinging bucket rotor in a Beckman L565 ultracentrifuge. This ultracentrifugation step separates free mitochondria from myelin and synaptosomes as these latter cell components band at the first and second interphases respectively, with free mitochondria being pelleted at the bottom. The mitochondrial pellet from each tube was resuspended in 10 ml of isolation medium and centrifuged at 9,800g for 10 min. The supernatant from each tube was rapidly decanted, pellets were combined and washed with 10 ml of bovine plasma albumin (BPA) medium (10 mg BPA in 20 ml isolation medium) and pelleted at 9,800g for 10 min. The purified mitochondria were resuspended in K^+ buffer (135 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose) at 6-8 mg protein/ml for $^{45}Ca^{2+}$ flux studies and in isolation medium at 8-10 mg protein/ml for respiratory control experiments. Although the majority of these mitochondria may not be from nerve terminals, brain mitochondria of synaptosomal and non-synaptosomal origin have similar Ca^{2+} transport properties (Nicholls, 1978). Mitochondrial protein was determined by the method of Lowry et al., (1951).

Measurements of $^{45}Ca^{2+}$ uptake and efflux by mitochondria. Flux studies using radiolabeled Ca^{2+} were done according to the method of Suszkiw et al. (1984) with slight modifications. Uptake of $^{45}Ca^{2+}$ by mitochondria was initiated by adding 50 μl of mitochondria in K^+ buffer (300-400 mg protein) to an equal volume of K^+ buffer supplemented with 50 μM $^{45}CaCl_2$, $\pm$ 2.0 mM ATP and $\pm$ MeHg at twice the desired final concentration. After allowing the solutions to mix for various time intervals (see figure legends), 2 ml of ice-cold K^+ buffer containing 1 mM EGTA was

added to stop $^{45}Ca^{2+}$ uptake. When effects of MeHg on $^{45}Ca^{2+}$ uptake were tested, MeHg was present in the K^+ buffer containing $^{45}Ca^{2+}$ before mitochondria were added. To test the effects of MeHg on release of $^{45}Ca^{2+}$ from mitochondria, the organelles were incubated with K^+ buffer containing labeled $^{45}Ca^{2+}$ (50 μM) for 60 sec before adding MeHg. MeHg was present for 10 sec before stopping the reaction. RR (20 μM) was tested for its effects on MeHg-induced release of $^{45}Ca^{2+}$ from mitochondria by adding it during $^{45}Ca^{2+}$ loading prior to adding MeHg.

Immediately after addition of the EGTA-supplemented K^+ buffer, samples were filtered under suction through Millipore filters (0.45 μm) and washed with two 5 ml aliquots of ice-cold K^+-EGTA buffer. Radioactivity retained on the filters represented $^{45}Ca^{2+}$ retained by mitochondria. 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. Radioactivity was determined in a Beckman LS 7000 liquid scintillation counter with a 70% efficiency for $^{45}Ca^{2+}$.

Measurement of respiratory rates and control. Mitochondrial oxygen consumption was measured polarographically with a Beckman oxygen electrode at 25°C in a closed vessel equipped with a stir bar. The respiratory medium used was that described by Ozawa et al. (1966): 0.3 M mannitol, 10 mM KCl, 10 mM Tris-HCl, 5 mM potassium phosphate and 0.2 mM EGTA. The respiratory substrates were 5 mM glutamate or a combination of 1 mM pyruvate + 2.5 mM malate. Each experiment was started by adding 1-2 mg of mitochondrial protein to the oxygraph vessel containing incubation medium and substrate. Mitochondria as well as other additions to the oxygraph were made through small injection ports. In experiments in which the effects of MeHg were tested, MeHg was added after

an equilibration period of 2 min. Three min after adding mitochondria, 300 μM ADP was added to initiate State 3 respiration. State 4 respiration was measured as the slower respiratory rate prior to addition of ADP or after its depletion. The respiratory control ratio is the ratio of the respiration rate in the presence of ADP (State 3) to that after utilization of ADP (State 4) (Chance and Williams, 1955).

Binding of Me[^{203}Hg] to mitochondria. Passive uptake of MeHg by mitochondria was measured by adding 50 μl of mitochondria in K^+ buffer (150-200 μg protein) to an equal volume of K^+ buffer containing Me[^{203}Hg]. Uptake of Me[^{203}Hg] was measured during intervals of 1,10,60,120 and 180 sec. The effects of RR on passive uptake of Me[^{203}Hg] by mitochondria were determined by comparing uptake of Me[^{203}Hg] in RR-free control experiments to uptake in experiments in which RR was added to mitochondria in K^+ buffer concurrently with MeHg. Uptake of Me[^{203}Hg] was stopped by rapidly filtering the samples through 0.45 μm Millipore filters under suction. The filters were washed twice with 5 ml of cold K^+ buffer. Filters were then collected and 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. Radioactivity retained on the filters was measured in a Searle model 1197 gamma counter with a 45% efficiency for Me[^{203}Hg].

Materials. Methylmercury chloride was purchased from K+K Rare and Fine chemicals (Plainview, NY). This chloride salt of MeHg is readily soluble in aqueous solutions and could be dissolved directly in the physiological buffers used in these experiments. Ficoll type 400-DL, $[K^+]_2$ Ethylenediamine tetraacetic acid (K^+ EDTA), ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), Trizma HCl, ATP, ADP, D-mannitol, pyruvate, malate, glutamate and ruthenium red were

from Sigma Chemical Co. (St. Louis, MO). N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from United States Biochemical Corporation (Cleveland, OH). $^{45}CaCl_2$ (15-20 mCi/g) and methyl[^{203}Hg]chloride (18 mCi/g) were purchased from New England Nuclear Co. (Boston, MA) and Amersham Corporation (Arlington Hts., IL). The scintillation cocktail used was Formula 963 from New England Nuclear (Boston, MA).

Animals. Adult male Sprague-Dawley rats (Harlan, 175-225g) were housed in plastic cages in a room which received 12 hrs of light per day. Room temperature was maintained at 22 to 24°C and relative humidity at 40 to 60%. Food (Purina Rat Chow) and water were provided ad libitum.

Statistical Analysis. Data were analyzed statistically using a randomized block analysis of variance (Steel and Torrie, 1960). Differences among treatment means were compared using Duncan's multiple range test and Dunnett's t-test. Differences were considered to be statistically significant at $P < .05$.

RESULTS

Respiratory control ratios (RCR's) were monitored as a means of assessing the functional integrity of isolated mitochondria. The RCR was measured as the ratio between the rates of substrate oxidation in the presence and absence of ADP. With 1 mM pyruvate + 2.5 mM malate as substrates for oxidation, the RCR was 3.9 ± 0.9 (n=5) for purified mitochondria. The RCR was 4.4 ± 1.4 (n=5) with 5 mM glutamate as substrate. These values are consistent with those reported by others (Booth and Clark, 1978; Dagani et al., 1988) using the Ficoll/sucrose procedure for isolating brain mitochondria and indicate that electron transport and oxidative phosphorylation are well-coupled in these organelles.

Mercurials alter cellular energy metabolism and impair respiration both in vitro (Fox et al., 1975; Verity et al., 1975; Sone et al., 1977; Von Burg et al., 1979; Kauppinen et al., 1989) and in vivo (Verity et al., 1975; O'Kusky, 1983). Since inhibition of mitochondrial respiration impairs the ability of the organelle to regulate intra- and extramitochondrial Ca^{2+} (Ashley et al., 1982; Heinonen et al., 1984; Kauppinen et al., 1988), it was of interest to test for effects of MeHg on respiration in our preparation of brain mitochondria. MeHg prevented the increased oxygen consumption which normally occurs following addition of ADP to mitochondria suspended in respiratory medium (Figure 1). At 2 μM, MeHg decreased ADP-induced respiration (State 3) by 15 ± 5% and reduced the RCR to 2.6 ± 0.9% (n=4). In several experiments, respiratory activity prior to addition of ADP (State 4) was elevated slightly by 2 μM MeHg, a result consistent with previous findings (Verity et al., 1975). Both resting and ADP-stimulated respiration were attenuated

markedly by 10 μM MeHg and were almost completely inhibited by 20 μM MeHg. As a result, RCR's at both concentrations of MeHg approached 1, indicating total loss of respiratory control. These data show that MeHg, in a dose-dependent manner, reduces the efficiency with which the free energy of oxidation is converted into the free energy of hydrolysis of ATP and that the functional integrity of these mitochondria may be disrupted.

Since mitochondria were discovered to have a large capacity for accumulating Ca^{2+}, investigators have proposed mitochondrial involvement in regulating intracellular Ca^{2+} (Lehninger et al., 1967). Sequestration of Ca^{2+} by mitochondria requires energy supplied by ATP or by respiration (Lehninger et al., 1967; Tjioe et al., 1970). To measure uptake of Ca^{2+} by mitochondria in our preparation from rat brain, suspensions of the organelles were incubated with $^{45}Ca^{2+}$ in the absence and presence of ATP. Uptake of Ca^{2+} occurred rapidly and saturated within 60-90 sec (Figure 2). Addition of 1.2 mM ATP to the incubation medium markedly enhanced uptake compared to ATP-free controls as shown previously by others (Tjioe et al., 1970; Rottenberg and Marbach, 1989).

MeHg could disrupt mitochondrial Ca^{2+} cycling and increase extramitochondrial Ca^{2+} by preventing uptake of Ca^{2+} by the organelle. The effects of MeHg on uptake of Ca^{2+} by mitochondria were tested by measuring uptake of $^{45}Ca^{2+}$ by mitochondria in the presence of MeHg during incubation intervals of various lengths. MeHg impaired uptake of $^{45}Ca^{2+}$ by mitochondria in a concentration-and time-dependent manner (Figure 3). At 10 μM, MeHg did not decrease $^{45}Ca^{2+}$ uptake during 10 sec of incubation but reduced uptake by over 30% at 30 and 90 sec and by over 50% during longer incubations of mitochondria

with $^{45}Ca^{2+}$. At 100 μM, MeHg significantly reduced $^{45}Ca^{2+}$ uptake by 65-80% irrespective of the duration of exposure tested. After reaching peak levels of nearly 3 nmoles/μg protein at 90 sec, $^{45}Ca^{2+}$ sequestered by untreated mitochondria declined steadily as observed to occur when brain mitochondria sequester Ca^{2+} in the absence of exogenous ATP (Nicholls and Scott, 1980; Hofstetter et al., 1981; Rottenberg and Marbach, 1989). ATP may enable mitochondria to retain Ca^{2+} by acting as a chelator of intramitochondrial Ca^{2+} (Carafoli et al., 1965), by protecting mitochondria against calcium-induced 'damage' (Nicholls and Scott, 1980) or by direct effects on the membrane (Hofstetter et al., 1981; Rottenberg and Marbach, 1989). Inhibition of $^{45}Ca^{2+}$ uptake by MeHg occurred even when the incubation medium was supplemented with ATP (Figure 4). 100 μM MeHg reduced uptake by 80-95% at all time points tested.

MeHg could also perturb cellular Ca^{2+} homeostasis by causing efflux of Ca^{2+} stored within mitochondria. Sulfhydryl reagents evoke Ca^{2+} release from isolated mitochondria (Scott et al., 1970; Brierley et al., 1978; Pfeiffer et al., 1979; Harris and Baum, 1980; Beatrice et al., 1984; Chavez and Holguin, 1988). Figure 5 shows results obtained in experiments in which mitochondria were incubated with $^{45}Ca^{2+}$ for 60 sec before being exposed to MeHg for 10 sec. Before adding MeHg, intramitochondrial levels of Ca^{2+} reached 2.6 $\pm$ 0.4 x 10^{-3} and 25 $\pm$ 6 x 10^{-3} nmoles/μg protein in the absence (Figure 5a) and presence (Figure 5b) of ATP, respectively. In each case, addition of MeHg caused a concentration-dependent efflux of Ca^{2+} from mitochondria. $^{45}Ca^{2+}$ associated with mitochondria was reduced significantly by MeHg at concentrations of 25 μM and higher in the absence of ATP and by 50 μM and above when $^{45}Ca^{2+}$ uptake was stimulated by ATP. MeHg did

not cause a complete loss of $^{45}Ca^{2+}$ from mitochondria even at the highest concentrations tested. Maximal $^{45}Ca^{2+}$ efflux occurred with 100 μM and nearly 200 μM MeHg in the absence and presence of ATP, respectively. It should be noted that in the absence of MeHg, intramitochondrial Ca^{2+} levels remained constant during the 10 sec period following the intial 60 sec incubation of mitochondria with $^{45}Ca^{2+}$. Thus in experiments with MeHg, Ca^{2+} released during this 10 sec period was due to the actions of MeHg.

RR, which inhibits Ca^{2+} uptake via the uniporter protein on the inner mitochondrial membrane (Moore, 1971) and inhibits efflux of Ca^{2+} that occurs through the reversed uniporter (Fiskum and Cockrell, 1978; Luthra and Olsen, 1977; Jurkowitz et al., 1983), blocks MeHg-induced increases in spontaneous quantal release of transmitter at the neuromuscular junction (Levesque and Atchison, 1987). RR may have blocked the effects of MeHg on spontaneous release of transmitter by inhibiting MeHg-induced release of Ca^{2+} from mitochondria. Accordingly, experiments were designed to determine whether RR could block MeHg-induced efflux of $^{45}Ca^{2+}$ from isolated mitochondria (Figure 6). Mitochondria were incubated with $^{45}Ca^{2+}$ and RR for 60 sec in the absence and presence of ATP. RR greatly reduced both nonstimulated and ATP-stimulated uptake of $^{45}Ca^{2+}$ from controls when it was added at the same time as $^{45}Ca^{2+}$. The inhibitory effect on $^{45}Ca^{2+}$ uptake was more pronounced in medium supplemented with ATP. Perhaps when Ca^{2+} uptake is driven by ATP, more enters through the uniporter as compared to when uptake occurs without ATP. Addition of 100 μM MeHg to mitochondria preincubated with $^{45}Ca^{2+}$ and RR did not evoke release of $^{45}Ca^{2+}$ from mitochondria. RR completely prevented the MeHg-induced efflux of

Ca^{2+} from mitochondria whether Ca^{2+} uptake was driven by ATP or not. Because RR inhibited uptake of $^{45}Ca^{2+}$, intramitochondrial levels of $^{45}Ca^{2+}$ were low at the time that MeHg was added. MeHg does not appear to deplete mitochondria totally of Ca^{2+} (Figure 5) and perhaps after treatment with RR, the concentration of $^{45}Ca^{2+}$ was too low to be acted upon by MeHg. Thus, mitochondria were loaded with $^{45}Ca^{2+}$ before adding RR (Figure 6b). This allowed for uptake of greater quantities of $^{45}Ca^{2+}$ by mitochondria. Still, exposure of mitochondria to MeHg for 10 sec did not significantly reduce $^{45}Ca^{2+}$ content following treatment with RR. Block of MeHg-induced release of Ca^{2+} by RR could be due to the inhibitory effects of RR on Ca^{2+} transport via the uniporter or perhaps RR simply prevented binding or access of MeHg to the mitochondria. To test this, suspensions of mitochondria were incubated with Me[^{203}Hg] for various time intervals in the presence and absence of RR (Figure 7). Binding of Me[^{203}Hg] occurred immediately, reaching 5.1 ± 1.6 x 10^3 nmoles/μg mitochondrial protein within 1 second, and increased to 7.9 ± 2.0 x 10^3 nmoles/μg protein after 3 min. RR did not inhibit the binding of Me[^{203}Hg] to the mitochondria. This suggests that RR blocked MeHg-induced release of $^{45}Ca^{2+}$ by a more specific effect on the Ca^{2+} release process.

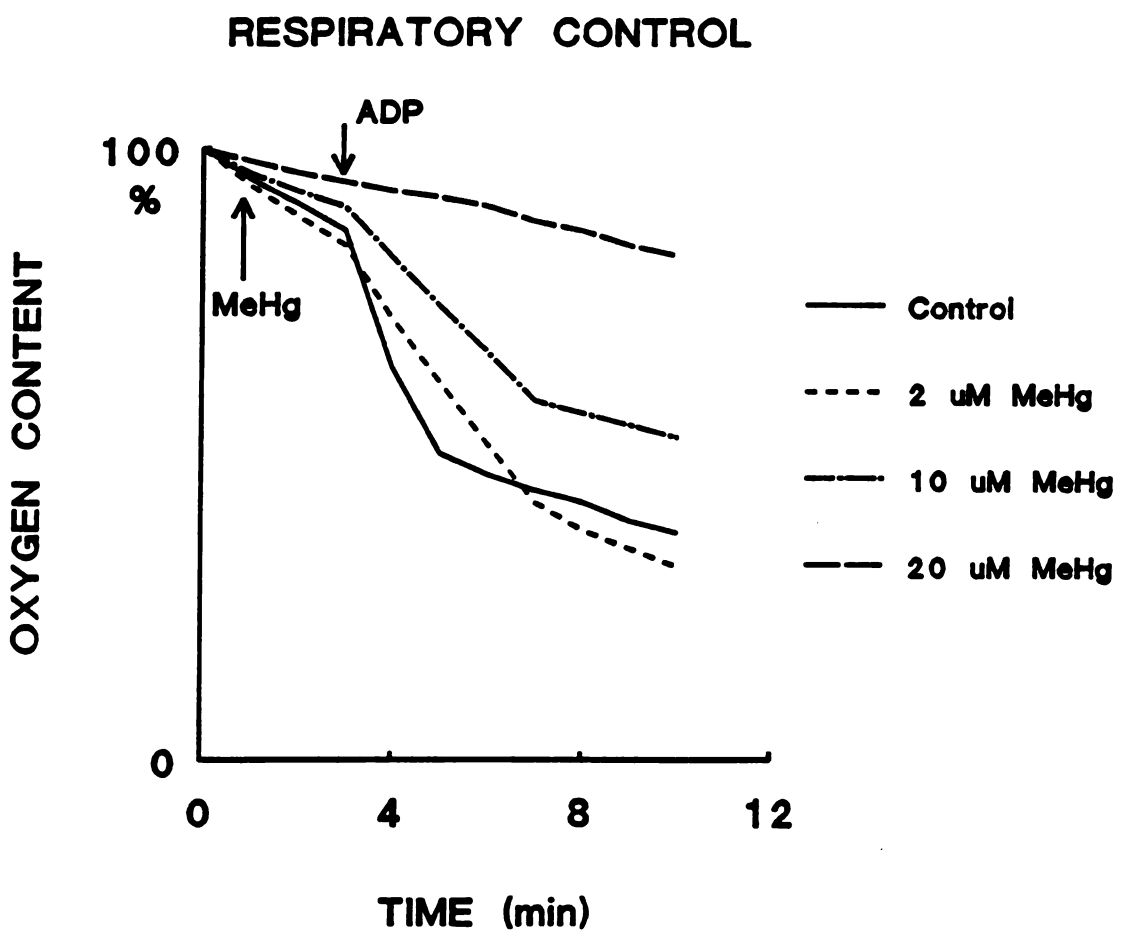

Figure 1. Representative traces of effects of MeHg on mitochondrial respiration. Each curve represents a separate experiment and depicts oxygen utilization by mitochondria over time. The oxygen content of the respiratory medium is shown on the y-axis and is considered to be 100% at the start of each experiment. The respiratory medium contained (mM): mannitol, 300; KCL, 10; Tris-HCL, 10; potassium phosphate, 5; EGTA, 0.2. Respiratory substrates were 1 mM pyruvate and 2.5 mM malate. Mitochondria (1.5-2.5 mg protein) were added at "time zero" and MeHg was added 1 min later. 300 μM ADP was added three min after mitochondria to initiate state 3 respiration. Resting or state 4 respiration was the slower respiratory rate following ADP depletion.

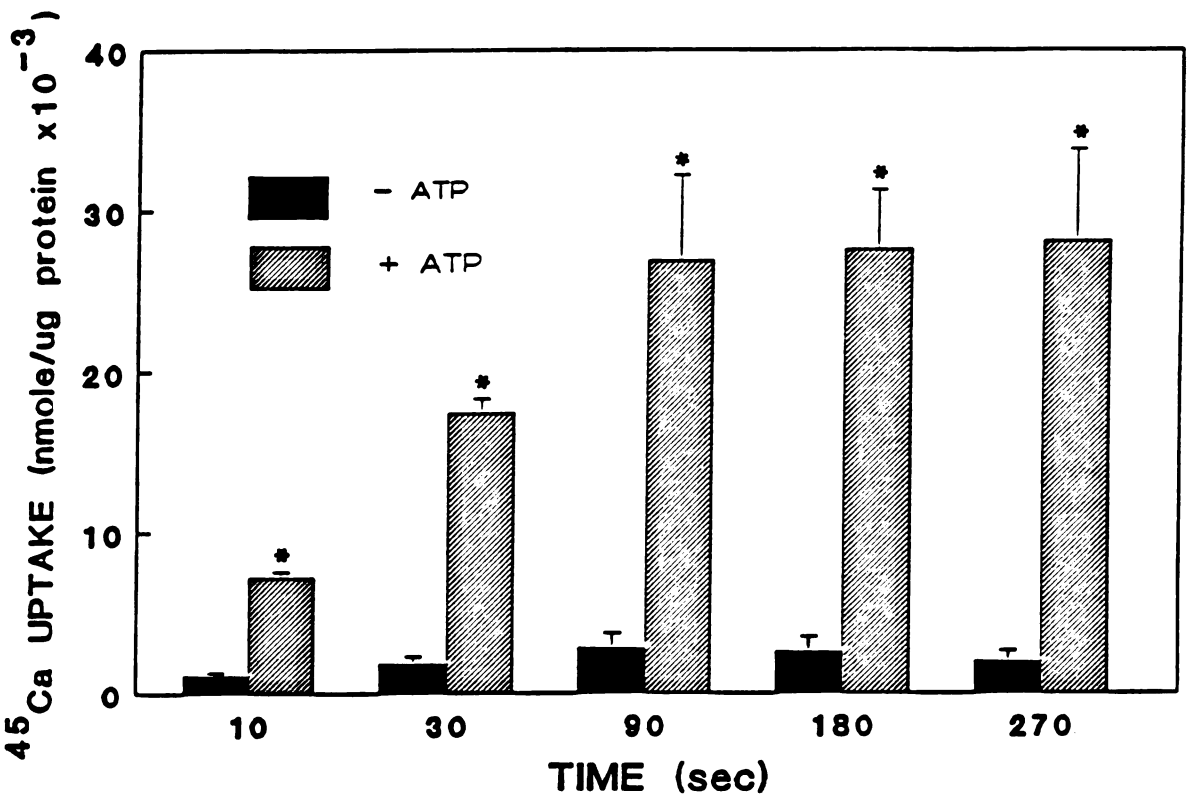

Figure 2. Time course of $^{45}Ca^{2+}$ uptake by mitochondria isolated from rat forebrain in the absence and presence of ATP. Mitochondria were added to K^+ HKR which contained 135 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 10 mM glucose and 50 μM $^{45}Ca^{2+}$ ± 1.2 mM ATP to initiate uptake. The uptake reaction was stopped by adding ice-cold quench buffer containing 1 mM EGTA after 10, 30, 90, 180 or 270 sec. Values are the mean ± SEM of five different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates a value significantly greater ($P \leq .05$) than $^{45}Ca^{2+}$ uptake without ATP.

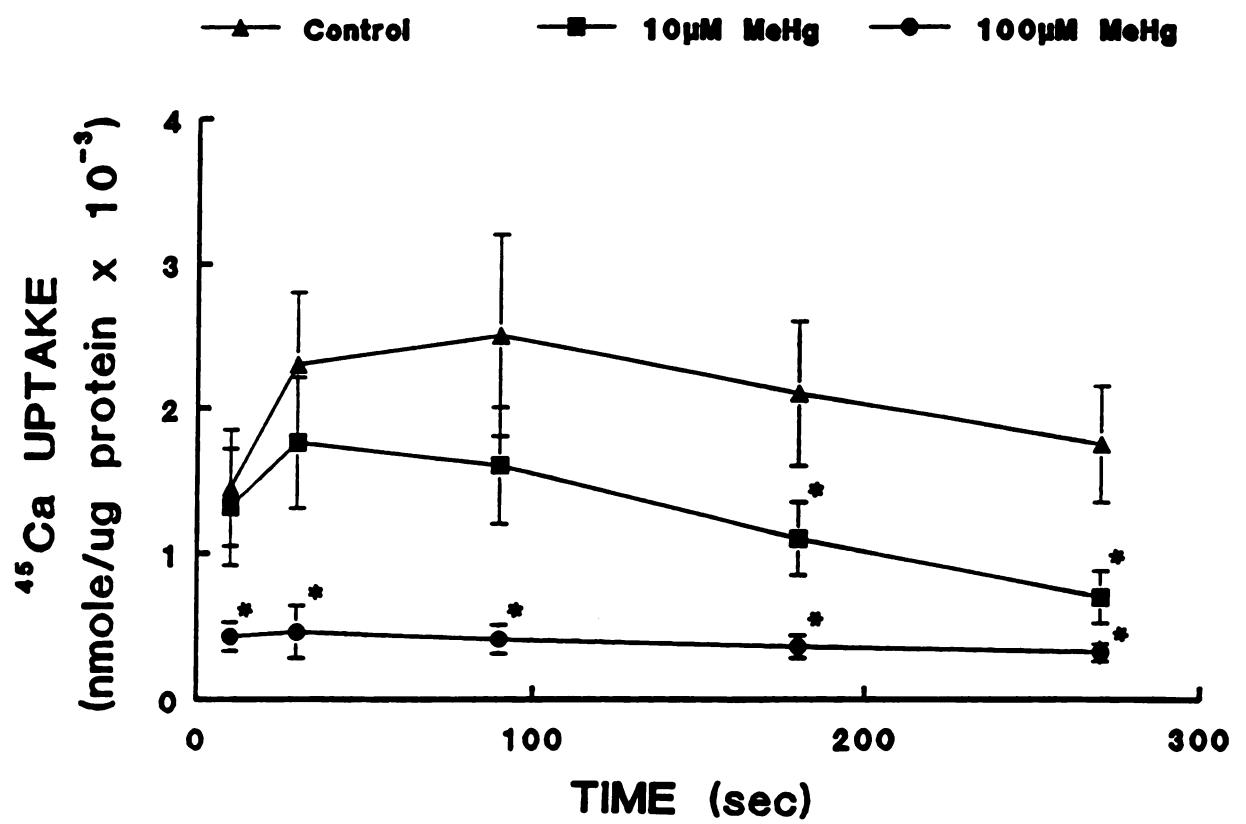

Figure 3. Time course of effects of MeHg (10 and 100 μM) on uptake of $^{45}Ca^{2+}$ by mitochondria. MeHg and $^{45}Ca^{2+}$ (50 μM) were added simultaneously to the suspension of mitochondria in K^+ HKR. The uptake reaction was quenched after 10, 30, 90, 180 or 270 sec of incubation. Values are the mean ± SEM of five different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates significantly less $^{45}Ca^{2+}$ uptake relative to MeHg-free controls ($P \leq .05$).

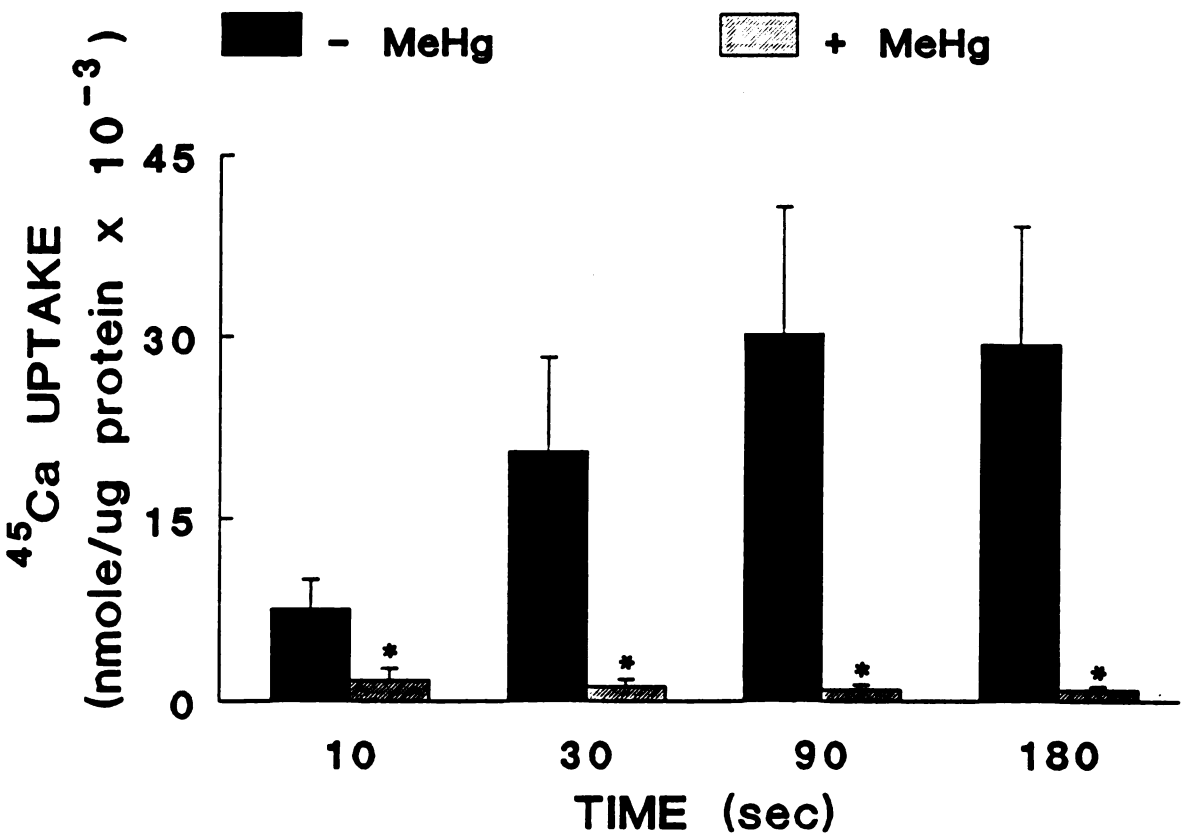

Figure 4. Time course of effects of MeHg (100 μM) on uptake of $^{45}Ca^{2+}$ (50 μM) by mitochondria in the presence of 1.2 mM ATP. When present, MeHg was added together with ATP at the start of the $^{45}Ca^{2+}$ uptake reaction. The uptake reactions were quenched after 10, 30, 90 and 180 sec. Values are the mean ± SEM of five different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates significantly less $^{45}Ca^{2+}$ uptake relative to MeHg-free controls ($P \leq .05$).

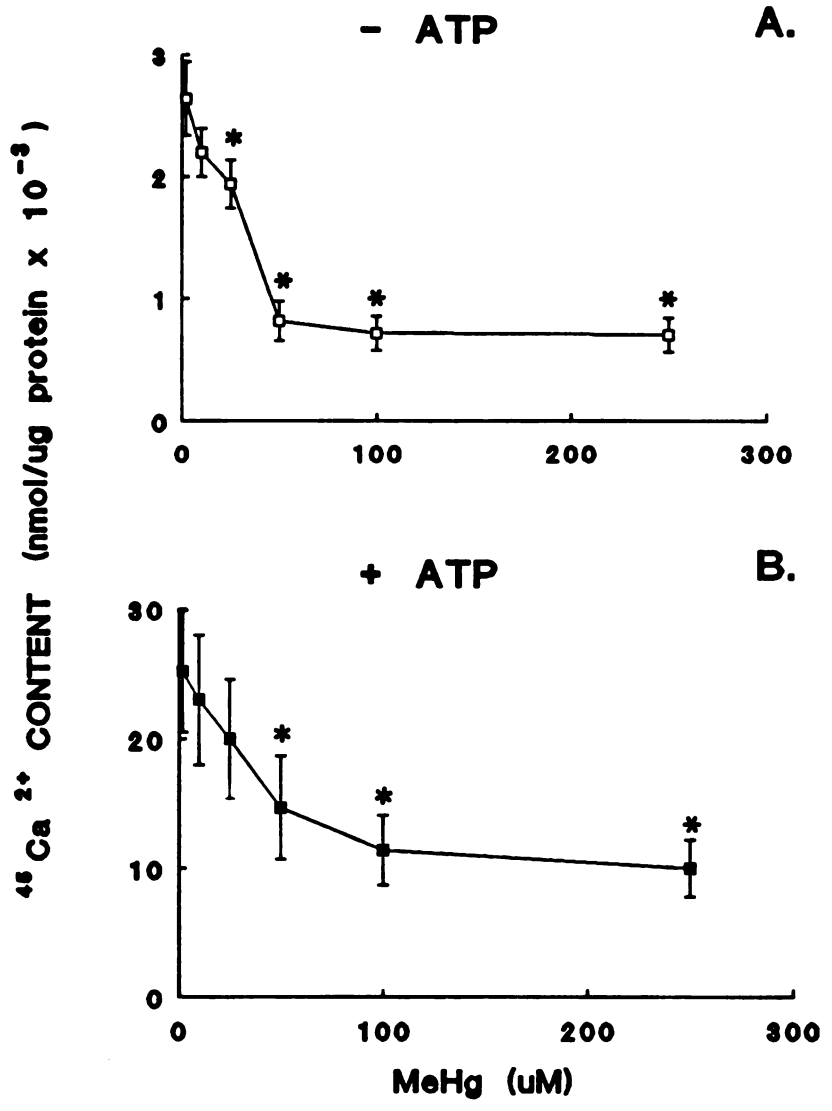

Figure 5. Effects of MeHg on release of $^{45}Ca^{2+}$ from mitochondria preloaded with $^{45}Ca^{2+}$ (50 μM) in the absence (A) or presence (B) of 1.2 mM ATP. Mitochondria were preloaded with $^{45}Ca^{2+}$ by incubating with $^{45}Ca^{2+}$ for 60 sec prior to addition of 2, 10, 25, 50, 100 and 250 μM MeHg. The reaction was quenched 10 sec after adding MeHg. Values refer to the amount of $^{45}Ca^{2+}$ remaining in the mitochondria and are the mean ± SEM of five determinations. Values for each experiment are the average of three replicates. The asterisk (*) indicates a significant reduction in intrasynaptosomal $^{45}Ca^{2+}$ content after treatment with MeHg compared to MeHg-free controls ($P \leq .05$).

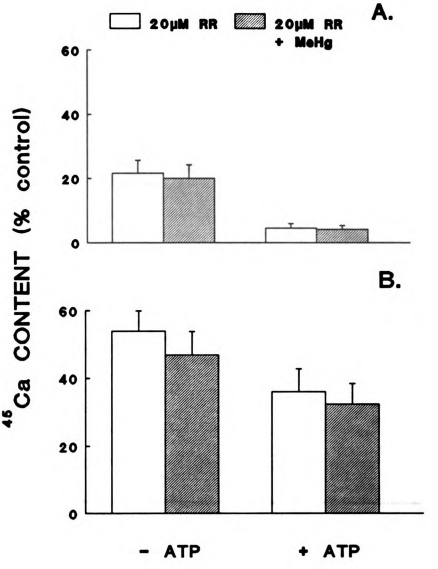

Figure 6. Effects of ruthenium red (RR, 20 μM) on MeHg (100 μM)-induced release of $^{45}Ca^{2+}$ from mitochondria preloaded for 60 sec with $^{45}Ca^{2+}$ (50 μM). RR was either present before (A) initiating the 60 second $^{45}Ca^{2+}$ loading reaction or was added 15 sec after (B) initiating loading. In both cases, MeHg was added immediately following the 60-sec loading period and the reaction was quenched 10 sec after adding MeHg. Experiments were performed in the absence and presence of 1.2 mM ATP. Results are expressed as the percentage of $^{45}Ca^{2+}$ retained within mitochondria after incubating in the presence of RR (open bars) or after incubating with RR and treating with MeHg (shaded bars), relative to parallel incubations without RR or MeHg. Values are the mean ± SEM of five experiments. Values for each experiment are the average of three replicates.

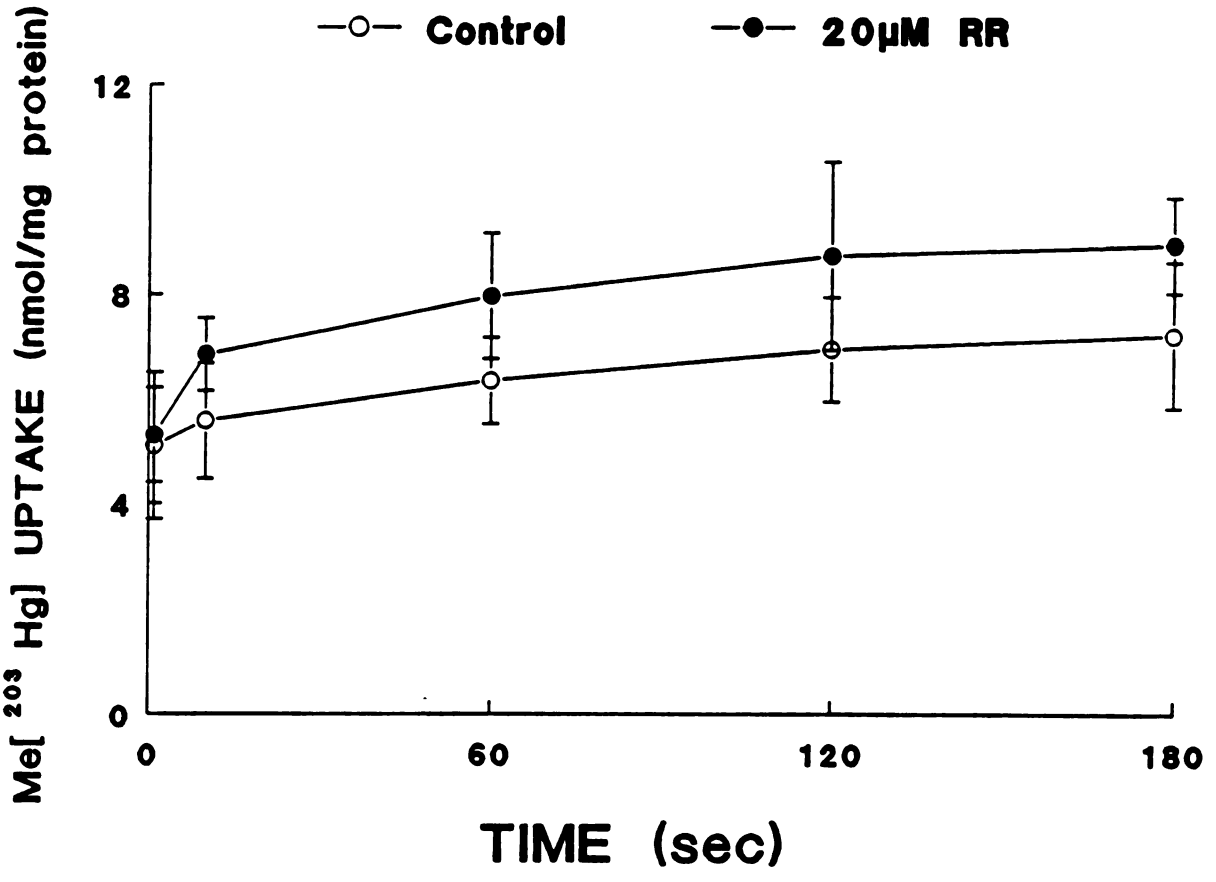

Figure 7. Time course of effects of RR (20 μM) on passive uptake of Me[^{203}Hg] (100 μM) during intervals of 1, 10, 60, 120 and 180 sec. Uptake was stopped by rapidly filtering the samples through 0.45 μm filters under suction. Values are the mean ± SEM of seven experiments. Values for each experiment are the average of three replicates.

DISCUSSION

The release of neurotransmitters from presynaptic nerve terminals is induced by increased $[Ca^{2+}]$. $[Ca^{2+}]$ can become elevated following depolarization of the nerve terminal, which causes increased plasma membrane permeability to Ca^{2+} with subsequent Ca^{2+} influx, or by disruption of Ca^{2+} sequestration by internal stores. Mitochondria are a major intraneuronal source of Ca^{2+} and presumably may play an important role in the regulation of $[Ca^{2+}]$ under conditions of Ca^{2+} loading (Nicholls, 1986). Inhibition of mitochondrial Ca^{2+} transport causes increased spontaneous release of transmitter. In the present work we investigated the effects of MeHg on isolated brain mitochondria to determine whether MeHg could interact with these organelles to impair their ability to sequester Ca^{2+}. MeHg impaired respiration and disrupted both uptake and retention of Ca^{2+} by mitochondria. Since spontaneous release of transmitter is increased by elevated $[Ca^{2+}]$ (Llinas and Nicholson, 1975), inhibition of these processes by MeHg in nerve terminals would likely result in stimulation of this form of transmitter release. RR blocked MeHg-induced release of $^{45}Ca^{2+}$ from isolated mitochondria in these experiments and prevented stimulation of spontaneous release of transmitter, as described in previous electrophysiological experiments (Levesque and Atchison, 1987). The results obtained with ruthenium red in the neurochemical and in the electrophysiological experiments indicate that MeHg may enhance spontaneous release of transmitter by disrupting mitochondrial Ca^{2+} buffering.

Because isolated mitochondria were used in the present study, potential diffusional barriers such as the plasma membrane were not present. Thus, MeHg

could easily reach and interact directly with mitochondrial membranes. In intact nerve terminals or in whole tissue preparations, MeHg would first have to permeate through membrane barriers before gaining entrance into the nerve terminal cytosol and interacting with intraneuronal mitochondria. Several lines of evidence from the literature indicate that MeHg can readily transverse biological membranes. The methyl group imparts sufficient lipophilicity upon the molecule to enable it to pass through lipid bilayers (Lakowicz and Anderson, 1980). Thus, the organomercurial should also penetrate cell membranes. MeHg can penetrate the blood-brain barrier and enter nerve cells from the bloodstream (Nordberg et al., 1970; Chang and Hartmann, 1972a,b). Once within the nerve cell, MeHg is bound primarily to membranous organelles such as mitochondria and Golgi complex. MeHg affects mitochondrial functions in situ (Fox et al., 1975; Verity et al., 1975; Cheung and Verity, 1981), suggesting that it can reach mitochondria. Finally, pathological changes in the ultrastructure of mitochondria from dendrites, axons and presynaptic terminals of cortical neurons have been observed after subcutaneous injection of rats with methylmercury (O'Kusky, 1983). It was concluded that the ultrastructural changes were due to inhibition of mitochondrial respiration by MeHg.

The effects of MeHg on uptake and release of Ca^{2+} could occur as a result of a MeHg-induced collapse of the mitochondrial membrane potential. Collapse of the electrical gradient across the inner mitochondrial membrane impairs uptake of Ca^{2+} and causes efflux via backflow of Ca^{2+} through the reversed uptake uniporter (Pozzan et al., 1977; Carafoli, 1982; Nicholls and Akerman, 1982). Because RR is a highly effective inhibitor of Ca^{2+} uptake via the uniporter, the efflux of Ca^{2+} via this pathway might be expected to be blocked by RR. Some

investigators have indeed reported that RR inhibits Ca^{2+} efflux via this pathway (Fiskum and Cockrell, 1978; Luthra and Olson, 1977; Jurkowitz et al., 1983) while others have shown this efflux to be insensitive to RR (Vasington et al., 1972; Puskin et al., 1976). The block of the MeHg-induced efflux of Ca^{2+} from mitochondria by RR observed in this study could indicate that the efflux caused by MeHg occurred as a result of a collapsed mitochondrial membrane potential and/or a reversed uniporter.

We, as well as others, (Verity et al., 1975; Sone et al., 1977; O'Kusky, 1983; Kauppinen et al., 1989), have found that MeHg inhibits mitochondrial respiration. MeHg has a high affinity for sulfhydryl groups (Clarkson, 1972) and perhaps it denatures or inactivates sulfhydryl-containing enzymes and electron carriers that are important for normal functioning of the energy transducing pathway (Verity, 1975; Nicholls, 1982). Along with inhibiting respiration, MeHg also decreases ATP production by mitochondria (Cheung and Verity, 1981; Kauppinen et al., 1989). Inhibition of respiration and ATP synthesis collapses the mitochondrial membrane potential causing uptake carrier reversal and Ca^{2+} efflux (Ashley et al., 1982; Kauppinen et al., 1989). In our study, inhibition of respiration was probably not the primary cause of MeHg-induced impairment of mitochondrial uptake and retention of Ca^{2+}. ATP can support uptake of Ca^{2+} by nonrespiring mitochondria. However, MeHg inhibited uptake and induced release of Ca^{2+} even in the presence of added ATP. Also, MeHg significantly reduced the mitochondrial Ca^{2+} content after only 10 sec of incubation with $^{45}Ca^{2+}$-loaded mitochondria. One would expect that a longer interval would be necessary before respiration and mitochondrial ATP levels would be reduced sufficiently to cause collapse of the mitochondrial membrane

potential and Ca^{2+} efflux. MeHg can increase free Ca^{2+} in synaptosomes before ATP levels are decreased, suggesting that mitochondrial energy production may be acutely less sensitive to MeHg than is $[Ca^{2+}]_i$. Rather than being the primary cause of impaired mitochondrial Ca^{2+} transport and increased $[Ca^{2+}]_i$, inhibition of respiration may be a consequence of some primary effect of MeHg on the mitochondrion which results in impaired uptake and release of Ca^{2+} by the organelle.

Sulfhydryl groups of membrane proteins may play a role in cation permeability and mitochondrial Ca^{2+} transport. Thiol reagents including N-ethylmaleimide (Ramachandran and Bygrave, 1978; Pfeiffer et al., 1979; Harris et al., 1979; Beatrice et al., 1980; Pfeiffer et al., 1983), diamide (Palmer and Pfeiffer, 1981; Vercesi, 1984), MeHg (Harris and Baum, 1980) and Hg^{2+} (Chavez and Holguin, 1988) produce an immediate release of Ca^{2+} from mitochondria. The sequence of events leading to the release of Ca^{2+} has been postulated to include an initial increase in membrane permeability, perhaps due to activation of phospholipase A_2 (Pfeiffer et al., 1979) or removal of membrane bound adenine nucleotides and Mg^{2+} (Harris et al., 1979), and an ensuing influx of K^+. Accumulation of K^+ collapses the mitochondrial membrane potential and ultimately results in Ca^{2+} release by reversed activity of the uptake uniporter. Involvement of the reversed uniporter in this Ca^{2+} efflux process is likely since the release of Ca^{2+} is inhibited by RR (Jurkowitz et al., 1983; Pfeiffer et al., 1983; Riley and Pfeiffer, 1986; Chavez and Holguin, 1988). Also, the K_i for K^+-induced Ca^{2+} release and for respiratory driven uniporter uptake of Ca^{2+} is the same (Rigoni et al., 1980). Several groups have demonstrated that MeHg increases the permeability of the

inner mitochondrial membrane to K^+ (Scott et al., 1970; Southard et al., 1974; Verity et al., 1975; Sone et al., 1977; Bogucka and Wojczak, 1979), but did not attempt to determine the effects of the intramitochondrial K^+ accumulation on Ca^{2+} release. The rapidity with which thiol reagents release Ca^{2+}, the sensitivity of the release to RR and the known effects of MeHg on the K^+ permeability of the inner mitochondrial membrane suggest that the mechanism underlying the effects of MeHg on Ca^{2+} release from mitochondria could involve binding to membrane thiols, increased K^+ permeability and membrane potential collapse. This could also be the principal mechanism by which MeHg inhibits respiration. Verity et al. (1975) suggested that synaptosomal respiration was inhibited by MeHg subsequent to increased permeability of the mitochondrial membrane to K^+.

MeHg was probably bound to thiol groups of the brain mitochondria used in the present study since bound Me[^{203}Hg] could be almost completely removed from mitochondria by treatment with the thiol reducing agent, dithiothreitol (results not shown). Binding of approximately 5 nmol of Me[^{203}Hg]/mg of mitochondrial protein occurred during the 10 sec interval that $^{45}Ca^{2+}$ loaded mitochondria were incubated with MeHg. Binding of 1 nmol of Hg^{2+}/mg of mitochondrial protein from heart (Chavez and Holguin, 1988) and 1 nmol of MeHg/mg of mitochondrial protein from kidney (Harris and Baum, 1979) induced release of Ca^{2+} from the mitochondria subsequent to collapse of the membrane potential. Chavez and Holguin (1988) tested RR for its effects on Ca^{2+} release and found that it blocked release induced by Hg^{2+}.

Although it seems likely that RR interacts with the Ca^{2+} uniporter to block the effects of MeHg on Ca^{2+} release, the actions of RR may be more complicated. RR

is a polysaccharide dye (Luft, 1971) which binds to proteins and lipids on membrane surfaces, but very little is known about the mode and consequences of its binding. RR may alter the structure or integrity of the membrane. Rigoni et al. (1980) suggested that effects of RR on mitochondrial Ca^{2+} efflux that they observed may have been due in part to changes of membrane structure and not only as a result of its binding to the Ca^{2+} uniporter. As mentioned, we tested for the possibility that RR blocked the effects of MeHg on mitochondrial buffering of Ca^{2+} by blocking the binding of MeHg to mitochondria and found that RR did not prevent the binding of Me[^{203}Hg]. Despite this, the possibility that RR blocked the effects of MeHg on Ca^{2+} release by a non-specific alteration of the membrane rather than by a specific interaction with the uniporter cannot be discounted. nl conclusion, MeHg inhibited ADP-stimulated respiration and respiratory control in isolated rat brain mitochondria. Also, MeHg disrupted mitochondrial transport of Ca^{2+} by inhibiting uptake and inducing Ca^{2+} efflux in the absence and presence of ATP. RR inhibited uptake of Ca^{2+} by mitochondria and blocked MeHg-induced efflux of Ca^{2+} from the organelle. The results with RR were taken as further evidence that the stimulatory effects of MeHg on spontaneous quantal release of transmitter seen at the neuromuscular junction are due to inhibition of mitochondrial Ca^{2+} sequestration by MeHg. The effects of MeHg on spontaneous transmitter release are also inhibited by RR.

The results with RR provide a link between the effects of MeHg observed in the present neurochemical study and previous electrophysiological studies. The proposal that perturbation of intracellular Ca^{2+} homeostasis by neurotoxicants can lead to altered or impaired Ca-mediated functions, such as transmitter release, is

reasonable and not unique (Pounds and Rosen, 1988). Several agents known to inhibit mitochondrial functions and impair Ca^{2+} sequestration by mitochondria also affect transmitter release (Alnaes and Rahamimoff, 1975; Blaustein et al., 1978; Martin and Miledi, 1978; Adam-Vizi and Ligeti, 1984; Bernath and Vizi, 1987). Also, mitochondria from brain and skeletal muscle are very similar with respect to the mechanisms underlying uptake and release of Ca^{2+} and to the response of Ca^{2+} transport processes to inhibitors (Nicholls and Crompton, 1980).

Disrupted intracellular Ca^{2+} homeostasis and elevated $[Ca^{2+}]_i$ could at least in part explain the two well recognized effects of MeHg on transmitter release; inhibition of synchronous evoked release and increased spontaneous quantal release. Precise regulation of Ca^{2+} is critical for both forms of release to occur normally (Katz and Miledi, 1967; Llinas and Nicholson, 1975; Silinsky, 1985). Spontaneous release is directly dependent on $[Ca^{2+}]_i$ and increases when Ca^{2+} is released from internal stores or when active extrusion of intracellular Ca^{2+} across the plasma membrane is blocked (Alnaes and Rahamimoff, 1975; Blaustein et al., 1978; Adam-Vizi and Ligetti, 1984; Adams et al., 1985). Synchronous evoked release can be inhibited by increased basal levels of ionized free Ca^{2+} in the presynaptic nerve terminal (Adams et al., 1985; Bernath and Vizi, 1987). In addition to affecting transmitter release, disturbances in intracellular Ca^{2+} homeostasis could affect other Ca^{2+}-dependent cellular functions in neuronal as well as in non-neuronal cells. Perhaps other pathophysiological consequences of MeHg intoxication (Chang, 1977) could be explained by perturbations of cellular Ca^{2+} homeostasis.

CHAPTER FIVE

STIMULATION OF ACETYLCHOLINE RELEASE FROM SYNAPTOSOMES BY METHYLMERCURY INDEPENDENTLY OF EXTRACELLULAR CALCIUM

ABSTRACT

Effects of MeHg, a potent neurotoxicant, on choline uptake and release of acetylcholine (ACh) in CNS were studied using [^{3}H]choline-loaded rat brain synaptosomes as a neuronal model. The primary goal was to assess the relative contribution of extracellular Ca^{2+} and nerve terminal mitochondria to the previously described stimulatory effects of MeHg on spontaneous release of ACh. In Ca^{2+}-containing solutions, 10 and 100 μM MeHg caused increases in release of [^{3}H]ACh from synaptosomes during 10-90 sec incubations. Excluding Ca^{2+} from the reaction medium diminished the effectiveness of both 10 μM and 100 μM MeHg for inducing [^{3}H]ACh release by about 30% and 25%, respectively. Ruthenium Red (RR) and N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035), inhibitors of mitochondrial Ca^{2+} transport, were tested for their ability to disrupt MeHg-induced release. RR alone increased [^{3}H]ACh release by 8-10% and 10-13% at 20 μM and 60 μM, respectively. RR-induced release was only slightly attenuated in Ca^{2+}-free solutions. Preincubation of loaded synaptosomes with RR reduced the stimulatory effect of MeHg on [^{3}H]ACh release by about 50% in the presence and absence of Ca^{2+}. YS035 had no effect on [^{3}H]ACh release when used alone but pretreatment of synaptosomes with YS035 reduced slightly stimulation of release by MeHg in the presence and absence of Ca^{2+}. MeHg depressed high affinity uptake of [^{3}H]choline into synaptosomes. Uptake was reduced by about 25% and 45% when synaptosomes were incubated with [3H]choline in the presence of 10 μM and 100 μM MeHg, respectively.

The results indicate that extracellular Ca^{2+} only partially contributes to MeHg-

induced spontaneous release of ACh. The results with RR and YS035, inhibitors of mitochondrial Ca^{2+} transport, suggest that MeHg interacts with mitochondria to induce release of bound intraterminal Ca^{2+} stores, resulting ultimately in stimulated release of ACh. Also, MeHg reduces choline uptake into nerve terminals. Thus, MeHg could interfere with cholinergic neurotransmission in CNS by affecting the regulatory step in ACh synthesis and by increasing spontaneous release of transmitter. This combination of events would result in a depletion of the readily releasable pool of ACh in axon terminals.

INTRODUCTION

Methylmercury (MeHg) is a lipophilic mercurial that exerts prominent neurotoxic effects after acute or chronic exposure (Takeuchi et al., 1968; Bakir et al., 1973; Chang, 1977). Characteristic neurotoxic signs include sensory disturbances, cerebellar ataxia, and generalized extremity weakness in exposed individuals (Hunter et al., 1940). The molecular and cellular mechanisms responsible for the effects of MeHg are not clear, but could be due to biochemical and physiological effects of MeHg on the nerve resulting in disruption of synaptic transmission with consequent sensory and motor defects. MeHg affects transmitter release from central (Bondy et al., 1979; Komulainen and Tuomisto, 1981; 1982; Saijoh et al., 1987; Minnema, 1989) and peripheral nerve endings (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982; Atchison et al., 1984). The effects of MeHg on transmitter release have been characterized electrophysiologically only at the neuromuscular junction (NMJ). One prominent effect of MeHg observed at this peripheral cholinergic synapse is a transient increase in, asynchronous, spontaneous quantal release of acetylcholine (ACh) (Barrett et al., 1974; Juang, 1976; Atchison and Narahashi, 1982). Spontaneous release of ACh is not dependent on depolarization-induced Ca^{2+} influx into the nerve terminal or axonal impulse propagation. The frequency of spontaneous release, however, is thought to be directly proportional to the free Ca^{2+} concentration in the nerve terminal ($[Ca^{2+}]$). $[Ca^{2+}]$ can be increased in the absence of external Ca^{2+} when Ca^{2+} is released from internal stores (Alnaes and Rahamimoff, 1975). MeHg increases spontaneous release of ACh at the NMJ in

the absence of extracellular Ca^{2+} suggesting a perturbation of subcellular mechanisms regulating $[Ca^{2+}]_i$ (Atchison, 1986; 1987). This finding prompted subsequent electrophysiological (Levesque and Atchsion, 1987; 1988) and neurochemical (Levesque and Atchison, submitted) studies that have implicated nerve terminal mitochondria as a source of Ca^{2+} for the increased spontaneous release of ACh induced by MeHg. Pretreatment of a neuromuscular preparation with ruthenium red (RR) or N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035), inhibitors of mitochondrial Ca^{2+} transport, completely prevented the stimulatory effects of MeHg on spontaneous release of ACh. Also, MeHg inhibited uptake of $^{45}Ca^{2+}$ by isolated mitochondria and induced release of $^{45}Ca^{2+}$ from preloaded mitochondria via a ruthenium red-sensitive mechanism. Taken together, these results suggest that MeHg is able to induce release of bound Ca^{2+} from mitochondria and that release of this pool of Ca^{2+} by MeHg may contribute to the increased spontaneous release of ACh observed at the NMJ.

The effects of MeHg on release of transmitter from central neurons have not been studied electrophysiologically but several neurochemical analyses have shown that MeHg increases spontaneous release of neurotransmitters from isolated nerve terminals (synaptosomes) (Bondy et al., 1979; Komulainen and Tuomisto, 1981; 1982; Minnema et al., 1989) and brain slices (Saijoh et al., 1987). Spontaneous release of ACh, dopamine, GABA, glycine and glutamate appear to be affected similarly by MeHg. Since the effects of MeHg on spontaneous release of transmitter from CNS preparations are not unique to a particular transmitter type, perhaps MeHg acts via a general mechanism common to all transmitter systems. One mechanism that could cause increased spontaneous release of different

neurotransmitters would be to increase [Ca^{2+}]. Using a fluorescent probe for Ca^{2+}, Komulainen and Bondy (1987) demonstrated that MeHg significantly increases [Ca^{2+}]. Moreover, a portion of the Ca^{2+} that is elevated by MeHg may come from intrasynaptosomal mitochondria (Komulainen and Bondy, 1987; Kauppinen et al., 1989; Minnema et al., 1989). It may well be that the mechanism responsible for MeHg's effects on spontaneous release of transmitter from synaptosomes, model CNS nerve endings, is similar to the mechanism that has been proposed (Levesque and Atchison, 1987; 1988) to underlie MeHg's effect at the NMJ, a peripheral cholinergic synapse.

Thus, in the present study, we sought to link the various observations regarding the increased release of transmitter and the suspected origin of the increased [Ca^{2+}]. The specific objective was to determine whether MeHg increased spontaneous release of ACh from central nerve terminals subsequent to an interaction with mitochondria to release Ca^{2+}. Effects of MeHg on release of [^{3}H]ACh from rat brain synaptosomes loaded with [^{3}H]choline were tested in the absence and presence of Ca^{2+}. Attempts were made to alter the MeHg-induced release of [^{3}H]ACh by pretreating labeled synaptosomes with RR and YS035 to inhibit transport of Ca^{2+} by mitochondria in situ. The effect of MeHg on choline uptake, a regulatory step in ACh synthesis (Simon et al., 1976), was also measured.

METHODS

Preparation of synaptosomes. Synaptosomes were isolated from forebrains of male Sprague-Dawley rats (Harlan, 175-225g) using a modification of the method of Booth and Clark (1978). This method, which utilizes Ficoll/sucrose discontinuous gradients, has two distinct advantages over sucrose gradient techniques: (a) suitable gradients are prepared while retaining iso-osmolality and (b) the time of centrifugation is shortened. This results in better functional integrity of the synaptosomes compared to other isolation techniques. The technique is apparently more suitable and reliable for studying the biochemical events induced by pharmacological or toxicological manipulation (Dagani et al., 1985). Rats were sacrificed by decapitation and the forebrains were rapidly removed and dropped into ice-cold isolation medium (0.32 M-sucrose/1 mM-potassium EDTA/10 mM-Tris/HCl, pH 7.4). All isolation procedures were carried out at 0-4°C. The tissue was minced and then homogenized in a Dounce-type homogenizer by 8 up-and-down strokes at 550 rpm (pestle clearance 0.1 mm). The homogenate was diluted to 60 ml with isolation medium and centrifuged at 1300g for 3 min in a Sorvall RC2-B refrigerated preparatory centrifuge. The supernatant from this step was centrifuged at 17,000g for 15 min, producing a crude mitochondrial/synaptosomal pellet which was resuspended in a total of 6 ml isolation medium. Then, 34 ml of 15% Ficoll/sucrose medium (15% (w/w) Ficoll, 0.32 M-sucrose, 50 μM-potassium EDTA, pH 7.4) was added. The crude suspension was then divided equally, introduced into separate 40 ml centrifuge tubes and above each, 12 ml of 7.5% Ficoll/sucrose medium (7.5% (w/w) Ficoll, 0.32 M-sucrose, 50 μM-potassium

EDTA, pH 7.4) was carefully layered. Finally, 5 ml of isolation medium was slowly layered to top off each tube. The tubes were centrifuged at 99,000g for 45 min in a SW27 swinging bucket rotor in a Beckman L565 ultracentrifuge. This ultracentrifugation step separates myelin, synaptosomes and mitochondria, as myelin and synaptosomes band at the first and second interphases respectively, and mitochondria pellet at the bottom. The myelin layer was carefully removed and the synaptosomes were gently sucked off from the interphase. The purified synaptosomes from each tube were diluted to 30 ml with Hepes-buffered Krebs Ringer (HKR) (145 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM D-glucose, 5 mM Hepes, pH 7.4) and centrifuged at 9,800g for 10 min to remove any contaminating myelin. The synaptosomal pellets were combined and resuspended in 5 ml of HKR at approximately 10 mg protein/ml for loading with [^{3}H]choline. This HKR and all other solutions used during subsequent steps were vigorously oxygenated before use. All buffers were prepared daily from stock solutions and osmolarity was maintained at 320 ± 10 mOsm. Synaptosomal protein was determined by the method of Lowry et al., (1951).

Labeling Intrasynaptosomal ACh. Intrasynaptosomal ACh was labeled via a modification of the method of Suszkiw and O'Leary (1983). The suspension of synaptosomes in 5 ml of HKR (approx. 10 mg protein/ml) was incubated in the presence of 100 nM [^{3}H]choline (85 ci/mmol) for 30 min at 30°C under O_2. After the loading incubation, synaptosomes were centrifuged at 10,000g for 10 min at 4°C. The loaded synaptosomes were then washed free of extrasynaptosomal radioactivity by two consecutive resuspensions in 5 ml cold HKR containing 20 μM hemicholinium, and repelleted (10,000g, 10 min, 4°C). Hemicholinium was used

to block the choline uptake carrier thereby inhibiting further uptake of choline. After washing, the final synaptosomal pellet was resuspended in oxygenated HKR (approx. 10 mg protein/ml) plus 50 μM eserine and 20 μM hemicholinium. Eserine was used to inhibit acetylcholinesterase and prevent breakdown of ACh in subsequent release experiments. All HKR solutions used in [^{3}H]ACh release experiments contained 20 μM hemicholinium and 50 μM eserine. In experiments in which Ca^{2+}-independent release of [^{3}H]ACh was measured, synaptosomes were loaded as described above but were washed with and resuspended in Ca^{2+}-free HKR. Ca^{2+}-free HKR was made by omitting $CaCl_2$, increasing $MgCl_2$ to 10 mM and reducing NaCl by 6 mM to maintain osmolarity. Previous studies in this lab (Atchison, 1986;1987) have shown such solutions to contain between 6-12 μM Ca^{2+} as contaminant when analyzed using inductively coupled plasma emission spectroscopy. As wash steps were performed at 4°C, synaptosomal suspensions were incubated at 30°C for 10 min before their use in release experiments.

Release of ACh. Release of [^{3}H]ACh from prelabeled synaptosomes was initiated by adding 100 μl aliquots (approx. 1 mg protein) of the labeled synaptosomes to 900 μl of HKR. After allowing the solutions to mix for various intervals (see figure legends), the synaptosomes were rapidly filtered through 0.65 μm Millipore filters under suction. The filters were washed twice with 3 ml of ice-cold HKR. The filtrates were collected and assayed for [^{3}H]ACh released. Ca^{2+}-independent release was determined as above except that labeled synaptosomes were washed and suspended in Ca^{2+}-free HKR and all solutions used during release incubations were Ca^{2+}-free. When MeHg, RR or YS035 were tested for their effects on [^{3}H]ACh release, aliquots of prelabeled synaptosomes were added to HKR buffers

containing these agents. All three were tested for effects on release in the presence and absence of Ca^{2+}. When RR and YS035 were tested for the ability to alter the effects of MeHg on release of [^{3}H]ACh, labeled synaptosomes were incubated in the presence of these agents for 10 min before aliquots of the synaptosomes were added to normal or Ca^{2+}-free HKR solutions containing MeHg. MeHg and the mitochondrial inhibitors were tested only for their effects on spontaneous release of [^{3}H]ACh. However, the response of the labeled synaptosomes to K^+-depolarization in the absence and presence of Ca^{2+} was assessed in separate control experiments. Depolarization-induced release of [^{3}H]ACh was initiated by adding 100 μl aliquots of prelabeled synaptosomes to 900 μl of high-K^+ HKR (K^+-HKR). K^+-HKR was similar to normal HKR except that the concentrations of Na^+ and K^+ were 90 mM and 60 mM, respectively. Ca^{2+}-independent, depolarization-induced release was measured in parallel by adding aliquots of synaptosomes to Ca^{2+}-free/10 mM Mg^{2+} K^+-HKR. The Ca^{2+}-dependent release was calculated by subtracting [^{3}H]ACh released in the Ca^{2+}-free/10 mM Mg^{2+} K^+-HKR from that released in the K^+-HKR containing normal concentrations of Ca^{2+} and Mg^{2+}.

Determination of [^{3}H]ACh. [^{3}H]ACh released into the filtrates was assayed by the method of Goldberg and McCaman (1973) as modified by Suskiw and O'Leary (1983). Briefly, 250 μl aliquots of the filtrates in scintillation vials were mixed with 1 ml of a phosphorylation medium to yield the following composition: 0.005 U choline kinase, 5 mM ATP, 10 mM $MgCl_2$ and 10 mM Na_2HPO_4 buffer, pH 7.9. The mixture was incubated for 15 min at 35°C to convert the free choline to phosphorylcholine. After 15 min, 1.0 ml of tetraphehylboron in heptanone (10

mg/ml) was added and the vials were vortexed vigorously to extract [^{3}H]ACh into the organic phase. Phosphorylated [^{3}H]choline is not extractable with tetraphenylboron and remains in the aqueous phase. After the aqueous and organic phases had separated, 10 ml of toluene-based scintillation fluor was added without disturbing the lower aqueous phase. Initially, internal acetyl[^{14}C]choline (approx. 1000 dpms) standards were added to all samples prior to the assays and its recovery was (>90%) used to calculate the original content of [^{3}H]ACh in the samples. To monitor the efficiency of the choline kinase-catalyzed phosphorylation of choline and separation of free [^{3}H]choline from [^{3}H]ACh, parallel control assays were run with acetylcholinesterase (10-15 U/ml) added to hydrolyze all ACh in the samples. Radioactivity in these samples was close to background (results not shown) indicating that the choline kinase assay procedure was highly efficient for separating [^{3}H]choline and [^{3}H]ACh (>90%). All radioactivity was estimated using a Searle model 6880 liquid scintillation spectrometer.

[^{3}H]Choline uptake measurements. Choline uptake was initiated by adding [^{3}H]choline to synaptosomes in HKR (approx. 10 mg/ml) at a final concentration of 100 nM. Synaptosomes were incubated in the presence of [^{3}H]choline for a total of 30 min in an oxygenated metabolic shaker at 30°C. The time course of [^{3}H]choline uptake during the 30 min incubation was determined by filtering 100 μl aliquots of synaptosomes every 5 min through 0.65 μm Millipore filters. After rinsing the filters twice with 2 ml of cold HKR, the filters were placed into glass vials and radioactivity was eluted by adding 1.5 ml of a Triton X-100/HCl solubilizer. After 10 min, 10 ml of aqueous scintillation cocktail was added and total tritium was estimated in a Searle model 6880 liquid scintillation spectrometer. Uptake of

[^{3}H]choline was determined in normal HKR, Na^+-free HKR (substituting N-methylglucamine for NaCl) or hemicholinium (20 μM)-containing HKR to assess the contribution of high affinity uptake of choline to total uptake. The effect of MeHg on [^{3}H]choline uptake was determined by adding MeHg to the suspension of synaptosomes in HKR before adding [^{3}H]choline.

Materials. Methylmercury chloride was purchased from K + K Rare and Fine Chemicals (Plainview, NY). [Acetyl-1-^{14}C]choline chloride (10 mCi/mmol) and [methyl-^{3}H]choline chloride (80 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA) and from Research Products International Corp (Mount Prospect, IL), respectively. Acetylcholinesterase (950 units/mg protein; electric eel Type III), choline phosphokinase (0.39 units/mg solid), ruthenium red, physostigmine sulfate, hemicholinium-3, Ficoll type 400-DL and 3-heptanone were all purchased from Sigma Chemical Co. (St. Louis, MO). Tetraphenylboron sodium was obtained from Aldrich Chemical Co. (Milwaukee, WI). YS035 [N,N-bis(3,4-dimethoxyphenethyl)-N-methylamine] was synthesized by the Organic Synthesis Lab of the Michigan State University Department of Chemistry according to the methods of Deana et al., (1984). YS035 was dissolved in ethanol and then added to HKR solutions so that the final concentration of ethanol was 0.04% (v/v). Control solutions contained an identical concentration of the vehicle.

Animals. Adult male Sprague-Dawley rats (Harlan, 175-225g) were housed in plastic cages in a room which received 12 hrs of light per day. Room temperature was maintained at 22 to 24°C and relative humidity at 40 to 60%. Food (Purina Rat Chow) and water were provided ad libitum.

Statistical Analysis. ACh release data were analyzed statistically using a

randomized block analysis of variance (Steel and Torrie, 1960). Differences among treatment means were compared using Duncan's and Dunnett's tests. Choline uptake and K^+-depolarization data were analyzed for significance using student's t test. Differences were considered to be significantly different at $p < .05$.

RESULTS

One drawback to using synaptosomes as model nerve terminals is that they contain a heterogeneous population of nerve terminals with respect to transmitter. In control experiments designed to determine whether our preparation of synaptosomes contained cholinergic nerve terminals, synaptosomes were analyzed for the presence of the hemicholinium-sensitive (Yamamura and Snyder, 1973; Barker and Mittag, 1975), high-affinity choline transporter (Haga and Noda, 1973; Yamamura and Snyder, 1973) that is selectively localized to cholinergic nerve terminals (Kuhar et al., 1973; Yamamura and Snyder, 1973). Synaptosomes were incubated in the presence of a low concentration of [^{3}H]choline (100 nM) to favor uptake via the high-affinity pathway and not by passive transport which have k_m's for choline of 1-4 μM and 40-80 μM, respectively (Haga and Noda, 1973; Yamamura and Snyder, 1975). Results of experiments in which [^{3}H]choline uptake was determined are illustrated in figure 1. Total tritium in the synaptosomes, as determined by filtration, increased rapidly and saturated within 10-15 min. After 15 min, the tritium content of the synaptosomes remained relatively constant. This may have been due to a balance between [^{3}H]choline uptake and release of tritium to the medium, as has been observed by others (Rowell and Duncan, 1981). Either inclusion of hemicholinium or removal of Na^+ inhibited the uptake of [^{3}H]choline by over 65% throughout the incubation period. This demonstrates that at the low concentration of choline used, most of the [^{3}H]choline is incorporated into cholinergic synaptosomes possessing high-affinity transport for choline. High affinity transport of choline is thought to be directly coupled to ACh synthesis

(Barker and Mittag, 1975; Barker et al., 1975; Simon et al., 1976) and is believed to be the rate limiting step for synthesis of this transmitter (Haga and Noda, 1973; Yamamura and Snyder, 1973). One possible mechanism by which MeHg could affect transmitter release would be to inhibit choline uptake and in so doing, decrease nerve terminal ACh levels. Results of experiments in which [^{3}H]choline uptake was determined in the presence of MeHg are shown in figure 2. At each time point tested throughout the 30 min incubation of synaptosomes with [^{3}H]choline, 10 μM and 100 μM MeHg reduced uptake by 15-20% and 40-45%, respectively. Based on this experiment, it cannot be determined whether the effect of MeHg was specific for either high or low affinity uptake. However, since high affinity uptake accounted for the majority of choline incorporated into synaptosomes, the large decrease in uptake produced by 100 μM MeHg may have been at least partly due to inhibition of high-affinity choline transport.

To determine further whether our preparation of synaptosomes retained normal functions, [^{3}H]choline-loaded synaptosomes were tested for the ability to release [^{3}H]ACh in response to depolarization. The time course of [^{3}H]ACh release from synaptosomes evoked by 55 mM K^+ in the presence and absence of 2.5 mM Ca^{2+} is shown in figure 3. To verify that the augmented efflux was in fact [^{3}H]ACh, [^{3}H]choline and [^{3}H]ACh were separated by choline kinase-tetraphenylboron extraction. This was done in all experiments involving release of [^{3}H]ACh. In solutions containing Ca^{2+} (shaded bars), the release of [^{3}H]ACh induced by K^+-depolarization was significantly greater at each time point than the spontaneous release in nondepolarizing HKR. Initially, K^+-evoked release of [^{3}H]ACh occurred rapidly, but after 30 sec, prolonged depolarization resulted in only a slight increase

in [^{3}H]ACh release. Depolarization of the synaptosomes for 30 sec released nearly 10% of the total tritium content of the [^{3}H]choline-loaded synaptosomes. Clearly, high K^+ alone did not stimulate release of [^{3}H]ACh in the absence of Ca^{2+} (dark bars). This confirms that, in this preparation, depolarization-induced release of ACh was Ca^{2+}-dependent.

Exposure of [^{3}H]choline-loaded synaptosomes to 10 μM or 100 μM MeHg caused a significant, concentration-dependent increase in spontaneous release of [^{3}H]ACh (Figure 4). The additional ACh release induced by MeHg over that occurring in its absence is expressed as a percent value from control samples incubated without MeHg. At 10 μM (Figure 4a), MeHg increased spontaneous release by 10% over control within 10 sec of being introduced into the suspension of labeled synaptosomes. Incubation of the synaptosomes in the presence of 100 μM MeHg (Figure 4b) resulted in a 30% increase in spontaneous [^{3}H]ACh release. At both concentrations of MeHg, release of [^{3}H]ACh increased only slightly with longer exposure times and reached a maximum at 90 sec. During longer incubations, MeHg-induced release began to decline towards control (results not shown). The increase in spontaneous release of [^{3}H]ACh induced by MeHg was attenuated but remained significant in the absence of Ca^{2+}.

RR, which blocks uptake and induces release of Ca^{2+} by mitochondria, and YS035, which blocks cellular uptake and release of Ca^{2+} by mitochondria, were tested for effects on spontaneous release of transmitter. RR significantly enhanced release of [^{3}H]ACh in a concentration-dependent manner in both the presence (Figure 5a) and absence (Figure 5b) of Ca^{2+}. Efflux of [^{3}H]ACh occurred rapidly and within 10 sec was significantly elevated compared to control. As shown, the

effect of RR on spontaneous release began to decline with increased time of exposure. The stimulatory effect of RR on [^{3}H]ACh release was reduced by only 10-20% in Ca^{2+}-free solutions. YS035, however, at concentrations up to 200 μM, did not alter release of [^{3}H]ACh from control either in the presence or absence of Ca^{2+} (Figure 6).

Aliquots of [^{3}H]choline-loaded synaptosomes were preincubated with the mitochondrial transport inhibitors for 10 min before adding MeHg. Preincubation of labeled synaptosomes with RR for 10 min significantly attenuated the stimulatory effects of MeHg on spontaneous release of [^{3}H]ACh in the presence (Figure 7a) and absence (Figure 7b) of Ca^{2+}. Although YS035 did not affect spontaneous release itself, pretreatment of labeled synaptosomes with this compound slightly reduced the ACh release induced by 10 μM or 100 μM MeHg (Figure 8a). This effect was independent of external Ca^{2+} (Figure 8b).

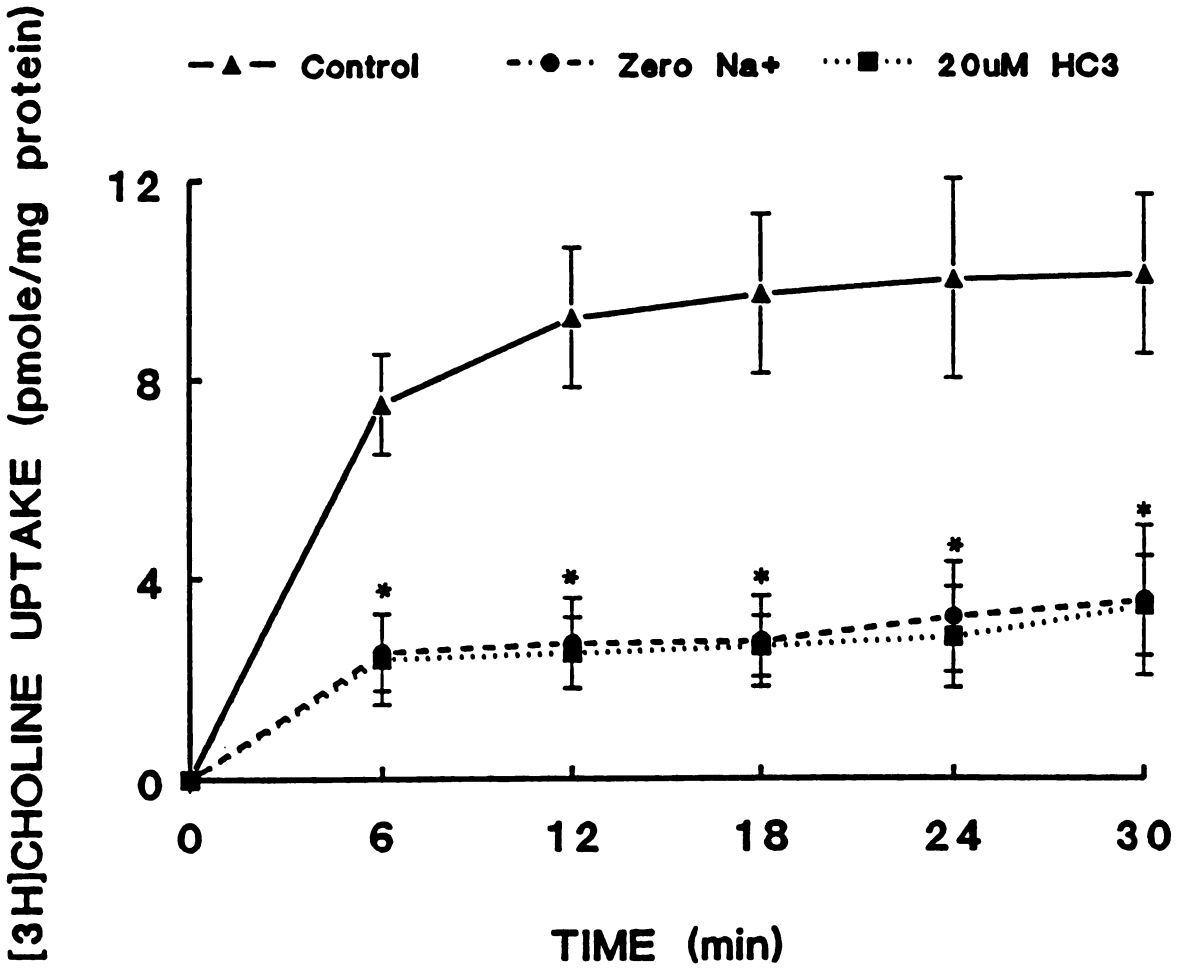

Figure 1. Time course of [^{3}H]choline uptake by synaptosomes incubated with 100 nM [^{3}H]choline in control ( ), Na^+-free ( ) and hemicholinium (20 μM)-containing ( ) HEPES-buffered Krebs Ringer solutions (HKR). [^{3}H]choline was added to synaptosomal suspensions (10 mg/ml) at 0 min and aliquots were filtered every 6 min to determine uptake. [^{3}H]choline uptake refers to the total tritium retained by filtered synaptosomes. Values are the mean $\pm$ SEM of four different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates a significant reduction from control uptake by synaptosomes incubated in "0-Na^+" and hemicholinium ($P \leq .05$).

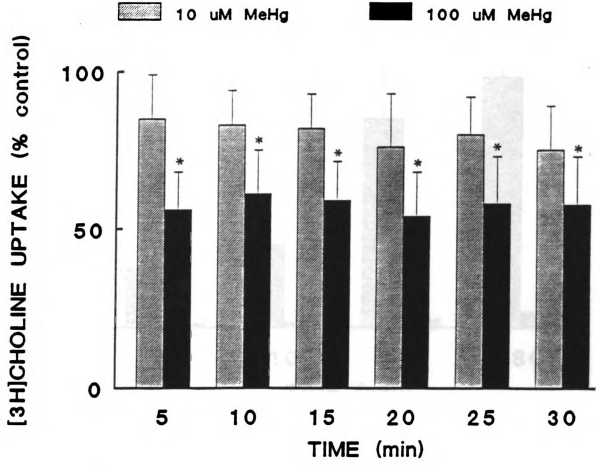

Figure 2. Time course of effects of 10 μM (shaded bars) and 100 μM (dark bars) MeHg on [^{3}H]choline uptake in HKR. MeHg was added to the synaptosomes (10 mg/ml) just prior to adding [^{3}H]choline. Aliquots of the synaptosomal suspensions were filtered every 5 min and total tritium retained on the filters was determined. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates a significant reduction in [^{3}H]choline uptake by MeHg compared to MeHg-free controls ($P \leq .05$).

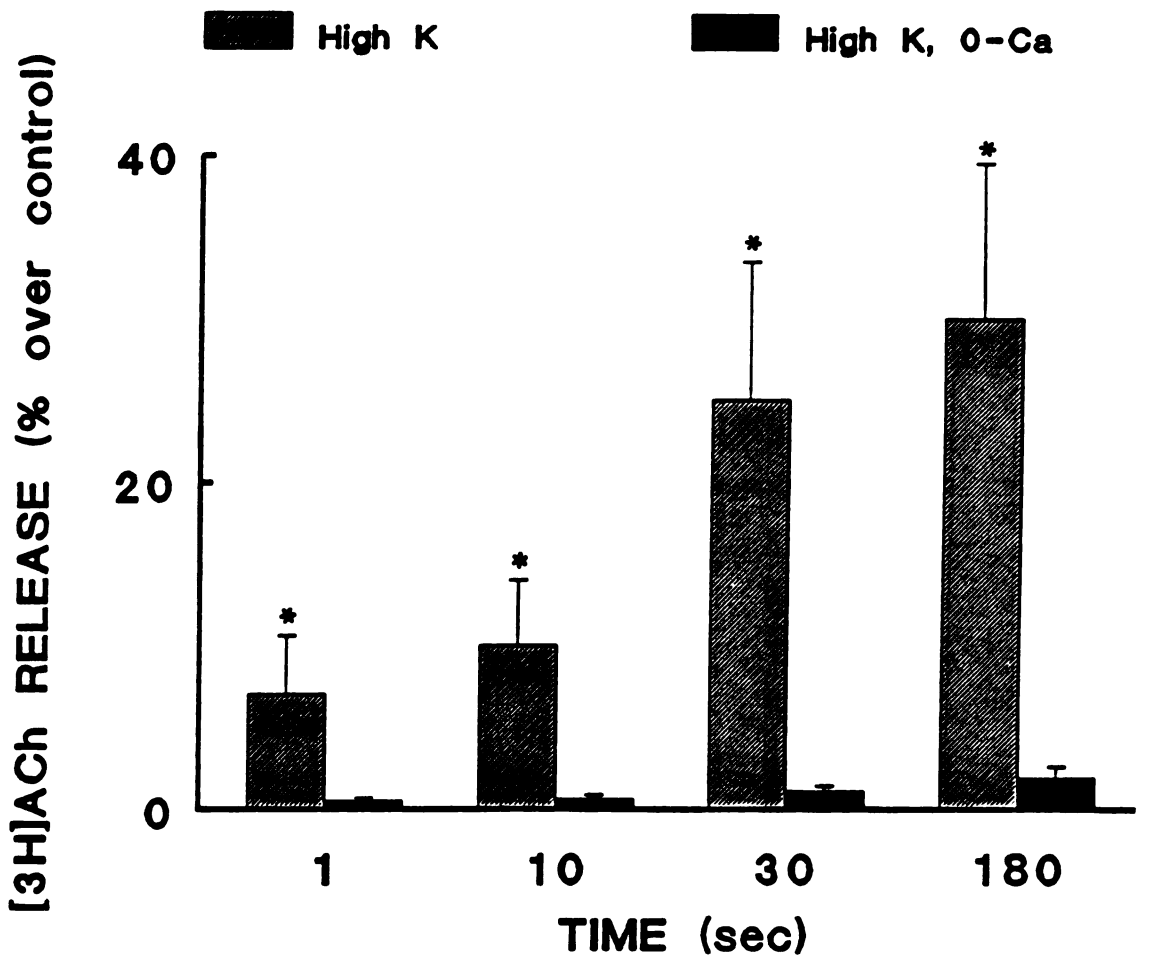

Figure 3. Time course of Ca^{2+}-dependent and independent release of [^{3}H]ACh evoked from synaptosomes by depolarizing with 55 mM K^+-HKR. Intrasynaptosomal ACh was labeled by incubating synaptosomes for 30 min with 100 nM [^{3}H]choline. Aliquots of labeled synaptosomes were depolarized with 55 mM K^+ HKR $\pm$ Ca^{2+} (2.5 mM) for 1, 10, 30 or 180 sec. Net depolarization-induced release of [^{3}H]ACh was determined by subtracting release in low K^+ HKR (5 mM) from release that occurred in high K^+ HKR. Results are expressed as the percentage of [^{3}H]ACh released in high K^+ HKR over basal [^{3}H]ACh efflux in low K^+ HKR. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates a significant increase relative to depolarization in the absence of Ca^{2+} ($P \leq .05$).

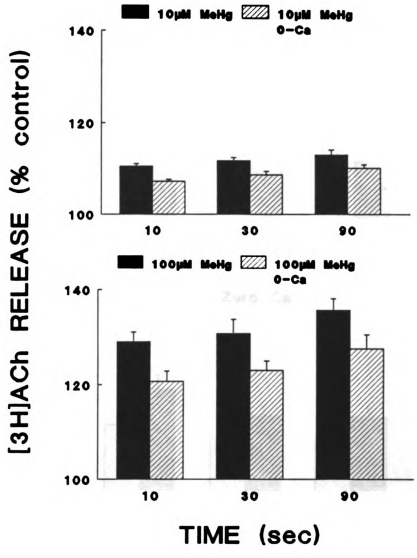

Figure 4. Effects of MeHg (10 or 100 μM) on release of [^{3}H]ACh from non-depolarized synaptosomes in the presence and absence of Ca^{2+} (2.5 mM). Aliquots of prelabeled synaptosomes were added to HKR (5 mM K^+) containing MeHg $\pm$ Ca^{2+} for 10, 30 or 90 sec. Results are expressed as the percentage of [^{3}H]ACh released in response to MeHg, relative to [^{3}H]ACh released during parallel incubations in the absence of MeHg. Values are the mean $\pm$ SEM of 10 different experiments. Values for each experiment are the average of three replicates. All values for both concentrations of MeHg in the presence and absence of Ca^{2+} are significantly greater than MeHg-free controls ($P \leq .05$).

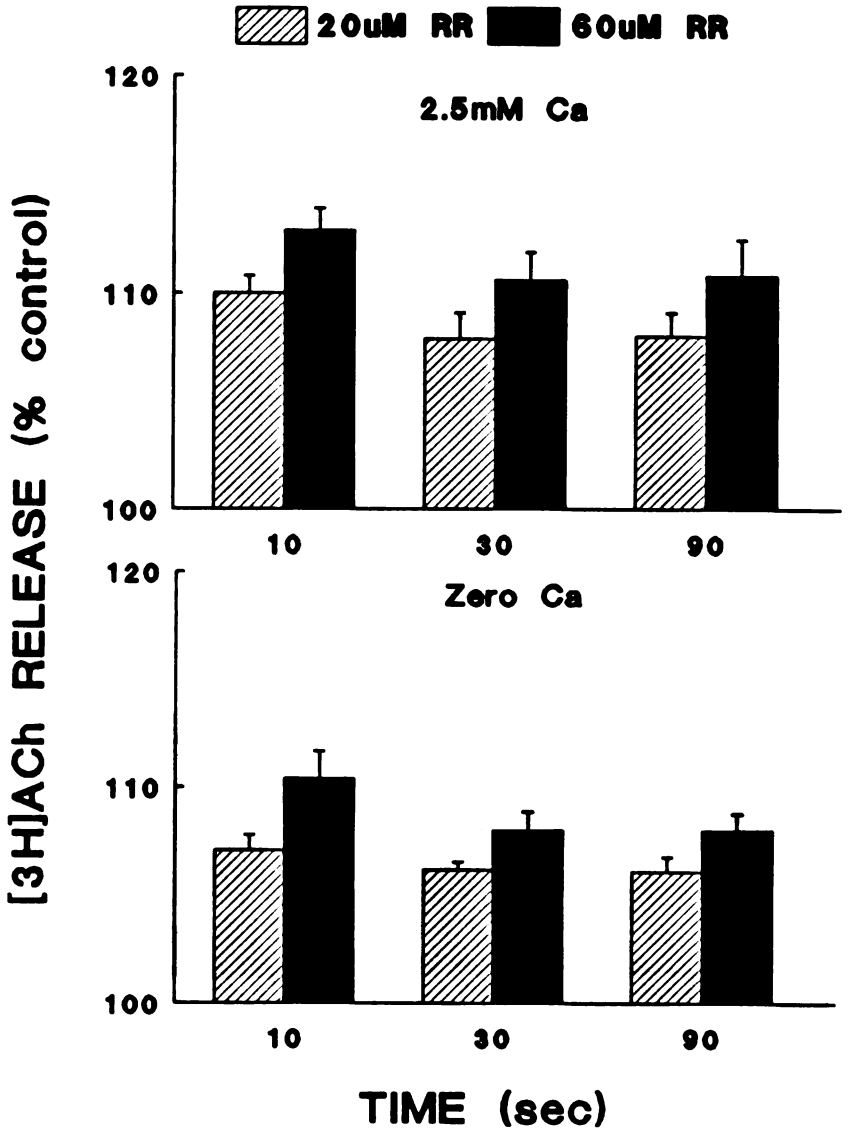

Figure 5. Effects of ruthenium red (RR, 20 or 60 μM) on release of [^{3}H]ACh in the absence and presence of Ca^{2+} (2.5 mM). Aliquots of prelabeled synaptosomes were added to HKR (5 mM K^+) containing RR $\pm$ Ca^{2+} for 10, 30 or 90 sec. Results are expressed as a percentage of [^{3}H]ACh released in response to RR, relative to [^{3}H]ACh released during parallel incubations in the absence of RR. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates. All values for both concentrations of RR in the presence and absence of Ca^{2+} are significantly greater than RR-free controls ($P \leq .05$).

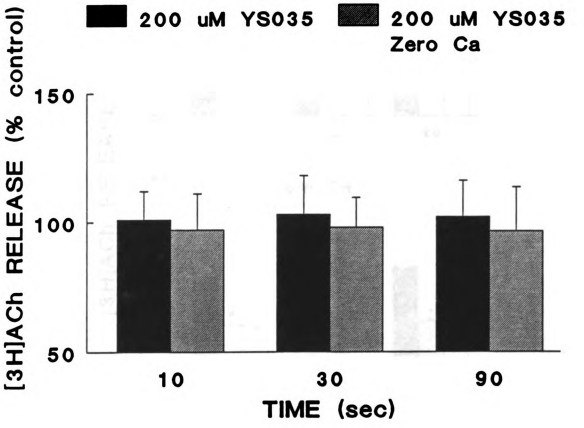

Figure 6. Effects of YS035 (200 μM) on release of [^{3}H]ACh in the absence and presence of Ca^{2+} (2.5 mM). Aliquots of prelabeled synaptosomes were added to HKR (5 mM K^+) containing YS035 $\pm$ Ca^{2+} for 10, 30 or 90 sec. Results are expressed as a percentage of [^{3}H]ACh released in response to YS035, relative to [^{3}H]ACh released during parallel incubations in the absence of YS035. Values are the mean $\pm$ SEM of three different experiments. Values for each experiment are the average of three replicates.

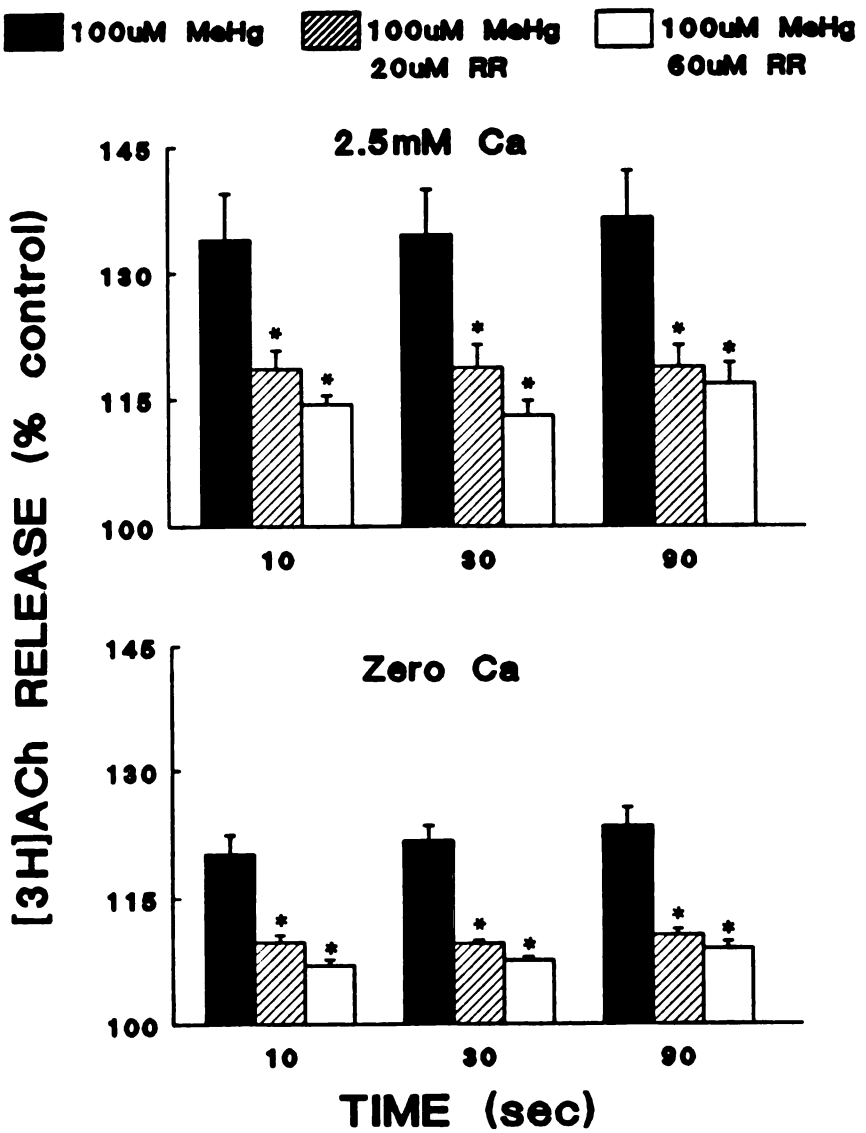

Figure 7. Effects of MeHg (100 μM) on release of [^{3}H]ACh from non-depolarized synaptosomes preincubated with RR (20 and 60 μM) in the absence and presence of Ca^{2+} (2.5 mM). Aliquots of prelabeled synaptosomes incubated with RR for 10 min were added to HKR (5 mM K^+) containing MeHg $\pm$ Ca^{2+} for 10, 30 or 90 sec. Results are expressed as the percentage of [^{3}H]ACh released by MeHg in the presence and absence of RR $\pm$ Ca^{2+} relative to [^{3}H]ACh released during parallel incubations without MeHg. Values are the mean $\pm$ SEM of seven different experiments. Values for each experiment are the average of three replicates. The asterisk (*) indicates a significant reduction in MeHg-induced release of [^{3}H]ACh from synaptosomes preincubated with RR ($P \leq .05$).

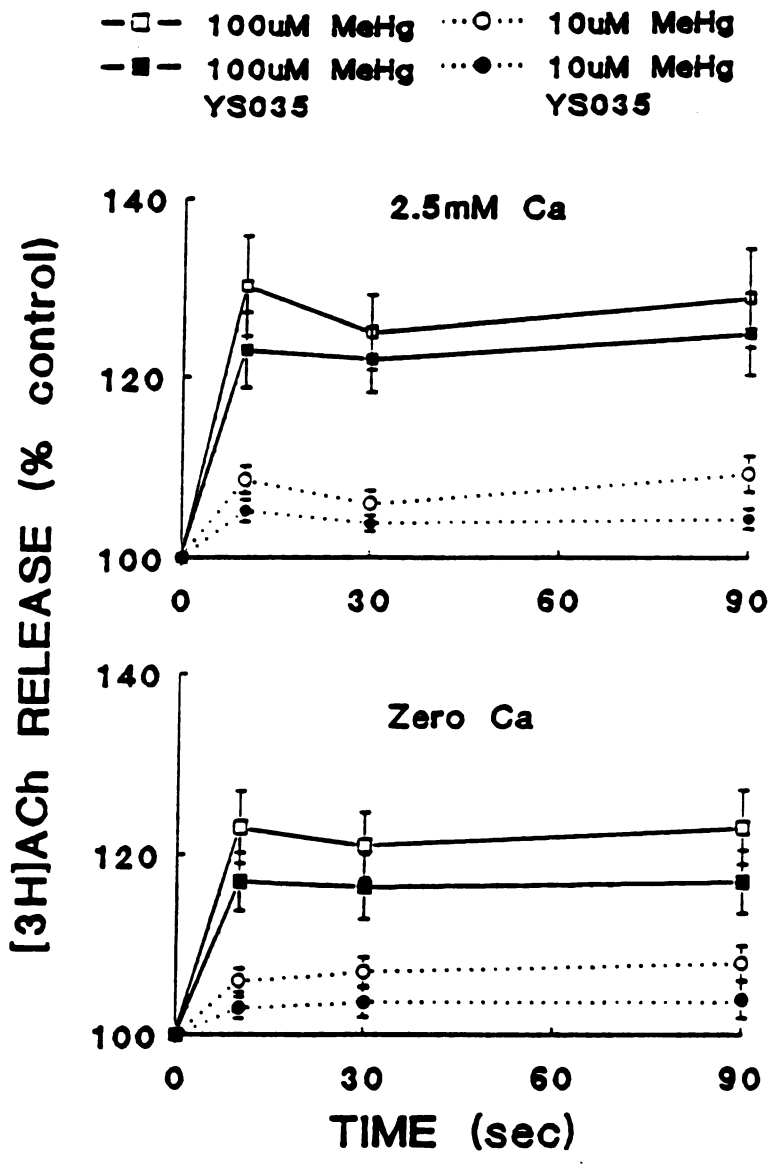

Figure 8. Effects of MeHg (10 or 100 μM) on release of [^{3}H]ACh from nondepolarized synaptosomes preincubated with YS035 (200 μM) in the absence and presence of Ca^{2+} (2.5 mM). Aliquots of prelabeled synaptosomes incubated with YS035 for 10 min were added to HKR containing MeHg $\pm$ Ca^{2+} for 10, 30 or 90 sec. Results are expressed as the percentage of [^{3}H]ACh released by MeHg in the presence and absence of YS035 $\pm$ Ca^{2+} relative to [^{3}H]ACh released in parallel MeHg-free controls. Values are the mean $\pm$ SEM of six different experiments. Values for each experiment are the average of three replicates.

DISCUSSION

Since MeHg has previously been shown to affect cholinergic neurotransmission in the peripheral nervous system and is a potent CNS neurotoxicant, we studied its effects on uptake of choline and release of ACh in the CNS using rat brain synaptosomes as model nerve terminals. The results indicate that the mechanisms underlying choline uptake and ACh release are potential targets for MeHg-induced disturbances in neurotransmission. MeHg partially inhibited uptake of [^{3}H]choline and induced spontaneous release of [^{3}H]ACh from [^{3}H]choline-loaded synaptosomes. Enhanced release of [^{3}H]ACh was slightly attenuated in Ca^{2+}-free solutions but was still significantly greater than MeHg-free controls. Preincubation of synaptosomes with inhibitors of mitochondrial Ca^{2+} transport reduced the effectiveness of MeHg for increasing spontaneous release of [^{3}H]ACh. These results indicate that the effects of MeHg on ACh release from central neurons, as measured neurochemically, are consistent with those obtained previously in electrophysiological studies at the NMJ.

The preparation of rat forebrain synaptosomes utilized in this study contained functional cholinergic nerve terminals since most of the [^{3}H]choline added was taken up via Na^+-dependent, hemicholinium-sensitive transport which is exclusively localized in cholinergic nerve terminals (Kuhar et al., 1973; Yamamura and Snyder, 1973). The degree of inhibition of choline uptake caused by Na^+ removal or hemicholinium was similar to those reported previously for high affinity uptake of choline by cholinergic nerve terminals (Guyenet et al., 1973; Yamamura and Snyder, 1973; Simon and Kuhar, 1976). Uptake of [^{3}H]choline via high affinity

transport implies that intrasynaptosomal ACh synthesis occurred normally since these two events are coupled (Barker et al., 1975; Simon et al., 1976). The preparation also retained normal functioning with respect to depolarization-induced transmitter release since [^{3}H]ACh was released from synaptosomes in response to K^+-induced depolarization only in Ca^{2+}-containing media (Blaustein, 1975; Murrin et al., 1977). Both the time course and the percentage of [^{3}H]ACh released in response to depolarization were consistent with results of a previous study utilizing a similar preparation (Suszkiw and O'Leary, 1983). The results of these control experiments indicate that the synaptosomes used in the present study retain fundamental characteristics of cholinergic nerve terminals and serve as an appropriate model for studying the effects of MeHg on cholinergic neurotransmission.

MeHg has been shown to inhibit uptake of several neurotransmitters including serotonin, dopamine, noradrenaline, glutamate and GABA (Bondy et al., 1979; Araki et al., 1981; Komulainen and Tuomisto, 1981) and the ACh precursor, choline (Bondy et al., 1979; Kobayashi et al., 1979; Saijoh et al., 1987). Choline uptake by synaptosomes was reduced significantly by MeHg in a concentration-dependent manner. Whether MeHg specifically inhibits high affinity choline uptake or affects uptake of choline indirectly has not been determined. High affinity choline transport is dependent on membrane potential and agents that depolarize the cell inhibit high affinity transport. Concentrations of MeHg above 30 μM have been shown to cause a time-dependent depolarization of synaptosomal membrane potentials (Kauppinen et al., 1989). Thus at 100 μM MeHg, perhaps inhibition of choline uptake was inhibited in part by membrane depolarization. This could

account for the much greater reduction in choline uptake induced by 100 μM than by 10 μM MeHg. Another possibility is that MeHg may not inhibit uptake but may rapidly induce the release of [^{3}H]ACh synthesized from [^{3}H]choline that is taken up during the incubation period. Thus, it would appear that less [^{3}H]choline was taken up in the presence of MeHg. Newly synthesized ACh is preferentially released from cholinergic nerve terminals (Molenaar et al., 1973). MeHg-induced release of [^{3}H]ACh at the same concentrations that it reduced [^{3}H]choline uptake. Moreover, the percent values by which MeHg inhibited uptake and induced release were similar at each concentration of MeHg. Increased release of monoamine neurotransmitters has been suggested to underlie the effects of MeHg on monoamine uptake into synaptosomes (Komulainen and Tuomisto, 1981). Whatever the mechanism, inhibition of this regulatory step in the synthesis of ACh by MeHg could affect neurotransmission at cholinergic synapses in the CNS.

The effects of MeHg on spontaneous release of ACh from CNS nerve terminals, as observed in the present study, are similar to those observed at the NMJ in that they are not abolished in the absence of external Ca^{2+}. Despite this similarity, there are differences in the effects of MeHg on ACh release from synaptosomes and the NMJ. MeHg stimulated spontaneous release of ACh from synaptosomes much more rapidly than from nerve terminals at the NMJ. The rapid stimulation of ACh release from synaptosomes by MeHg has been observed by others (Bondy et al., 1979; Minnema et al., 1989). The delayed onset of the effect of MeHg at the NMJ may be due to the fact that the nerve terminals from which ACh release was measured were imbedded in muscle tissue. There is little doubt that MeHg is sufficiently lipophilic to diffuse through plasma membranes and gain

entrance into cells (Nordberg et al., 1970; Chang and Hartmann, 1972; Verity et al., 1975; Lakowicz and Anderson, 1980). Since we are proposing that the effects of MeHg on spontaneous release are due to intraterminal actions, MeHg would have to diffuse through non-neuronal tissue barriers before reaching and entering the nerve terminal. It may take longer to reach the nerve terminal and the concentration that gains entrance may be less if a significant amount of MeHg becomes bound to non-neuronal tissue. This explanation seems possible since increasing the concentration of MeHg, and thus increasing the driving force for diffusion, dramatically shortens the latency preceding the onset of the effect of MeHg at the NMJ (Atchison and Narahashi, 1982). Non-neuronal diffusional barriers are not present in the synaptosomal preparation and nerve terminal membranes are readily accessible to MeHg. Therefore, MeHg may reach it's putative intraterminal target more quickly and at a higher concentration in synaptosomes than in a neuromuscular preparation.

There also appear to be differences with respect to concentration-dependence when comparing the effects of MeHg at the NMJ and on synaptosomes. The effects of MeHg on spontaneous release of ACh in the present study and in other neurochemical studies (Bondy et al., 1979; Minnema et al., 1989) appear to be concentration-dependent. Increasing the concentration of MeHg increased the percentage of ACh released relative to untreated controls. In studies at the NMJ, the peak effect of MeHg on spontaneous quantal ACh release did not increase when the concentration of MeHg was increased from 10 μM to 100 μM (Atchison et al., 1984). A possible explanation for this discrepancy may involve the different techniques used to measure ACh release. In neurochemical

studies with synaptosomes, no distinction can be made between spontaneous quantal and non-quantal release of transmitter. Any ACh released from the synaptosomes, whether quantal or not, is detected by the enzymatic assay used to measure ACh released. In the electrophysiological experiments at the NMJ, only effects of MeHg on spontaneous quantal release of transmitter were measured. Moreover, the latter technique only measures the result of interaction of ACh with its receptor, and obviously much of the released quantal ACh never reaches the receptor. Perhaps the effects of MeHg on quantal release, as detected electrophysiologically, are maximal at concentrations lower than 100 μM. If the higher concentration of MeHg (100 μM) increased non-quantal release of ACh, it would only be detected by the neurochemical measurements and not by the electrophysiological experiments used previously. It is not known whether MeHg increased non-quantal release of ACh from synaptosomes in the present experiment. A portion of intrasynaptosomal ACh is unbound in the cytosol (Rowell and Duncan, 1981). Non-quantal release of ACh could occur by non-specific leakage of cytosolic ACh from synaptosomes. Minnema et al. (1989) observed that MeHg increases [^{3}H]deoxyglucose leakage from synaptosomes and suggested that perhaps MeHg also causes transmitter leakage by increasing membrane permeability. In that study, the membrane effects of MeHg were probably not extensive since they were completely reversible and the synaptosomes released transmitter in response to K^+-depolarization after treatment with MeHg. Whether increased deoxyglucose efflux can be taken to indicate increased non-specific ACh efflux is uncertain. Komulainen and Bondy (1987) reported that 30 μM MeHg did not alter synaptosomal plasma membrane permeability but that 100 μM MeHg

increased the permeability of membranes to small ions. This concentration of MeHg did not cause leakage of an intrasynaptosomal Ca^{2+}-sensitive dye. We observed that partially permeabilizing the plasma membranes of [^{3}H]choline-loaded synaptosomes with low concentrations of Saponin causes [^{3}H]ACh leakage (results not shown). Treatment of [^{3}H]choline-loaded synaptosomes with Saponin after they have been incubated with 10 μM or 100 μM MeHg caused a much greater increase in release of [^{3}H]ACh than that caused by MeHg alone. This indicates that MeHg did not extensively perturb membrane integrity. During incubations of similar duration to those in the present study in which 100 μM MeHg increased [^{3}H]ACh release, 100 μM MeHg significantly blocks uptake of $^{45}Ca^{2+}$ into synaptosomes (Atchison et al., 1986; Shafer and Atchison, 1989). This suggests that at least during short incubation periods MeHg does not cause excessive membrane leakiness. Based on current information, it cannot be determined whether MeHg increases non-specific leakage of ACh from nerve terminals. Although, non-quantal release of transmitter can occur by non-specific leakage of transmitter from the nerve terminal, other mechanisms are also involved and this form of transmitter release is a much more complicated process than leakage or passive diffusion of transmitter from the axon terminal (Polak et al., 1981). Non-quantal release of ACh may be produced by the ACh transport system in synaptic vesicles (Vyskocil et al., 1989). Non-quantal release of transmitter occurs under normal conditions and accounts for much of the ACh released spontaneously from neuromuscular preparations (Mitchell and Silver, 1963; Fletcher and Forester, 1975; Miledi et al., 1980; Vyskocil et al., 1989) as well as from synaptosomes (Heuser and Lennon, 1973). Thus, it is possible that MeHg could affect non-quantal release

of transmitter by mechanisms other than increasing the permeability of plasma membranes to ACh.

MeHg stimulates spontaneous release of ACh from synaptosomes (present study, Minnema et al., 1989), brain slices (Saijoh et al., 1987) and the NMJ (Atchison, 1986; 1987) in the absence of external Ca^{2+}. MeHg may stimulate spontaneous release by releasing Ca^{2+} from bound intracellular stores. Intraterminal mitochondria are a likely target for this effect of MeHg. Although the role of mitochondria in the overall regulation of cytosolic Ca^{2+} is not clear, nerve terminal mitochondria can sequester and store large quantities of Ca^{2+} (Scott et al., 1980; Nicholls and Akerman, 1981; Nicholls, 1986). Intrasynaptosomal mitochondria from nerve terminals that have been isolated in both normal Ca^{2+} and Ca^{2+}-free media have been shown to contain Ca^{2+} (Scott et al., 1980). MeHg inhibits mitochondrial respiration (Verity et al., 1975; Sone et al., 1977; O'Kusky, 1983; Cheung and Verity, 1981) and ATP production (Sone et al., 1977; Kauppinen et al., 1989). In synaptosomes, inhibition of these mitochondrial functions results in increased $[Ca^{2+}]$ (Ashley et al., 1982; Heinonen et al., 1984). MeHg also induces an immediate efflux of $^{45}Ca^{2+}$ from isolated mitochondria (Harris and Baum, 1980; Levesque and Atchison, submitted). If MeHg produced this efflux of Ca^{2+} from mitochondria in situ, then $[Ca^{2+}]$ would become elevated. Komulainen and Bondy (1987) used a fluorescent probe for Ca^{2+} to show that MeHg significantly increases $[Ca^{2+}]$ in synaptosomes incubated in Ca^{2+}-free solutions, however, the source of this Ca^{2+} was not examined in detail. This Ca^{2+} may have come from mitochondria since MeHg was less effective in elevating $[Ca^{2+}]$ in synaptosomes that were pretreated to inhibit uptake and induce release of Ca^{2+} from intrasynaptosomal

mitochondria.

The present results with RR and perhaps YS035 provide further evidence for a link between release of mitochondrial Ca^{2+} and the stimulation by MeHg of spontaneous release of transmitter from the nerve terminal. RR inhibits Ca^{2+} transport into or out of the mitochondrion via the Ca^{2+} uniport protein located on the inner mitochondrial membrane (Moore, 1971; Fiskum and Cockrell, 1978; Luthra and Olson, 1977; Jurkowitz et al., 1983). RR readily penetrates plasma membranes and can enter into nerve fibers (Singer et al., 1972). RR may have reduced the effectiveness of MeHg for inducing ACh release from synaptosomes by preventing MeHg from releasing Ca^{2+} from mitochondria. Pretreatment of $^{45}Ca^{2+}$-loaded mitochondria with RR blocks the release of $^{45}Ca^{2+}$ from the organelles that MeHg induces in the absence of RR (Levesque and Atchison, submitted). The similar results obtained with RR in the presence and absence of external Ca^{2+} imply that RR blocked the stimulatory effect of MeHg on ACh release by preventing release of mitochondrial Ca^{2+} and not by altering plasma membrane Ca^{2+} transport. Pretreatment of [^{3}H]choline-loaded synaptosomes with RR could also reduce the effect of MeHg on transmitter release by inducing the efflux of a pool of Ca^{2+} from mitochondria (Carafoli, 1982) upon which MeHg could act. Specific mitochondrial Ca^{2+} efflux pathways, not associated with the uniporter, are insensitive to RR and RR induces a net efflux of Ca^{2+} from mitochondria (Moore, 1971; Vasington et al., 1972). The RR-induced release of Ca^{2+} from the mitochondria has been suggested to underlie it's stimulation of spontaneous transmitter release observed at the NMJ (Alnaes and Rahamimoff, 1975; Bernath and Vizi, 1987; Levesque and Atchison, 1987) and from brain slices (Gomez and Farrell, 1985). RR probably induced

spontaneous release of [^{3}H]ACh from synaptosomes in the present study by this mechanism since it retained it's ability to stimulate release even in the absence of external Ca^{2+}.

YS035 inhibits Ca^{2+} entry into synaptosomes and inhibits release of Ca^{2+} from isolated mitochondria (Deana et al., 1984). Unlike RR, YS035 by itself did not stimulate [^{3}H]ACh release from synaptosomes. Based on the known stabilizing effects of YS035 on [Ca^{2+}], this agent probably does not increase [Ca^{2+}] and would not be expected to increase transmitter release via a Ca^{2+}-dependent mechanism when used alone. If anything, the presumed ability of YS035 to prevent increases in or cycling of intracellular Ca^{2+} might be expected to reduce transmitter release. This may explain the inhibitory effect of YS035 on the frequency of spontaneous release of transmitter at the NMJ (Levesque and Atchison, 1988). In the present study, YS035 only slightly reduced the ability of MeHg to stimulate release of [^{3}H]ACh from synaptosomes. This effect of YS035 was probably due to inhibition of mitochondrial Ca^{2+} transport rather than plasma membrane Ca^{2+} fluxes since it occurred in Ca^{2+}-free media. YS035 apparently blocks mitochondrial Ca^{2+} release induced by different treatments with variable efficiency (Deana et al., 1984). Perhaps the MeHg-induced efflux of Ca^{2+} from mitochondria occurs by a mechanism that is only partially sensitive to YS035.

The effectiveness of RR and YS035 for blocking MeHg-induced increases in spontaneous release of transmitter differed in the present study and in previous experiments at the NMJ (Levesque and Atchison, 1987; 1988). Both RR and YS035 completely blocked MeHg-induced increases in spontaneous quantal release of ACh at the NMJ. Perhaps the inhibitory effects of these agents were

partially due to postsynaptic effects. Whether YS035 has such effects has not been determined but RR is thought to affect postsynaptic responses to neurotransmitters (Robertson and Wann, 1987). The synaptosomal preparation only contains presynaptic nerve terminals and results would not be affected if RR or YS035 had postsynaptic actions. Another explanation could be likely if MeHg increases non-quantal release or leakage of ACh in addition to stimulating quantal release. Considering their somewhat specific effects on Ca^{2+} transport, RR and YS035 may inhibit only quantal release induced by MeHg. Non-quantal release is not as sensitive to $[Ca^{2+}]_i$ as is quantal release (Vyskocil et al., 1989). Thus, in the presence of RR and YS035 only non-quantal release of ACh would occur. As was mentioned, ACh released in this manner would be detected only by the neurochemical methods utilized in the present experiments and not by the electrophysiological measurements made in the studies at the NMJ.

The effect of MeHg on [^{3}H]ACh release from synaptosomes was somewhat attenuated in Ca^{2+}-free solutions. A reduced effect of MeHg in the absence of Ca^{2+} was also observed at the NMJ. This implies that external Ca^{2+} contributes at least in part to the elevated $[Ca^{2+}]_i$ for the stimulatory effect of MeHg on spontaneous release of transmitter. Komulainen and Bondy (1987) showed that although MeHg significantly increases synaptosomal free Ca^{2+} levels in the absence of external Ca^{2+}, the maximum increase in $[Ca^{2+}]_i$ is less than that observed in Ca^{2+}-containing media. The mechanism by which MeHg caused Ca^{2+} entry into nerve terminals is unclear and it is not known whether this occurred in the present experiments. It is doubtful that MeHg elicits Ca^{2+} influx through voltage-sensitive Ca^{2+} channels since the increase in synaptosomal $[Ca^{2+}]$ induced by MeHg is not blocked by

verapamil (Komulainen and Bondy, 1989). Also, the MeHg-induced increase in spontaneous quantal release of ACh at the NMJ is not prevented by Co^{2+} (Miyamoto, 1983) or high Mg^{2+} (Atchison and Narahashi, 1982). It has been suggested that 100 μM MeHg increases the permeability of synaptosomal plasma membranes to Ca^{2+} (Komulainen and Bondy, 1987). The putative effects on membrane permeability were not observed with 30 μM MeHg or lower concentrations. This would not explain the apparent partial dependence on external Ca^{2+} of the effect of 10 μM MeHg on [^{3}H]ACh release observed in the present study. In addition, 100 μM MeHg has been shown to block uptake of $^{45}Ca^{2+}$ into synaptosomes during time intervals similar to those during which MeHg stimulated [^{3}H]ACh release in the present study (Atchison et al., 1986; Shafer and Atchison, 1989). There is no conclusive evidence indicating whether MeHg caused Ca^{2+} influx through the plasma membrane in the present study and the contribution of external Ca^{2+} to the stimulatory effects of MeHg on spontaneous release of ACh remains to be determined.

Another possible explanation for the reduced effect of MeHg on spontaneous release of ACh in Ca^{2+}-free solutions may be that there is less intrasynaptosomal bound Ca^{2+}. Incubating synaptosomes in Ca^{2+}-free solutions reduces intramitochondrial Ca^{2+} content (Scott et al., 1980). In the present study, [^{3}H]choline-loaded synaptosomes were washed twice and resuspended in Ca^{2+}-free media before they were used in Ca^{2+}-free release experiments. If there is less bound Ca^{2+} within the nerve terminal for MeHg to release, the maximum increase in $[Ca^{2+}]$ would be less than if mitochondria contained a larger quantity of Ca^{2+}. Since spontaneous release of ACh is strongly dependent on $[Ca^{2+}]$, the quantity

of ACh released by MeHg under these conditions would be reduced. The effect of RR on [^{3}H]ACh release was also reduced in Ca^{2+}-free solutions. Rather than increase Ca^{2+} influx into synaptosomes, RR may block entry of Ca^{2+} through voltage-gated channels (Taipale et al., 1989). This suggests that the reduced effect of RR on [^{3}H]ACh release in Ca^{2+}-free solutions may not be due to the absence of external Ca^{2+} but is probably related to it's effects on mitochondrial Ca^{2+}. Reduced intraterminal bound Ca^{2+} levels may also explain the attenuated effect of MeHg in Ca^{2+}-free media at the NMJ since bathing the neuromuscular preparation in Ca^{2+}-free solutions markedly reduced the Ca^{2+} content of the tissue (Atchison, 1986).

In Summary, the results indicate that MeHg may interfere with cholinergic neurotransmission in the CNS by reducing ACh synthesis and by stimulating spontaneous release of ACh. The effects of MeHg on transmitter release from CNS nerve endings are similar to those observed previously at the NMJ. MeHg may stimulate spontaneous transmitter release subsequent to disrupting intraterminal Ca^{2+} homeostasis and elevating $[Ca^{2+}]$. The results indicate that MeHg may induce release of Ca^{2+} from nerve terminal mitochondria. Perturbation of $[Ca^{2+}]$ by MeHg may underlie it's effects on other transmitter systems in both the peripheral and central nervous systems. Moreover, release of neurotransmitters is only one example of a Ca^{2+}-dependent process. If MeHg indeed alters cellular Ca^{2+} regulation by interfering with transmembrane Ca^{2+} fluxes or Ca^{2+} buffering by intracellular organelles, one could predict effects of MeHg on other Ca^{2+}-dependent cellular functions in neuronal as well as in non-neuronal cells.

CHAPTER SIX

CHARACTERISTICS OF BINDING OF METHYLMERCURY TO ISOLATED MITOCHONDRIA AND SYNAPTOSOMES

ABSTRACT

Binding characteristics of the neurotoxicant methylmercury (MeHg) to synaptosomes and mitochondria isolated from rat brain were studied using radiolabeled Me[^{203}Hg]. The primary objectives were to examine possible modes of entry of MeHg into nerve endings and to assess the effects of ruthenium red (RR) and N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035) on binding of Me[^{203}Hg] to mitochondria and synaptosomes. Binding of MeHg was determined by incubating synaptosomes or mitochondria with the radiolabeled organomercurial, filtering and measuring the radioactivity retained on the filters. Binding of Me[^{203}Hg] to synaptosomes occurred rapidly and saturated at about 3.8 $\pm$ 0.5 x 10^{-2} nmoles/μg protein within 10 sec. Depolarizing synaptosomes with high K^+ (55 mM) did not alter the time course or amount of Me[^{203}Hg] bound by synaptosomes relative to parallel non-depolarized controls. Partially permeabilizing synaptosomal plasma membranes with saponin, a detergent that disrupts the structural integrity of cholesterol-rich membranes, reduced the binding of Me[^{203}Hg] to synaptosomes. Me[^{203}Hg] rapidly became bound to mitochondria and reached peak levels of approximately 8.2 $\pm$ 2.0 nmoles/mg protein within 60 sec. Saponin did not affect binding of Me[^{203}Hg] to mitochondria, which have membranes that are low in cholesterol. Pretreatment of mitochondria and synaptosomes with RR or YS035 had no effect on binding of Me[^{203}Hg]. The thiol reagents, D-penicillamine (D-PEN), glutathione (GSH) and dithiothreitol (DTT), reduced the binding of Me[^{203}Hg] by over 90%. Treatment of synaptosomes with D-Pen or GSH, which are not as readily membrane-permeable, after Me[^{203}Hg]

binding had already occurred, reduced total Me[^{203}Hg] bound by 35-45%. DTT, a membrane-permeable thiol reagent , removed over 80% of the bound Me[^{203}Hg] from synaptosomes. After incubating mitochondria with Me[^{203}Hg], subsequent treatment with D-Pen or GSH resulted in the loss of about 35% of the bound MeHg, while DTT removed over 65%. Permeabilizing synaptosomal membranes with saponin increased the ability of D-Pen and GSH, but not DTT, to remove bound Me[^{203}Hg]. Saponin pretreatment did not alter the effects of any of the thiol reagents on binding of Me[^{203}Hg] to mitochondria. The results with saponin and the thiol reagents indicate that in addition to binding externally, MeHg may gain entrance into synaptosomes. Entry of MeHg into synaptosomes may occur primarily via passive diffusion rather than voltage-dependent membrane channels since K^+-induced depolarization did not increase binding. RR and YS035 do not occlude entry of MeHg into synaptosomes and do not inhibit its binding to mitochondria. As expected, much of the MeHg bound to synaptosomes and mitochondria is apparently bound to sulfhydryl groups.

INTRODUCTION

Methylmercury (MeHg) is a potent environmental neurotoxicant that transiently stimulates spontaneous release of transmitter at both central and peripheral synapses. Increased spontaneous release of neurotransmitter has been attributed to presynaptic actions of MeHg (Atchison and Narahashi, 1982). MeHg may interact with intraneuronal mitochondria to induce release of bound Ca^{2+} into the nerve terminal cytoplasm, ultimately resulting in stimulated spontaneous release of neurotransmitter (Levesque and Atchison, 1987; 1988). In order for MeHg to affect neurotransmitter release subsequent to an interaction with intraneuronal mitochondria, MeHg would have to penetrate the plasma membrane and enter the nerve terminal cytoplasm, a hypothesis consonant with existing data. For example, MeHg crosses the blood-brain barrier and becomes localized within central neurons when given parenterally (Nordberg et al., 1970; Chang and Hartmann, 1972a,b). When administered to isolated neuronal preparations, MeHg binds to membranous organelles within the cytoplasm (Chang et al., 1977). MeHg also affects mitochondrial functions in situ (Fox et al., 1975; Verity et al., 1975) and in vivo (O'Kusky, 1983), suggesting that it can penetrate cells and reach the mitochondrion. The mechanisms by which MeHg traverses cell membranes are not clear. Studies of the permeability of MeHg across model lipid bilayers (Lakowicz and Anderson, 1980) and membrane phospholipids (Leblanc et al., 1984) indicate that MeHg crosses such surfaces rapidly, presumably via passive diffusion. Perhaps the aliphatic methyl group confers sufficient lipophilicity upon the molecule to permit its entry through the cell membrane via passive

diffusion. Passive diffusion may not be the only mechanism by which MeHg may enter cells. Results of electrophysiological experiments at the neuromuscular junction (NMJ) suggest that MeHg may additionally enter the nerve terminal through voltage-dependent Ca^{2+} channels (Atchison, 1986; 1987). This conclusion was based on the marked shortening of the time preceding the onset and time to peak stimulation of spontaneous release of ACh induced by MeHg after activating Ca^{2+} channels, in Ca^{2+}-deficient solutions.

Synaptosomes were used as model nerve terminals in the present study in attempts to show that MeHg gains entrance into nerve terminals and to determine by which pathway MeHg enters the cell. Passive uptake of MeHg was measured by incubating synaptosomes and mitochondria with Me[^{203}Hg] during various incubation times. To test whether MeHg could enter synaptosomes, uptake of Me[^{203}Hg] by synaptosomes under normal conditions was compared to uptake after permeabilization of the plasma membrane with saponin, a cholesterol-specific detergent (Birk and Peri, 1980). The non-permeable thiol reagents, D-penicillamine (D-PEN) and glutathione (GSH) (Chui and Grady, 1981; Meister and Anderson, 1983), and a membrane permeable thiol reagent, dithiothreitol (DTT) (Cleland, 1964), were used to determine the nature of the binding of MeHg to synaptosomes and mitochondria and to assess further whether MeHg binds internally. These agents maintain sulfhydryl groups and also chelate MeHg. To determine the importance of uptake of MeHg into nerve terminals through voltage-dependent membrane ion channels, the effects of K^+-induced depolarization on uptake of Me[^{203}Hg] by synaptosomes was measured. The cholesterol-specific detergent saponin and thiol reagents with different membrane permeabilities were used to

ascertain whether MeHg entered the synaptosomes or became bound externally.

MeHg-induced stimulation of spontaneous release of ACh from nerve terminals (Levesque and Atchison, 1987; 1988) is blocked by pretreatment with the mitochondrial Ca^{2+} transport inhibitors (Moore , 1971; Deana et al., 1984), ruthenium red (RR) and N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035). It was presumed that these agents blocked the effects of MeHg by inhibiting the interaction of MeHg with intraterminal mitochondria to elevate free-Ca^{2+} within the axon terminal. Alternatively, RR and YS035 may have prevented access of MeHg to the nerve terminal cytoplasm and hence inhibited accumulation of MeHg at it's proposed intraterminal target site. It is also possible that these agents inhibit binding of MeHg to mitochondria or to some site on the mitochondrial membrane at which MeHg may act. To test these possibilities, passive uptake of Me[^{203}Hg] by synaptosomes and mitochondria was measured in the absence and presence of RR and YS035.

METHODS

Preparation of mitochondria and synaptosomes. Mitochondria and synaptosomes were isolated from forebrains of male Sprague-Dawley rats (Harlan, 175-225g) using a modification of the methods of Booth and Clark (1978) as described previously (Chapters 4,5). Briefly, forebrains were quickly removed from decapitated rats, dropped into ice-cold isolation medium (0.32 M sucrose; 1 mM potassium EDTA; 10 mM Tris /HCl; pH 7.4) and homogenized by 8 up-and-down strokes at 550 rpm. The homogenate was centrifuged at 1,300g for 3 min to remove blood cells and connective tissue. The supernatant was centrifuged at 17,000g for 15 min to produce a crude mitochondrial/synaptosomal pellet, which was resuspended in 15% Ficoll/sucrose medium (15% (w/w) ficoll, 0.32 M sucrose, 50 μM potassium EDTA, pH 7.4) and placed into an ultracentrifuge tube. A 7.5% Ficoll/sucrose solution (7.5% (w/w) Ficoll, 0.32 M sucrose, 50 μM potassium EDTA, pH 7.4) was carefully layered on top of the resuspended crude preparation; isolation medium was used to fill the top portion of the centrifuge tube. The tubes were then centrifuged at 99,000g for 45 min to separate free mitochondria from synaptosomes. The synaptosomes, which banded at the interface between the 7.5 and 15% Ficoll layers, were collected and resuspended in Hepes-buffered Krebs Ringer (HKR) (145 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM D-glucose, 10 mM Hepes, pH 7.4) and centrifuged at 9,800g for 10 min. The purified synaptosomes were resuspended in HKR (5-10 mg protein/ml) for use in binding experiments. Mitochondria, which pelleted at the bottom of the ultracentrifuge tubes, were resuspended in 10 ml of isolation medium and centrifuged at 9,800g

for 10 min. The pellets were resuspended in bovine plasma albumin medium (10 mg BPA in 20 ml isolation medium) and repelleted at 9,800g for 10 min. The purified mitochondria were resuspended in K^+ buffer (135 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 10 mM glucose) at 5-10 mg protein/ml for use in binding studies. All isolation steps were carried out at 4°C. All buffers were prepared daily from stock solutions and osmolarity was maintained at 320 $\pm$ 10 mOsm. Mitochondrial and synaptosomal protein was determined by the method of Lowry et al. (1951).

Binding of Me[^{203}Hg] to mitochondria and synaptosomes. Passive uptake of MeHg by mitochondria or synaptosomes was measured by adding 50 μl of mitochondria in K^+ buffer (150-200 μg) or synaptosomes in HKR (150-200 μl) to an equal volume of the same buffer containing 200 μM Me[^{203}Hg] (twice the desired final concentration). Mitochondria and synaptosomes were incubated with Me[^{203}Hg] for various time intervals (see figures) before stopping the binding/uptake reaction by diluting the samples with 2 ml of ice-cold buffer and then rapidly filtering through 0.45 μm Millipore filters under suction. The filters were washed with two 5 ml aliquots of buffer. Any Me[^{203}Hg] retained on the filters was bound to mitochondria or synaptosomes since washing completely removed all Me[^{203}Hg] when it was passed through filters in control experiments without mitochondria or synaptosomes. Filters were placed into scintillation vials containing 1.5 ml of Triton X-100/HCl solubilizer; 10 ml of scintillation cocktail were added after 10 min. Radioactivity retained on the filters was measured in a Searle model 1197 gamma counter with a 45% efficiency for Me[^{203}Hg]. The effects of RR and YS035 on passive uptake of Me[^{203}Hg] by mitochondria or synaptosomes were determined by comparing uptake of Me[^{203}Hg] in the absence of these agents to

uptake in experiments in which RR or YS035 were added to mitochondria and synaptosomes at the same time as Me[^{203}Hg]. The effects of the thiol reagents D-PEN, DTT and GSH on binding of Me[^{203}Hg] to mitochondria or synaptosomes were tested in experiments in which these agents were present in the quench solutions used to stop the uptake reactions. Thus, at the zero time points, these agents were present before initiating Me[^{203}Hg] uptake. At all other time points tested, the thiol reagents were not added until the reaction was quenched after uptake had occurred. Immediately after quenching with the thiol reagents, samples were filtered under suction. To determine the contribution of uptake of MeHg through voltage-dependent membrane ion channels to total synaptosomal uptake, the effects of K^+-induced depolarization on Me[^{203}Hg] uptake were measured. Synaptosomes were depolarized in solutions containing 55 mM K^+ HKR. The high K^+ buffer was the same as K^+ HKR except that the concentration of sodium was reduced (95 mM) to account for the elevated K^+. Total voltage-dependent and -independent uptake of Me[^{203}Hg] were determined during 1, 10, 30 and 60 sec incubations. Depolarization-independent uptake was taken as the uptake which occurred during incubations with normal (5 mM) K^+ HKR.

Synaptosomal plasma membrane permeabilization. Synaptosomal membranes were partially permeabilized by adding 0.1% (w/v) Saponin to the K^+ HKR containing the labeled MeHg prior to adding synaptosomes to start the Me[^{203}Hg] uptake/binding reactions. Saponin is a detergent that has a high affinity for cholesterol groups and will `punch' holes in, but not lyse, cholesterol-rich membranes such as the synaptosomal plasma membrane (Birk and Peri, 1980).

Materials. Methyl[^{203}Hg]chloride (18 mCi/g) was purchased from Amersham

Corporation (Arlington Hts., IL). This chloride salt of MeHg is readily soluble in aqueous solutions and Me[^{203}Hg]-containing buffers were made daily by adding small quantities of a concentrated Me[^{203}Hg] stock solution. Ficoll type 400-DL, dipotassium ethylenediamine tetraacetic acid (K^+ EDTA), Trizma HCl, ruthenium red, saponin, dithiothreitol, glutathione and D-penicillamine were purchased from Sigma Chemical Co. (St. Louis, MO). N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) was purchased from United States Biochemical Corporation (Cleveland, OH). YS035 was synthesized by the Organic Synthesis Laboratory of the Michigan State University Department of Chemistry according to the methods of Deana et al., (1984). A stock solution of YS035 dissolved in ethanol was used to prepare buffers containing YS035. The final concentration of ethanol in buffers did not exceed 0.04% (v/v) and solutions used in parallel control experiments contained an identical concentration of the vehicle.

RESULTS

If MeHg can utilize voltage-dependent Ca^{2+} channels for entry into nerve terminals, as has been suggested previously (Atchison, 1987), depolarization of synaptosomes might increase uptake of Me[^{203}Hg] over non-depolarized controls. To test this, the effects of K^+-induced depolarization on uptake of Me[^{203}Hg] during 1, 10, 30 and 60 sec intervals was measured (Figure 1). Uptake of Me[^{203}Hg] by synaptosomes in high $[K^+]_e$ was compared to uptake in parallel controls with low $[K^+]_e$. Uptake by synaptosomes in both high and low $[K^+]_e$ occurred very rapidly, reaching maximal levels within 1-10 sec. There was no difference between uptake of Me[^{203}Hg] in high and low K^+. Thus, uptake of Me[^{203}Hg] by synaptosomes was not increased subsequent to activation of voltage-dependent membrane ion channels by incubating synaptosomes in high $[K^+]_e$ HKR. Even when a large volume of cold buffer was added to dilute the samples and inhibit the uptake reaction before adding synaptosomes (0 sec time points), between 60-70% of the maximal Me[^{203}Hg] binding occurred. This may indicate that most of the uptake occurred via passive diffusion rather than by carrier- or channel-mediated mechanisms.

RR and YS035 may block the stimulatory effects of MeHg on spontaneous release of ACh (Levesque and Atchison, 1987; 1988) by preventing uptake of MeHg by synaptosomes or mitochondria. If this mechanism underlies the effects of these agents, then uptake of Me[^{203}Hg] by synaptosomes or mitochondria should be reduced by these agents. Adding RR (20 μM) to the reaction medium containing Me[^{203}Hg] prior to addition of synaptosomes did not reduce uptake of

Me[^{203}Hg] in either high or low [K^+]$_e$ HKR from control uptake in the absence of RR (Figure 1). Passive uptake of Me[^{203}Hg] by mitochondria was not decreased by RR (20 μM) during 1, 10, 60, 120 and 180 sec incubations (Figure 2). YS035 (200 μM; 1 mM) had no effect on uptake of Me[^{203}Hg] by depolarized or non-depolarized synaptosomes, compared to YS035-free controls (Figure 3). The same concentrations of YS035 also had no effect on passive uptake of Me[^{203}Hg] by mitochondria during 10, 30 and 60 sec intervals (results not shown). Thus, neither RR or YS035 appeared to inhibit uptake of Me[^{203}Hg] by synaptosomes or mitochondria.

There is evidence in the literature indicating that MeHg can traverse cell membranes. In the present study, attempts were made to determine whether MeHg was able to enter into synaptosomes or whether it became bound externally. If the synaptosomal plasma membrane is a sufficient barrier to prevent MeHg from entering the cytosol, increasing the permeability of this barrier might allow for entry of MeHg and may ultimately result in an increase in the amount of MeHg bound to synaptosomal protein. To examine this, saponin (0.1% w/v) was added to HKR solutions used in the Me[^{203}Hg] uptake reactions to permeabilize the synaptosomal plasma membrane. Inclusion of saponin in the reaction buffers actually reduced uptake of Me[^{203}Hg] by synaptosomes (Figure 4). Unlike the plasma membrane of synaptosomes, mitochondrial membranes have a low cholesterol content and are not permeabilized by saponin. As expected, saponin had no effect on uptake of Me[^{203}Hg] by mitochondria (Figure 5). This indicates that the saponin-induced reduction in Me[^{203}Hg] uptake by synaptosomes was due to it's effects on the membrane rather than a direct interaction with MeHg. The synaptosomal plasma

membrane may not prevent entry of MeHg into the cytosol since permeabilizing the membrane with saponin did not enhance binding of MeHg.

To determine further whether MeHg becomes bound within synaptosomes and mitochondria and to examine the nature of the binding sites, the thiol-containing compounds D-PEN, GSH and DTT were tested for their effects on binding of Me[^{203}Hg]. D-PEN and GSH are less permeable than DTT and do not enter cells as readily as does DTT. MeHg has a high affinity for binding to sulfhydryl groups and the majority of MeHg associated with synaptosomal and mitochondrial protein may be bound to sulfhydryl groups (Clarkson, 1972). The thiol reagents are able to remove bound MeHg by maintaining sulfhydryl groups in the reduced form or by chelating MeHg directly. D-PEN and GSH would be excluded from the intracellular space and thus may only remove MeHg bound externally, while DTT, the more permeable reagent, could possibly chelate any MeHg that may be bound internally. The effects of these reagents on Me[^{203}Hg] bound to synaptosomes are shown in Figure 6. Thiol reagents were present in the quench buffers used to stop the Me[^{203}Hg] uptake reactions. The results are expressed as the percentage of Me[^{203}Hg] still bound to synaptosomes after quenching with the thiol reagents relative to parallel controls in which the quench buffers contained no thiol reagents. All three agents completely prevented uptake of Me[^{203}Hg] by synaptosomes when the quench solutions were added prior to adding synaptosomes ("zero time" points). Quenching the uptake reaction with either D-PEN or GSH, after incubating the synaptosomes with Me[^{203}Hg] for 10 or 30 sec, reduced the amount of Me[^{203}Hg] bound to synaptosomes by about 40%. DTT was much more effective at removing bound Me[^{203}Hg], reducing the total

amount bound by almost 90%. If the difference in the effectiveness of these agents for removing bound Me[^{203}Hg] was related to the fact that D-PEN and GSH are less permeable than DTT, then perhaps partially permeabilizing the synaptosomal plasma membrane with saponin would enhance the effectiveness of D-PEN and GSH. As shown in Figure 6, both D-PEN and GSH were able to remove much more bound Me[^{203}Hg] from synaptosomes after permeabilization with saponin, while the effect of DTT was unchanged. Similar experiments were done with mitochondria and the results are shown in Figure 7. As in synaptosomes, DTT was more effective than D-PEN and GSH at reducing the amount of Me[^{203}Hg] retained by mitochondria. Quenching with DTT resulted in the loss of over 65% of bound Me[^{203}Hg], while D-PEN and GSH removed 30-35%. Since mitochondrial membranes are relatively insensitive to the permeabilizing effects of saponin, it was not surprising to find that the effects of the thiol reagents were no different in the presence of saponin than in it's absence. This may be taken as evidence that the enhancement of the effects of D-PEN and GSH after treatment of synaptosomes with saponin were indeed related to the increased permeability of the plasma membrane.

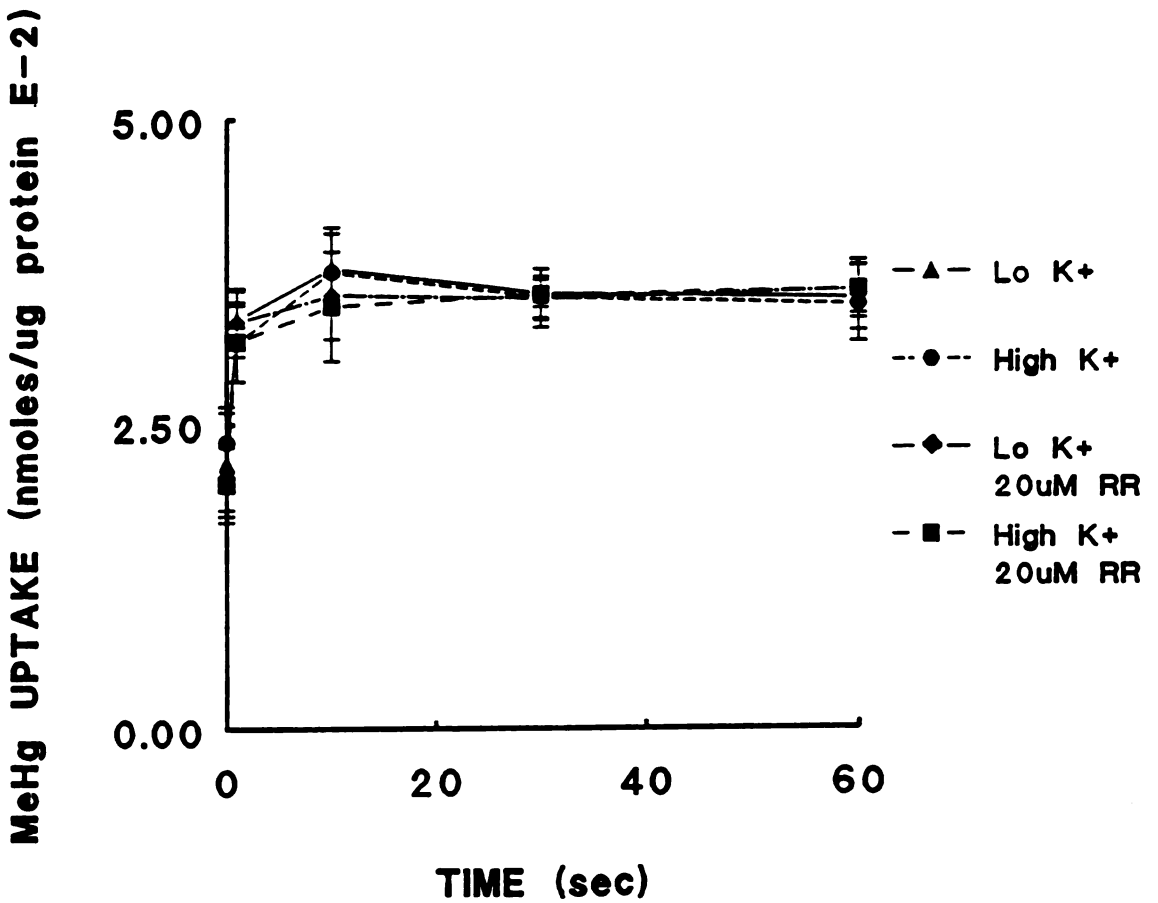

Figure 1. Time course of Me[^{203}Hg] (100 μM) uptake by depolarized (55 mM, high $[K^+]_e$) and non-depolarized (5 mM, lo $[K^+]_e$) synaptosomes in the absence and presence of 20 μM ruthenium red. Me[^{203}Hg] and RR were present in the HKR buffer (145 or 95 mM NaCl, 5 or 55 mM KCL, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM d-glucose, 5 mM Hepes, pH 7.4) to which synaptosomes were added to initiate the uptake reactions. Me[^{203}Hg] uptake was measured during intervals of 1, 10, 30 and 60 sec. The uptake reactions were stopped by diluting with cold quench buffer either before initiating uptake (time zero) or immediately after the various incubation intervals. The samples were filtered under suction immediately after quenching. Me[^{203}Hg] uptake refers to the total gamma radioactivity (^{203}Hg) retained on the filters. Values are the mean $\pm$ SEM of six different experiments. Values for each experiment are the average of three replicates.

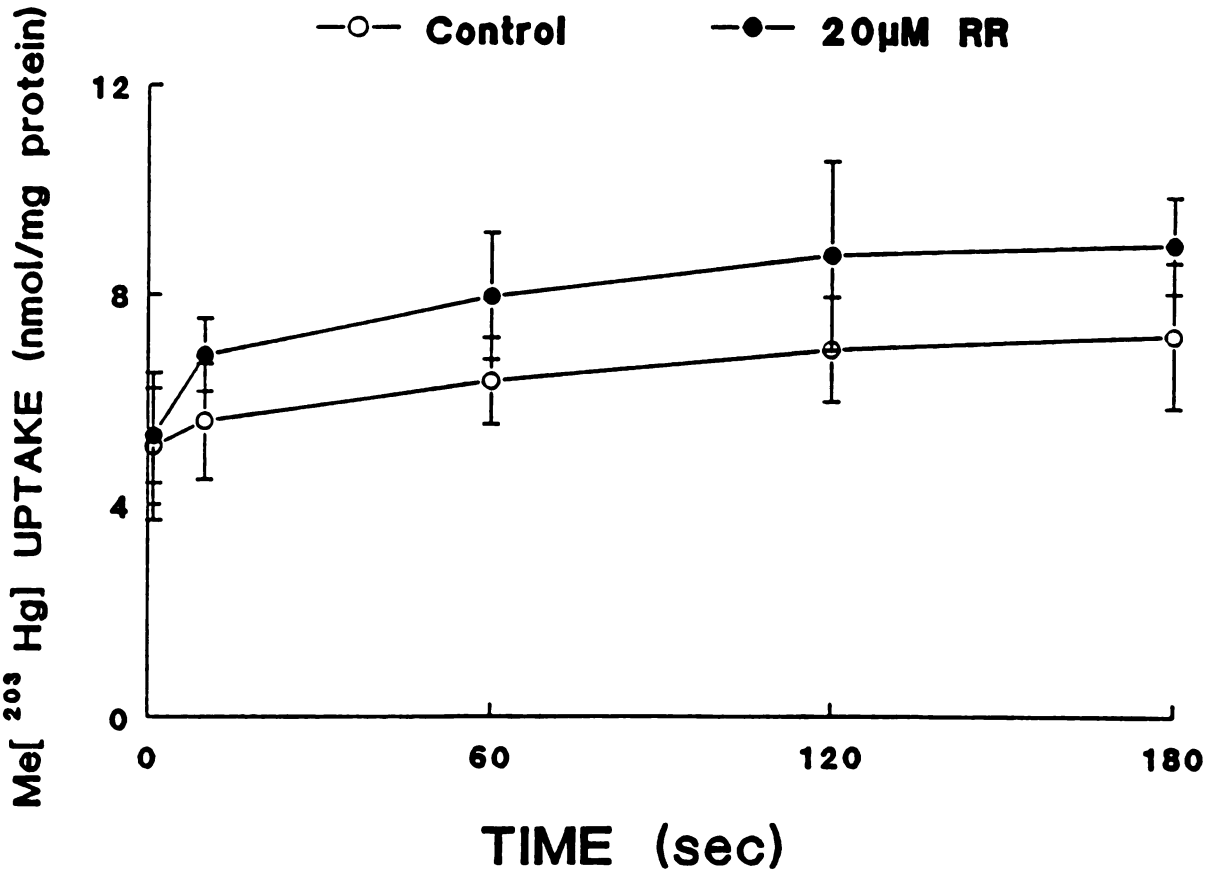

Figure 2. Time course of effects of RR (20 μM) on passive uptake of Me[^{203}Hg] (100 μM) during intervals of 1, 10, 60, 120 and 180 sec. RR was present in the K^+ buffer (135 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 10 mM glucose, pH 7.4) to which mitochondria were added to initiate uptake. Uptake was stopped by diluting with cold K^+ and then suction filtering. Me[^{203}Hg] uptake refers to the total gamma radioactivity (^{203}Hg) retained on the filters. Values are the mean $\pm$ SEM of seven experiments. Values for each experiment are the average of three replicates.

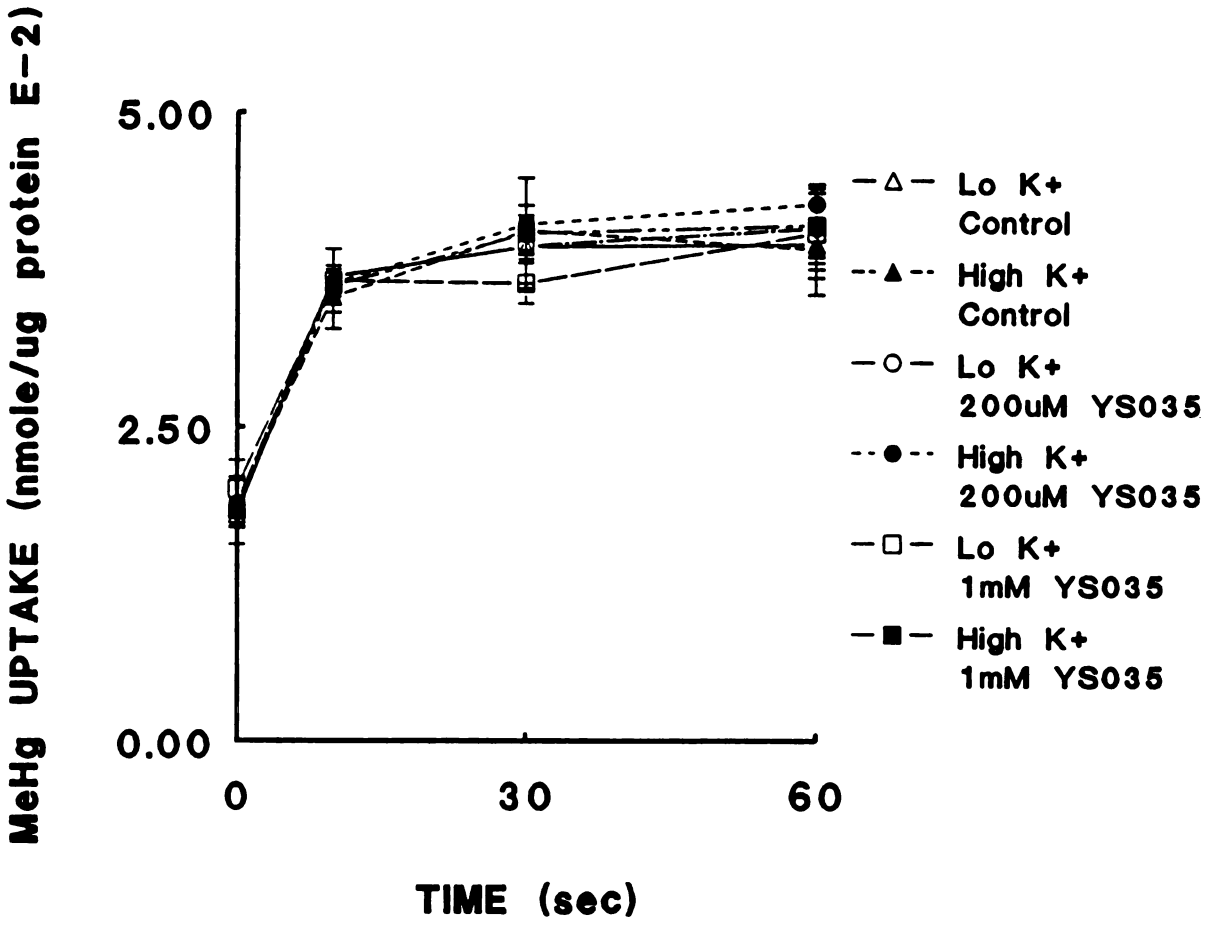

Figure 3. Time course of Me[^{203}Hg] (100 μM) uptake by depolarized (55 mM, high $[K^+]_e$) and non-depolarized (5 mM, lo $[K^+]_e$) synaptosomes in the absence and presence of 200 μM or 1 mM N,N-bis(3,4-dimethoxyphenylethyl)-N-methylamine (YS035). Me[^{203}Hg] and YS035 were present in the HKR buffer to which synaptosomes were added to initiate the uptake reactions. Other details are the same as in Figure 1. Me[^{203}Hg] uptake refers to the total gamma radioactivity (^{203}Hg) retained on the filters. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates.

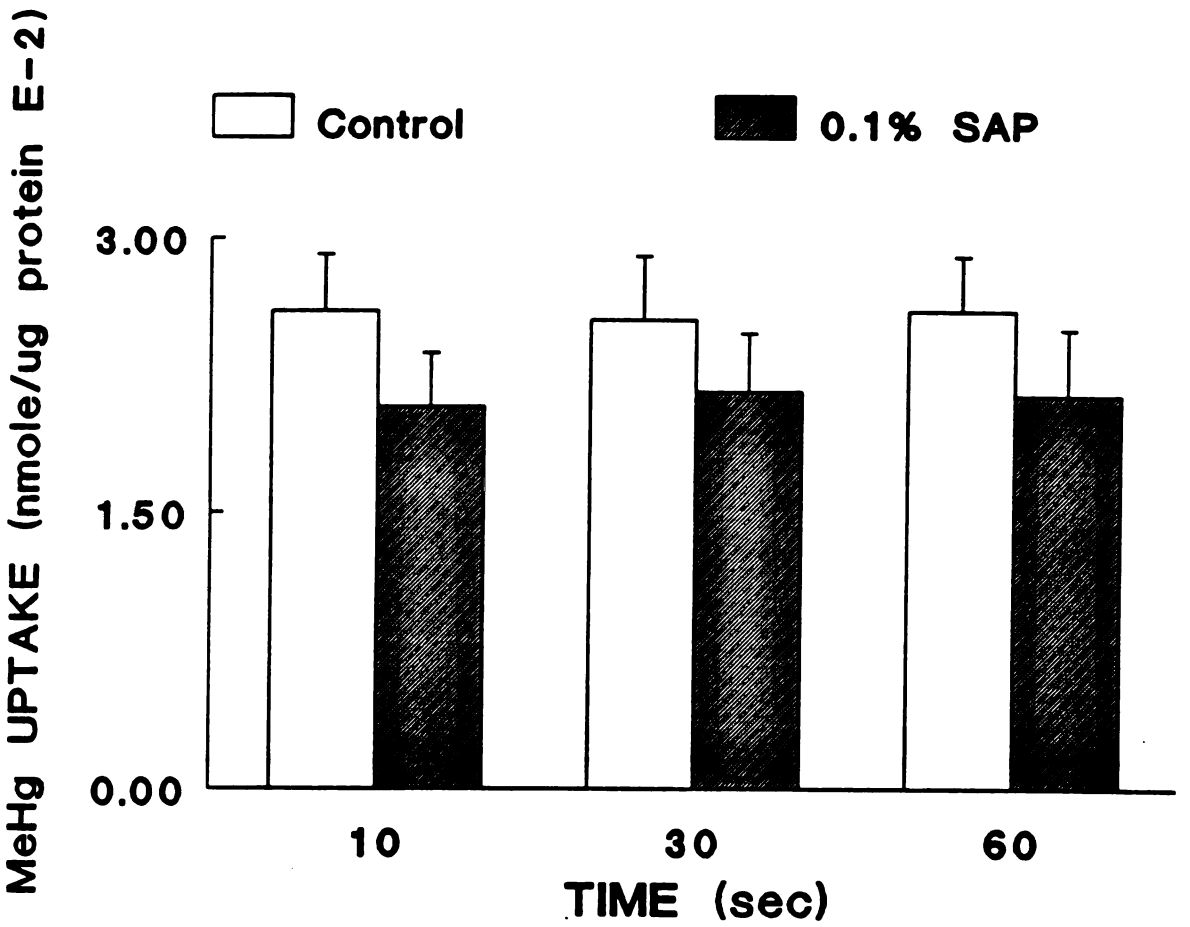

Figure 4. Effects of saponin 0.1% on passive uptake (low $[K^+]_e$ HKR) of Me[^{203}Hg] (100 μM) by synaptosomes. Saponin was dissolved in the HKR buffer containing Me[^{203}Hg] before synaptosomes were added to initiate uptake. The uptake reactions were quenched after 10, 30 or 60 sec. Other details are the same as in Figure 1. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates.

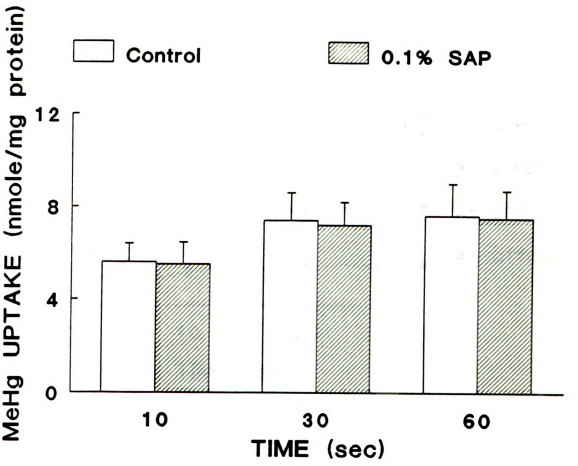

Figure 5. Effects of saponin 0.1% on passive uptake of Me[^{203}Hg] (100 μM) by mitochondria in K$^+$ buffer. Saponin was dissolved in the K$^+$ buffer containing Me[^{203}Hg] before mitochondria were added to initiate uptake. The uptake reactions were quenched after 10, 30 or 60 sec. Other details are the same as in Figure 2. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates.

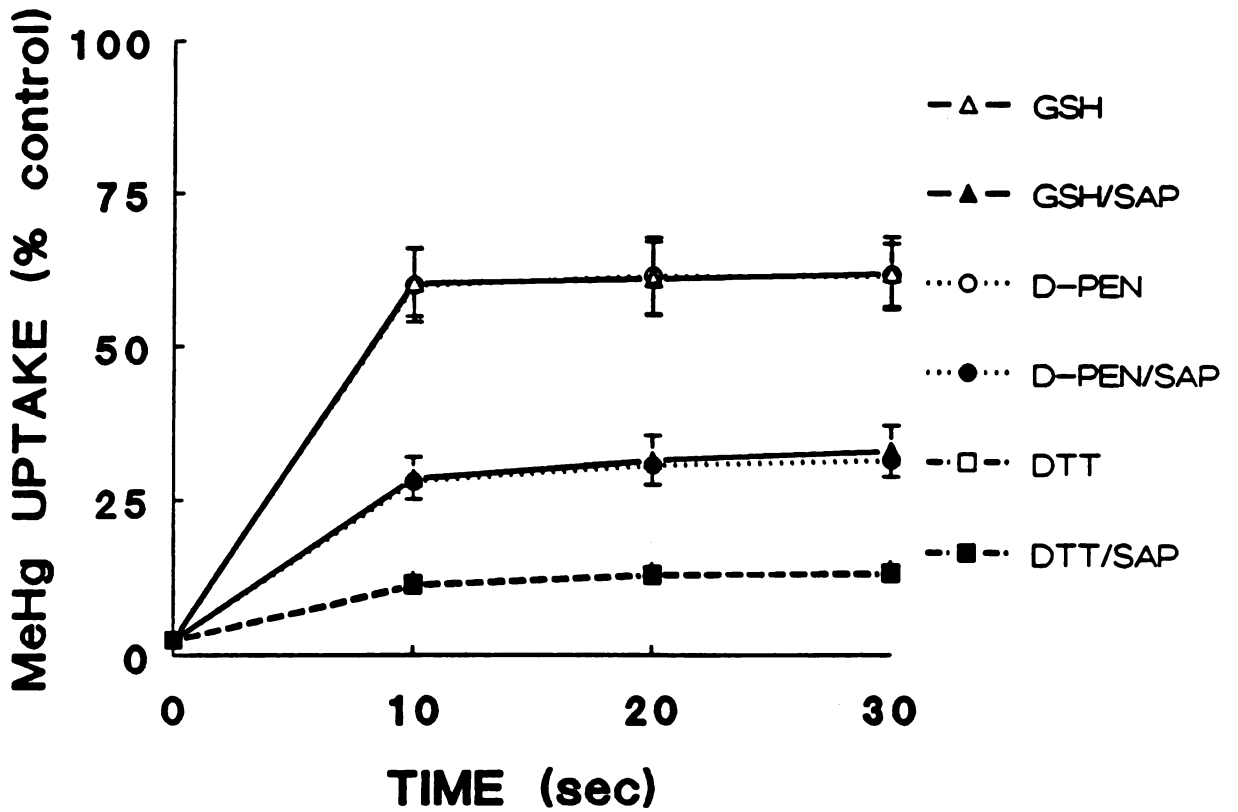

Figure 6. Me[^{203}Hg] retained by synaptosomes in the presence and absence of 0.1% saponin after stopping the uptake reactions with quench solutions containing d-penicillamine (d-PEN; 1 mM), glutathione (GSH; 1 mM) or dithiothreitol (DTT; 1mM). Saponin was dissolved in the HKR buffer (5 mM K^+) containing Me[^{203}Hg] (100 μM) before adding synaptosomes to initiate uptake. The uptake reaction was quenched with cold HKR containing the thiol reagents before adding synaptosomes (0 sec) or after incubating synaptosomes with Me[^{203}Hg] for 10, 20 or 30 sec. Results are expressed as a percentage of Me[^{203}Hg] retained by synaptosomes in the presence of the thiol reagents or saponin, relative to Me[^{203}Hg] retained by synaptosomes in parallel controls without saponin or thiol reagents. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates.

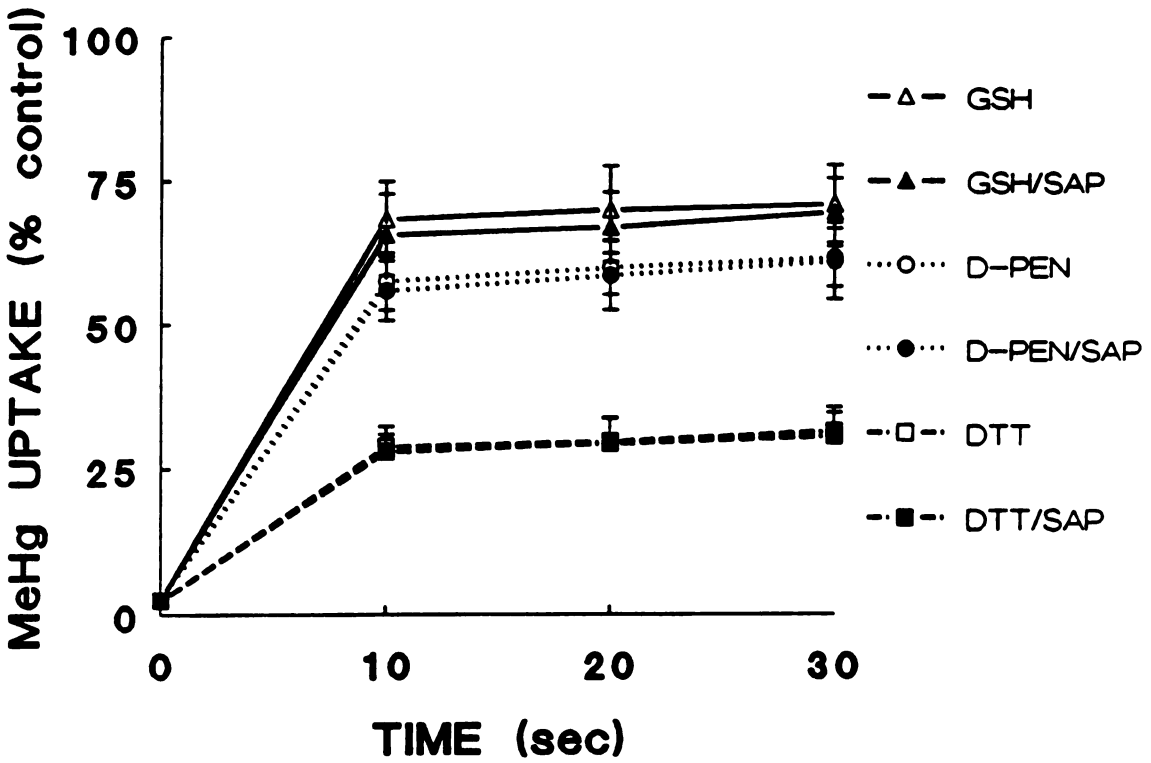

Figure 7. Me[^{203}Hg] retained by mitochondria in the presence and absence of 0.1% saponin after stopping the uptake reactions with quench solutions containing d-penicillamine (d-PEN; 1 mM), glutathione (GSH; 1 mM) or dithiothreitol (DTT; 1mM). Saponin was dissolved in the K^+ buffer containing Me[^{203}Hg] (100 μM) before adding mitochondria to initiate uptake. The uptake reaction was quenched with cold K^+ buffer containing the thiol reagents before adding mitochondria (0 sec) or after incubating mitochondria with Me[^{203}Hg] for 10, 20 or 30 sec. Results are expressed as a percentage of Me[^{203}Hg] retained by mitochondria after treatment with the thiol reagents or saponin, relative to Me[^{203}Hg] retained by mitochondria in parallel controls without saponin or thiol reagents. Values are the mean $\pm$ SEM of five different experiments. Values for each experiment are the average of three replicates.

DISCUSSION

Effects of MeHg on neurotransmission have been partly attributed to disruption of Ca^{2+} buffering by mitochondria within the presynaptic nerve terminal (Atchison, 1987; Levesque and Atchison, 1987; 1988). The impetus for this proposal came from the observations a) that MeHg stimulated spontaneous release of ACh, a process that is strongly dependent on increased $[Ca^{2+}]_i$, in the absence of $[Ca^{2+}]_e$ and b) that this effect of MeHg could be blocked by RR and YS035, inhibitors of mitochondrial Ca^{2+} transport. The mechanism proposed for this effect of MeHg implies that it acts intracellularly, however, this has not been demonstrated directly. In the present study, Me[^{203}Hg] was used to examine the characteristics of binding of MeHg to synaptosomes and mitochondria. The results indicate that: 1) MeHg may enter the nerve terminal; 2) entry of MeHg may occur primarily via passive diffusion; 3) much of the MeHg associated with mitochondria and synaptosomes is bound to internal and external sulfhydryl groups and 4) RR and YS035 do not prevent uptake or binding of MeHg to synaptosomes or mitochondria.

The proposal that MeHg enters the synaptosomes via passive diffusion is not consistent with a previous suggestion (Atchison, 1987) that MeHg may utilize voltage-dependent Ca^{2+} channels for entry into nerve terminals. However, it is quite possible that MeHg can utilize both pathways to enter the nerve terminal. In the present study, Me[^{203}Hg] rapidly became bound to synaptosomes even when attempts were made to inhibit uptake by adding a relatively large volume of cold HKR prior to adding Me[^{203}Hg]. In addition to entering the synaptosomes via

passive diffusion, much of the MeHg may have become bound non-specifically to sulfhydryl groups on the external surface of the plasma membrane. Perhaps passive diffusion and non-specific binding accounted for such a large majority of the Me[^{203}Hg] associated with synaptosomes that potential increases in bound Me[^{203}Hg] due to depolarization-induced entry were obscured. This could especially be likely if only a small quantity of MeHg enters subsequent to Ca^{2+} channel activation. Another possibility may be that a fraction of the MeHg entering the nerve terminal via passive diffusion in the absence of depolarization, may utilize Ca^{2+} channels that are activated when the terminal is depolarized. Thus, total uptake would be the same under both conditions. Atchison (1987) observed that a Ca^{2+} channel agonist hastened the onset of stimulation of spontaneous release of neurotransmitter by MeHg and that the effect of the agonist was more pronounced in the absence of external Ca^{2+}. This finding led to the suggestion that MeHg may compete with Ca^{2+} for entry through Ca^{2+} channels. In the present study, depolarization-dependent entry of MeHg was measured in solutions containing 1 mM Ca^{2+} and 100 μM Me[^{203}Hg]. Perhaps Ca^{2+}, which was present at a higher concentration, occluded entry of Me[^{203}Hg] into the synaptosomes. More definitive experiments are necessary to define clearly the role of the putative pathways which MeHg may utilize for gaining entry into nerve terminals.

The notion that MeHg entered synaptosomes rather than binding externally was based on the observations that: 1) saponin, which increases the permeability of the plasma membrane, did not increase the amount of Me[^{203}Hg] retained by synaptosomes; 2) DTT, a membrane-permeable thiol reagent, was more effective in removing Me[^{203}Hg] from synaptosomes than were D-PEN and GSH, which are

less permeable thiol reagents and 3) partially permeabilizing the synaptosomal membrane with saponin increased the effectiveness of D-PEN and GSH but not DTT for decreasing the quantity of Me[^{203}Hg] bound to synaptosomes. If MeHg was unable to pass through the synaptosomal plasma membrane, we presumed that the amount of MeHg bound to synaptosomes could be increased subsequent to permeabilizing the membrane with saponin and exposing sulfhydryl groups on cytosolic proteins to which MeHg may bind. Rather than enhance binding of MeHg, treatment with saponin actually decreased the amount of MeHg bound to synaptosomes. It is possible that permeabilization of the membrane with saponin may have released MeHg bound to sulfhydryl groups on intrasynaptosomal proteins that were small enough to leak out through the permeabilized membrane. The synaptosomes were washed after filtering and this may have enhanced such leakage. Alternatively, saponin may have altered the surface of the synaptosomes to decrease the number of binding sites for MeHg. It is doubtful that saponin interacted directly with the MeHg molecule to decrease it's binding to synaptosomes since saponin had no effect on the binding of MeHg to mitochondria. The mitochondrial membranes are not affected by saponin since they have a low cholesterol content. Thus, the reduction in the amount of MeHg bound to synaptosomes in the presence of saponin is most likely due to the effect of this detergent of the plasma membrane.

The thiol reagents removed MeHg bound to synaptosomes and mitochondria when they were present in quench solutions used to stop the Me[^{203}Hg] uptake/binding reactions. This finding may indicate that much of the MeHg is bound to internal and external sulfhydryl groups. These agents probably

acted by chelating MeHg, since they contain sulfhydryl groups, and perhaps by reducing sulfhydryl groups to which MeHg was bound. The effectiveness of these agents for removing bound MeHg from synaptosomes paralleled the ease with which they cross the plasma membrane. DTT, the most readily permeable thiol reagent used, was most effective for reducing the amount of MeHg retained by synaptosomes. Treatment of synaptosomes with saponin increased the effectiveness of D-PEN and GSH for removing MeHg, presumably by enabling these agents to gain access to the synaptoplasm. This interpretation seems likely since saponin did not alter the effect of DTT on synaptosomes and had no effect on the results obtained with any of the thiol reagents on binding of MeHg to mitochondria.

In conclusion, the results of the present study suggest that MeHg may readily gain access to the nerve terminal cytosol via passive diffusion. Much of the MeHg bound to either synaptosomes or mitochondria is bound to sulfhydryl groups and can be removed by treatment with thiol-containing reagents. Whether passive diffusion is the only pathway by which MeHg may enter nerve terminals cannot be determined from these results and other possible pathways should not be ruled out. The results also show that the previously observed effects of RR and YS035 on MeHg-induced transmitter release were not due to block of binding or uptake of MeHg into synaptosomes or mitochondria.

CHAPTER SEVEN

CONCLUDING DISCUSSION

The primary objective of this work was to investigate the cellular mechanisms underlying the stimulatory effects of methylmercury on spontaneous release of ACh. The hypothesis proposed was that MeHg disrupts the action of intraterminal Ca^{2+} buffers to store Ca^{2+} leading to increased $[Ca^{2+}]$. In turn, this leads to increased spontaneous release of neurotransmitter.

Preliminary intracellular microelectrode recording experiments were designed to delineate potential sources of bound intraterminal Ca^{2+} which could be mobilized by MeHg. Agents which inhibit the Ca^{2+} transport mechanism in mitochondria, but not those that inhibit Ca^{2+} transport by SER, completely prevented MeHg from increasing spontaneous quantal release of ACh. Thus, the results of the electrophysiological studies at the NMJ suggested an interaction between MeHg and mitochondria to induce release of bound Ca^{2+} stores into the nerve terminal cytoplasm, resulting ultimately in stimulated release of ACh.

Ca^{2+} regulation in intact cells is a complex process and it is difficult to obtain a clear picture of potential interactions between MeHg and specific intracellular Ca^{2+} storage sites from observations made on intact tissue. Thus, to follow up on the electrophysiological data implicating mitochondria as a source of Ca^{2+} for the increased spontaneous release of ACh produced by MeHg, subsequent neurochemical experiments were designed to obtain detailed information regarding the direct effects of MeHg on Ca^{2+} transport by mitochondria isolated from rat brain. The results indicated that MeHg impairs the functional integrity of mitochondria and disrupts the ability of mitochondria to take up and retain Ca^{2+}. MeHg-induced release of Ca^{2+} from mitochondria was inhibited by the same agents that blocked the stimulatory effects of MeHg on spontaneous release of ACh in

the electrophysiological studies. Thus, neurochemical studies utilizing isolated mitochondria provided further evidence that the stimulatory effects of MeHg on spontaneous quantal release of ACh at the NMJ are due to inhibition of mitochondrial Ca^{2+} sequestration.

Since MeHg has been shown to affect cholinergic neurotransmission at both central and peripheral synapses, experiments utilizing synaptosomes were used to link neurochemically the effects of MeHg on spontaneous release of ACh from central nerve terminals and effects on Ca^{2+} buffering. The results indicated that extracellular Ca^{2+} contributes only partially contributes to MeHg-induced release of ACh from central nerve terminals. Preincubation of synaptosomes with inhibitors of mitochondrial Ca^{2+} transport reduced the effectiveness of MeHg for increasing spontaneous release of ACh. Thus, the effects of MeHg on ACh release from CNS nerve endings are similar to those observed at the NMJ, with respect to Ca^{2+}-dependence and mitochondrial involvement.

In a final study, the binding characteristics of MeHg to synaptosomes and mitochondria were examined using radiolabeled MeHg. The results indicated that MeHg readily gains access to the nerve terminal cytosol via passive diffusion. Also, much of the MeHg bound to either synaptosomes or mitochondria is bound to sulfhydryl groups.

Taken together, the results provided evidence that MeHg enters the nerve terminal and induces release of bound Ca^{2+} from mitochondria and that release of this pool of Ca^{2+} by MeHg contributes to the increased spontaneous release of ACh induced by MeHg at both peripheral and central synapses. Thus, the preliminary studies which utilized the NMJ as a model synapse in conjunction with

the neurochemical studies of effects of MeHg on nerve terminal Ca^{2+} regulation and spontaneous release of neurotransmitter have provided useful information regarding the mechanisms underlying effects of MeHg on synaptic transmission at both the physiological and biochemical levels.

Disrupted intracellular Ca^{2+} homeostasis and elevated $[Ca^{2+}]$ could explain at least in part explain the two well recognized effects of MeHg on neurotransmitter release: inhibition of synchronous evoked release and increased spontaneous quantal release. Precise regulation of Ca^{2+} is critical for both forms of release to occur normally (Katz and Miledi, 1967; Llinas and Nicholson, 1975; Silinsky, 1985). Synchronous evoked release of neurotransmitter can be inhibited by elevated basal levels of ionized free Ca^{2+} in the presynaptic nerve terminal (Adams et al., 1985; Bernath and Vizi, 1987). Spontaneous quantal release is directly dependent on $[Ca^{2+}]$ and increases when Ca^{2+} is released from internal stores or when active extrusion of Ca^{2+} across the plasma membrane is blocked (Alnaes and Rahamimoff, 1975; Blaustein et al., 1978; Adams et al., 1985). MeHg induces release of Ca^{2+} from mitochondria and disrupts mitochondrial respiration. The latter effect of MeHg could lead to depletion of ATP which would ultimately impair extrusion of Ca^{2+} from the nerve terminal cytosol by ATP-dependent plasma membrane Ca^{2+}. MeHg and other mercurials not only affect synaptic function at cholinergic synapses, as observed in the present study, but cause analogous changes in release of non-cholinergic neurotransmitters from both peripheral and central synapses (Borowitz, 1974; Bondy et al., 1979; Nakazato et al., 1979; Tuomisto and Komulainen, 1981; Minnema et al., 1989). Since effects of MeHg on synaptic transmission are not unique to a particular transmitter type, perhaps MeHg

acts via a general mechanism common to all transmitter systems. One such mechanism which could affect release of different transmitters would be perturbation of $[Ca^{2+}]_i$. Thus, the mechanisms responsible for the effects of MeHg on ACh release, as described in this thesis, may be similar to the mechanisms underlying the effects of MeHg at other chemical synapses in the peripheral and central nervous systems.

An unexpected benefit resulted from the above mentioned studies, which with further examination, could shed light on the final known characteristic of MeHg on ACh release at the NMJ. This is the secondary block of spontaneous release of ACh. Results of choline uptake experiments indicate a reduction by MeHg in this rate-limiting step in ACh synthesis. Normally, quantal release of ACh occurs from the so-called "immediately available store" of ACh, that pool which is newly-synthesized. If MeHg blocks de novo synthesis of ACh by inhibiting substrate availability, this could ultimately be reflected in a reduction and/or frank block of quantal exocytosis, particularly in light of the marked increases in MEPP frequency normally induced by MeHg prior to block of MEPP frequency. This series of events, in turn, might explain the dramatically lower rate of MEPP frequency induced by La^{3+} in MeHg-poisoned NMJs.

The histopathological findings in MeHg intoxication are quite variable but neuronal cells of the peripheral and central nervous systems appear to be primary targets. Neuronal degeneration has been observed in the brain and periphery (Chang, 1977). The molecular and cellular mechanisms underlying pathological lesions that occur with MeHg intoxication undoubtedly occur in response to more subtle biochemical or physiological effects on nerve cell bodies or processes. In

addition to affecting transmitter release, MeHg-induced disturbances in Ca^{2+} buffering by mitochondria could affect other Ca^{2+}-dependent cellular functions in neuronal cells. Perhaps other pathophysiological consequences of MeHg intoxication may be due to perturbations of cellular Ca^{2+} homeostasis. The pathogenetic effects of MeHg have been attributed in part to disruption of cellular metabolism leading eventually to cell death (Chang, 1977). It is commonly assumed that calcium, which normally serves important functions as a membrane stabilizer, metabolic regulator, second messenger and promotor of cell development and repair, also can mediate toxic cell death (Trump et al., 1981; Pounds and Rosen, 1988). Disturbances in cellular Ca^{2+} homeostasis with cell Ca^{2+} overload have been associated with cell injury and death (Trump and Berezesky, 1985). Perturbations in $[Ca^{2+}]$ and the Ca^{2+} messenger system may place the regulation of cellular processes out of the normal range of physiological control. Prolonged elevation of $[Ca^{2+}]$ may cause cell death due to exhaustion in handling free intracellular Ca^{2+}. Also, increased $[Ca^{2+}]$ may accelerate reactions that are deleterious to the survival of the cell. In particular, Ca^{2+} may activate enzymes degrading cell structure, including lipases, proteases and endonucleases (Orrenius et al., 1988). Thus, an immediate effect of MeHg-induced increases in $[Ca^{2+}]$ would be increased spontaneous release of neurotransmitters, as observed in the present studies, and perhaps a more long term consequence of elevated Ca^{2+} would be cell death and tissue degeneration, as observed in histopathological studies.

The mechanisms proposed above for the neurotoxic effects of MeHg are applicable to many different cells, and still not all cells are affected by MeHg, even

in the brain. Different characteristics of nervous tissue from other tissues such as strict dependence on glucose, high oxygen utilization and excitability, render it especially susceptible to toxic insult. There are several explanations which may account for the variable sensitivity of certain types of nervous tissue within the brain and periphery to MeHg. Due to high affinity of mercuric ions towards sulfhydryl and disulfide groups, the biochemical basis of toxicological effects of mercury is generally sought through mercury-sulfur interactions (Clarkson, 1972). It is possible that large amounts of sulfhydryl groups on some cells may act as inert sites and offer a quenching effect on the action of MeHg inside the cell, rendering a higher mercury tolerance. Alternatively, perhaps some cells are more vulnerable than others because they contain higher concentrations of Ca^{2+}-sensitive enzymes degrading cell structure, such as proteases and lipases (Seisjo and Bengtsson, 1989). This may be likely with certain neuronal cells since these enzymes are normally involved in cell plasticity. Finally, perhaps some cells may be better able than others to buffer or extrude the $[Ca^{2+}]$ presumably elevated by MeHg. Also, certain cells may be able to adapt to or compensate for MeHg-induced changes in $[Ca^{2+}]$. Until more is known about the specific subcellular effects of MeHg, it will be difficult to explain beyond speculation, the differential sensitivity of nervous tissue to the toxic effects of MeHg.

In conclusion, the work presented in this thesis provides evidence in support of the hypothesis that MeHg stimulates spontaneous release of transmitter subsequent to disrupting nerve terminal $[Ca^{2+}]$ homeostasis. Intraneuronal mitochondria are a primary target for this effect of MeHg. In addition to affecting transmitter release, disturbances in intracellular Ca^{2+} homeostasis could affect other

Ca^{2+}-dependent cellular functions in neuronal and non-neuronal cells. Because of the high affinity of MeHg for sulfhydryl groups and the lipophylicity of the molecule, one could expect that MeHg has other subcellular actions in addition to those that affect cellular Ca^{2+} regulation, which may produce neurotoxic effects. Thus, although perturbation by MeHg of subcellular processes regulating $[Ca^{2+}]_i$ may underlie some of the neurotoxic effects of MeHg, other biochemical and physiological processes may also be affected by MeHg.

BIBLIOGRAPHY

BIBLIOGRAPHY

ABRAMSON, J. J., TRIMM, J. L., WEDEN, L., and SALAMA, G. (1983). Heavy metals induce rapid Ca^{2+} release from sarcoplasmic reticulum vesicles isolated from skeletal muscle. Pros.Natl. Acad. Sci. **80,** 1526-1530.

ADAMS, D. J., TAKEDA, K., and UMBACH, J. A. (1985). Inhibitors of calcium buffering depress evoked transmitter release at the squid giant synapse. J. Physiol. (Lond.) **369,** 45-159.

ADAM-VIZI, V. and LIGETI, E. (1984). Release of acetylcholine from rat brain synaptosomes by various agents in the absence of external calcium ions. J. Physiol. (Lond.) **353,** 505-521.

AKERMAN, K. E. (1978). Changes in membrane potential during Ca^{2+} ion influx and efflux across the mitochondrial membrane. Biochim. Biophys. Acta **502,** 359-366.

AKERMAN, K. E. O., and NICHOLLS, D. G. (1981). Calcium transport by intact synaptosomes. Influence of ionophore A23187 on plasma membrane potential, plasma membrane calcium transport, mitochondrial membrane potential, respiration, cytosolic free calcium concentration and noradrenaline release. Eur. J. Biochem. **115,** 67-73.

AKERMAN, K. E., and NICHOLLS, D. G. (1983). Physiological and bioenergetic aspects of mitochondrial Ca^{2+} transport. Rev. Physiol., Biochem., Pharmacol. **95,** 152-201.

ALLY, A., PHIPPS, J., and MILLER, D. R. (1984). Interactions of methylmercury chloride with cellular energetics and related processes. Toxicol. Appl. Pharmacol. **76,** 207-218.

ALNAES, E., and RAHAMIMOFF, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. J. Physiol. (Lond.) **248,** 285-306.

ARAKI, K., WAKABAYASHI, M., SAKIMURA, K., KUSHIYA, E., OZAWA, H., KUNAMOTO, T., and TAKAHASHI, Y. (1981). Decreased uptake of GABA by dorsal ganglia in methylmercury-treated rat. Neurotoxicology **2,** 557-561.

ASHLEY, R. H., BRAMMER, M. J. and MARCHBANKS, R. (1982). Measurement of intrasynaptosomal free calcium by using the fluorescent indicator quin-2. Biochem. J. **219,** 149-158.

ATCHISON, W. D. (1986). Extracellular calcium-dependent and -independent effects of methylmercury on spontaneous and potassium-evoked release of acetylcholine at the neuromuscular junction. J. Pharmacol. Exp. Ther. **237,** 672-680.

ATCHISON, W. D. (1987). Effects of activation of sodium and calcium entry on spontaneous release of acetylcholine induced by methylmercury. J. Pharmacol. Exp. Ther. **241,** 131-139.

ATCHISON, W. D. (1988). Effects of neurotoxicants on synaptic transmission: Lessons learned from electrophysiological studies. Neurotox. and Teratol. **10,** 393-416.

ATCHISON, W. D., CLARK, A. W., and NARAHASHI, T. (1984). Presynaptic effects of methylmercury at the mammalian neuromuscular junction. In: Cellular and Molecular Neurotoxicology (Narahashi, T., ed.). Raven Press, New York, pp. 23-43.

ATCHISON, W. D., JOSHI, U., and THORNBURG, J. E. (1986). Irreversible suppression of calcium entry into nerve terminals by methylmercury. J. Pharmacol. Exp. Ther. **238,** 618-624.

ATCHISON, W. D., and NARAHASHI, T. (1982). Methylmercury-induced depression of neuromuscular transmission in the rat. Neurotoxicology **3,** 37-50.

BAKER, P. F., and CRAWFORD, A. C. (1975). A note on the mechanism by which inhibitors of the sodium pump accelerate spontaneous release of transmitter from motor nerve terminals. J. Physiol. (Lond.) **247,** 209-226.

BAKER, P. F., HODGKIN, A. L., and RIDGWAY, E. B. (1971). Depolarization and Ca^{2+} entry in squid giant axons. J. Physiol.(Lond.) **218,** 709-755.

BAKIR, J., DAMLUJI, S., AMIN-ZAKI, L., MURTAKHA, M., KHALIDI, A., AL-RAWI, N. Y., TIKRITI, S., DHAHIR, H. I., CLARKSON, T. W., SMITH, J. C., and DOHERTY, R. A. (1973). Methylmercury poisoning in Iraq. Science **181,** 230-240.

BARKER, L. A., DOWNDALL, M.J., and MITTAG, T. W. (1975). Comparative studies on synaptosomes: high-affinity uptake and acetylation of N-[Me-3H]choline and N-[Me-3H]N-hydroxyethyl-pyrrolidinium. Brain Res. **86,** 343-348.

BARKER, L. A., and MITTAG, T. W. (1975). Comparative studies of substrates and inhibitors of choline transport and choline acetyltransferase. J. Pharmacol. Exp. Ther. **192,** 86-94.

BARRETT, J., BOTZ, D., and CHANG, D. B. (1974). Block of neuromuscular transmission by methylmercury. In: Behavioral Toxicology, Early Detection of Occupational Hazards (Xintaras, C., Johnson, B. L. and de Groot, I., eds.). U.S. Dept. HEW Document, Vol. 5, pp. 277-287, Washington, D.C.

BARSTAD, J. A. B. and LILLEHEIL, G. (1968). Transversely cut diaphragm preparation from rat. Arch. Int. Pharmacodyn. Ther. **175,** 373-390.

BARTOLOME, J., TREPANIER, P., CHAIT, E. A., SEIDLER, F. J., DESKIN, R., and SLOTKIN, T. A. (1982). Neonatal methylmercury poisoning in the rat: Effects on development of central catecholamine neurotransmitter systems. Toxicol. Appl. Pharmacol. **65,** 92-99.

BEATRICE, M. C., PALMER, J. W., and PFEIFFER, D. R. (1980). The relationship between mitochondrial membrane permeability, membrane potential, and retention of Ca^{2+} by mitochondria. J. Biol. Chem. **255,** 8663- 8671.

BEATRICE, M. C., STEERS, D. L., and PFEIFFER, D. R. (1984). The role of glutathione in the retention of Ca^{2+} by liver mitochondria. J. Biol. Chem. **259,** 1279-1287.

BERNATH, S., and VIZI, E. S. (1987). Inhibitory effect of ionized free intracellular calcium enhanced by ruthenium red and m-chloro-carbonylcyanide phenyl hydrazone on the evoked release of acetylcholine. Biochem. Pharmacol. **36,** 3683-3687.

BIESOLD, D. (1974). Isolation of brain mitochondria. In Research Methods in Neurochemistry (N. Marks and R. Rodnight, Eds.), Vol. 2, pp. 39-52. Plenum, New York/London.

BINAH, O., MEIRI, U., and RAHAMIMOFF, H. (1978). The effects of $HgCl_2$ and mersalyl on mechanisms regulating intracellular calcium and transmitter release. Europ. J. Pharmacol. **51,** 453-457.

BINDOLI, A., and FLEISCHER, S. (1983). Induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum by sulfhydryl reagents and chlorpromazine. Arch. Biochem. Biophys. **221,** 458-466.

BIRK, Y., and PERI, I. (1980). Saponin. In Toxic. Const. Plant (I. E. Liener, ed.), pp. 161-182. Academic Press, New York, 2nd ed.

BIRKS, R. I., and COHEN, M. W. (1968). The action of sodium pump inhibitors on neuromuscular transmission. Proc. R. Soc. Lond. (Biol.) **170,** 381-399.

BLAUSTEIN, M. P. (1975). Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals **in vitro.** J. Phys. **247**, 617-655.

BLAUSTEIN, M. P., and ECTOR, A. L. (1976). Carrier-mediated sodium-dependent and calcium-dependent calcium efflux from pinched-off presynaptic nerve terminals (synaptosomes) in vitro. Biochim. Biophys. Acta **419**, 295-308.

BLAUSTEIN, M. P., KENDRICK, N. C., FRIED, R. C., and RATZLAFF, R. W. (1977). Calcium metabolism at the mammalian presynaptic nerve terminal: Lessons from the synaptosome. In society for Neuroscience Symposia, Vol. II (Cowan, M. and Ferrendelli, J. A., eds.). Bethesda, MD: Society for Neurosciences, pp. 172-194.

BLAUSTEIN, M. P., MCGRAW, C. F., SOMLYO, A. V., and SCHWEITZER, E. S. (1980). How is the cytoplasmic calcium concentration controlled in nerve terminals? J. Physiol. (Paris). **76**, 459-470.

BLAUSTEIN, M. P., RATZLAFF, R. W., and KENDRICK, N. K. (1978a). The regulation of intracellular calcium in presynaptic nerve terminals. Ann. N.Y. Acad. Sci., **307**, 195-211.

BLAUSTEIN, M. P., RATZLAFF, R. W., and SCHWEITZER, E. S. (1978b). Calcium buffering in presynaptic nerve terminals. II. Kinetic properties of the nonmitochondrial Ca^{2+} sequestration mechanism. J. Gen. Physiol. **72**, 43-67.

BLIOCH, Z. L., GLAGOLEVA, I. M., LIBERMAN, E. A., and NENASHEN, V. A. (1968). A study of the mechanism of quantal transmitter release at a chemical synapse. J. Physiol. (Lond.) **V199**, 11-35.

BOGUCKA, K., and WOJTCZAK, L. (1979). On the mechanism of mercurial-induced permeability of the mitochondrial membrane to K^+. FEBS Lett. **100**, 301-304.

BONDY, S. C., ANDERSON, C. L., HARRINGTON, M. E., and PRASAD, K. N. (1979). The effects of organic and inorganic lead and mercury on neurotransmitter high-affinity transport and release mechanisms. Environ. Res. **19**, 102-111.

BOOTH, R. F., and CLARK, J. B. (1978). A rapid method for the preparation of relatively pure, metabolically competent synaptosomes from rat brain. Biochem. J. **176**, 365-370.

BOROWITZ, J. L. (1974). Mechanism of adrenal catecholamine release by divalent mercury. Toxicol. Appl. Pharmacol. **28**, 82-87.

BRAND, M. D., REYNAFARJE, B., and LEHNINGER, A. L. (1976). Re-evaluation of the H^+/site ratio of mitochondrial electron transport with the oxygen pulse technique. J. Biol. Chem. **251,** 5670-5679.

BRANISTEANU, D. D., PROCA, B. and HAULICA, I. D. (1979). Dual action of ouabain on transmitter release at neuromuscular junctions of the frog. J. Pharmacol. Exp. Ther. **209,** 31-36.

BRASELTON, W. E., MEERDINK, G. L., STOWE, H. D., and TONSAGER, S. R. (1981). Experience with multielement analysis in diagnostic clinical toxicology and nutrition. In: Proceedings of the 24th Annual Meeting, American Association of Veterinary Laboratory Diagnosticians, pp. 111-126.

BRIERLEY, G. P., KNIGHT, V. A., and SETTLEMIRE, C. T. (1978). Ion transport by heart mitochondria. XII. Activation of monovalent cation uptake by sulfhydryl group reagents. J. Biol. Chem. **243,** 5035-5043.

BULBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. Br. J. Pharmacol. **1,** 38-61.

CARAFOLI, E. (1967). In vivo effect of uncoupling agents on the incorporation of Ca^{2+} and Sr^{2+} into mitochondria and other subcellular fractions of rat liver. J. Gen. Physiol. **50,** 1849-1864.

CARAFOLI, E. (1979). The calcium cycle of mitochondria. FEBS Lett. **104,** 1-5.

CARAFOLI, E. (1982a). The transport of calcium across the inner membrane of mitochondria. In: Membrane Transport of Calcium (Carafoli, E., ed.), pp. 109-140. Academic Press, London.

CARAFOLI, E. (1982b). The regulation of intracellular calcium. Adv. Exp. Med. Biol. **151,** 461-472.

CARAFOLI, E. (1988). Intracellular calcium regulation, with special attention to the role of the plasma membrane calcium pump. J. Cardio. Pharmacol. **12,** S77-S84.

CARAFOLI, E., and CROMPTON, M. (1976). In: Calcium in Biological Systems (Duncan, C. J., ed.), Symp. No. 30 Soc. Exptl. Biol., pp. 89-115. Cambridge University Press, England.

CARAFOLI, E., and CROMPTON, M. (1978). The regulation of intracellular calcium by mitochondria. Ann. N.Y. Acad. Sci. **307,** 269-284.

CARAFOLI, E., PRESTIPINO, G. F., CECCARELLI, D., and CONTI, F. (1974a). In: Membrane Proteins in Transport and Phosphorylation (Azzone, G. F., Helingenberg, M., Quagliariello, E. and Siliprandi, N., eds.), pp. 85-90. North-Holland, Amsterdam.

CARAFOLI, E., and SCOTTOCASA, G. L. (1974). In: Dynamics of Energy-Transducing Membranes (Ernster, L., Eastabrook, R. W. and Slater, E. C., eds.), pp. 455-469. Elsevier, Amsterdam.

CARAFOLI, E., TIOZZO, R., LUGLI, G., CROVETTI, F., and KRATZING, C. (1974b). The release of Ca^{2+} from heart mitochondria by sodium. J. Mol. Cell. Cardiol. **6,** 361-371.

CHANCE, B., and WILLIAMS, G. R. (1955). Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. 383-393.

CHANG, L. W. (1977). Neurotoxic effects of mercury - a review. Environ. Res. **14,** 329-373.

CHANG, L. W., and HARTMANN, H. A. (1972a). Ultrastructural studies of the nervous system after mercury intoxication. I. Pathological changes in the nerve cell bodies. Acta Neuropathol. of (Berlin) **20,** 122-138.

CHANG, L. W., and HARTMANN, H. A. (1972b). Ultrastructural studies of the nervous system after mercury intoxication. II. Pathological changes in the nerve fibers. Acta Neuropathol. of (Berlin) **20,** 316-334.

CHAPPELL, J. B., and CROFTS, A. R. (1965). Calcium ion accumulation and volume changes of isolated liver mitochondria. Calcium ion-induced swelling. Biochem. J. **95,** 378-386.

CHAVEZ E., and HOLGUIN, J. A. (1988). Mitochondrial calcium release as induced by Hg^{2+}. J. Biol. Chem. **263,** 3582-3587.

CHEUNG, M., and VERITY, M. A. (1981). Methylmercury inhibition of synaptosome protein synthesis: The role of mitochondrial dysfunction. Environ. Res. **24,** 286-298.

CHIESI, M., and INESI, G. (1979). The use of quench reagents for resolution of single transport cycles in sarcoplasmic reticulum. J. Biol. Chem. **254,** 10370-10377.

CHIOU, C. Y., and MALAGODI, M. H. (1975). Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscles. Brit. J. Pharmacol. **53,** 279-285.

CHIU, C. C., and GRADY, L. T. (1981). in Analytical Profiles of Drug Substances (ed., K. Florey), pp. 601-637. Academic Press, New York.

CLARKSON, T. W. (1972). The pharmacology of mercury compounds. Annu. Rev. Pharmacol. **12,** 75-106.

CLELAND, W. W. (1964). Dithiothreitol. A new protective reagent for sulfhydryl groups. Biochemistry **3,** 480-482.

CROMPTON, M., CAPANO, M., and CARAFOLI, E. (1976). The sodium-induced efflux of Ca^{2+} from heart mitochondria. A possible mechanism for the regulation of mitochondrial calcium. Eur. J. Biochem. **69,** 453-462.

DAGANI, F., MARZATICO, F., and CURTI, D. (1988). Oxidative metabolism of nonsynaptic mitochondria isolated from rat brain hippocampus: A comparative regional study. J. Neurochem. 50, **1233-1236.**

DAGANI, F., ZANADA, F., MARZATICO, F., and BENZI, G. (1985). Free mitochondria and synaptosomes from single rat forebrain. A comparison between two known subfractionation techniques. J. Neurochem. **45,** 653-656.

DANKO, S., KIM, D. H., SRETER, F. A., and IKEMOTO, N. (1985). Inhibitors of Ca^{2+} release from the isolated sarcoplasmic reticulum. II. The effects of dantrolene on Ca^{2+} release induced by caffeine, Ca^{2+} and depolarization. Biochim. Biophys. Acta **816,** 18-24.

DEANA, R., PANATO, L., CANCELLOTTI, F. M., QUADRO, G., and CALZIGNA, L. (1984). Properties of a new calcium ion antagonist on cellular uptake and mitochondrial efflux of calcium ions. Biochem. J. **218,** 899-905.

DEBISE, R., GACHON, P., and DURAND, R. (1978). Correlation between glutamate and Ca^{2+} uptake in rat liver mitochondria. FEBS Lett. **85,** 25-29.

DEL CASTILLO, J., and KATZ, B. (1954). Quantal components of the end plate potential. J. Physiol. (Lond.) **124,** 560-573.

DE PIERRE, J. W., and ERNSTER, L. (1977). Enzyme topology of intracellular membranes. Annu. Rev. Biochem. **46,** 201-262.

DESNOYERS, P. A., and CHANG, L. W. (1975). Ultrastructural changes in rat hepatocytes following acute methylmercury intoxication. Environ. Res. **9,** 224-239.

DRAHOTA, Z., CARAFOLI, E., ROSSI, C. S., GAMBLE, R. L., and LEHNINGER, A. C. (1965). The steady state maintenance of accumulated Ca^{2+} in rat liver mitochondria. J. Biol. Chem. **240,** 2712-2720.

DURANT, N. N., LEE, C. and KATZ, R. L. (1980). The action of dantrolene on transmitter mobilization at the rat neuromuscular junction. Eur. J. Pharmacol. **68,** 403-408.

DUSZYNSKI, J., and WOJTCZAK, L. (1977). Effect of Mg^{2+} depletion of mitochondria on their permeability to K^{+}: The mechanism by which ionophore A23187 increases K+ permeability. Biochem. Biophys. Res. Commun. **74,** 417-424.

ELDER, J. A., and LEHNINGER, A. L. (1973). Respiration-dependent transport of carbon dioxide into rat liver mitochondria. Biochemistry **12,** 976-982.

ELMQVIST, D., and FELDMAN, D. S. (1965a). Effects of sodium pump inhibitors on spontaneous acetylcholine release at the neuromuscular junction. J. Physiol. (Lond.) **181,** 498-505.

ELMQVIST, D., and FELDMAN, D. S. (1965b). Calcium dependence of spontaneous acetylcholine release at mammalian motor nerve terminals. J. Physiol. (Lond.) **181,** 487-497.

EROGLU, L., and KEEN, P. (1977). Active uptake of $^{45}Ca^{2+}$ by a microsomal fraction prepared from rat dorsal roots. J. Neurochem. **29,** 905-909.

FATT, P., and KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. J. Physiol. (Lond.) **115,** 320-370.

FATT, P., and KATZ, B. (1952). Spontaneous subthreshold activity of motor nerve endings. J. Physiol. (Lond.) **117,** 109-128.

FISKUM, G., and COCKRELL, R. S. (1978). Ruthenium red sensitive and insensitive Ca^{2+} transport in rat liver and Ehrlich Ascities tumor cell mitochondria. FEBS Lets **92,** 125-128.

FLETCHER, P., and FORRESTER, T. (1975). The effect of curare on acetylcholine from mammalian motor nerve terminals and an estimate of quantum content. J. Physiol. (Lond). **251,** 131-144.

FOWLER, B. A., and WOODS, J. S. (1977). The transplacental toxicity of methylmercury to fetal rat liver mitochondria. Morphometric and biochemical studies. Lab. Invest. **36,** 122-130.

FOX, J. H., PATEL-MANDLIK ,. and COHEN, M. M. (1975). Comparative effects of organic and inorganic mercury on brain slice respiration and metabolism. J. Neurochem. **24,** 757-762.

FRANCIS, K. T. (1978). The effect of dantrolene sodium on the efflux of Ca^{45} from rat heavy sarcoplasmic reticulum. Res. Commun. Chem. Pathol. Pharmacol. **21,** 573-576.

GLAGOLEVA, I. M., LIBERMAN, Y. A., and KHASHAYEV, Z. Kh. M. (1970). Effect of uncoupling agents of oxidative phosphorylation on the release of acetylcholine from nerve endings. Biofizika **15,** 76-83.

GOLDBERG, A. M., and McCAMAN R. E. (1973). The determination of picomole amounts of acetylcholine in mammalian brain. J. Neurochem. **20,** 1-8.

GOMEZ, M. V., and FARRELL, N. (1985). The effect of tityustoxin and ruthenium red on the release of acetylcholine from slices of cortex of rat brain. Neuropharmacology **24,** 1103-1107.

GOVIER, W. C., and HOLLAND, W. (1964). Effects of ouabain on tissue calcium and calcium exchange in pacemaker in turtle heart. Amer. J. Physiol. **207,** 195-198.

GREENAWALT, J. W., ROSSI, C. S., and LEHNINGER, A. L. (1964). Effect of active accumulation of calcium and phosphate ions on the structure of rat liver mitochondria. J. Cell. Biol.**23,** 21-38.

GRYNKIEWICZ, G., POENIE, M., and TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260,** 3440-3450.

GUNTER, T. E., GUNTER, K. K., PUSKIN, J. S., and RUSSELL, P. R. (1978). Efflux of Ca^{2+} and Mn^{2+} from rat liver mitochondria. Biochemistry **17,** 339-345.

GUYENET, P., LEFRESNE, P., ROSSIER, J., BEAUJOUAN, J. C., and GLOWINSKI, J. (1973). Inhibition by hemicholinium-3 of [^{14}C]acetylcholine synthesis and [^{3}H]choline high-affinity uptake in rat striatal synaptosomes. Mol. Pharmacol. **9,** 630-639.

HAGA, T., and NODA, H. (1973). Choline uptake systems of rat brain synaptosomes. Biochim. Biophys. Acta **291,** 564-575.

HARRIS, E. J. (1978). The importance of CO_2 for Ca^{2+} uptake by some mitochondria. Nature (Lond.) **274,** 820-821.

HARRIS, E. J., AL-SHAIKHALY, M., and BAUM, H. (1979). Stimulation of mitochondrial calcium ion efflux by thiol-specific reagents and by thyroxine. Biochem. J. **182,** 455-464.

HARRIS, E. J., and BAUM, H. (1980). Production of thiol groups and retention of calcium ions by cardiac mitochondria. Biochem. J. **186,** 725-732.

HEATON, G. M., and NICHOLLS, D. G. (1976). The Ca^{2+} conductance of the inner membrane of rat over mitochondria and the determination of the Ca^{2+} electrochemical gradient. Biochem. J. **156(3)**,635-646.

HEINONEN, E., AKERMAN, K. E. O., and KAILA, K. (1984). Depolarization of the mitochondrial membrane potential increases free cytosolic calcium in synaptosomes. Neurosci. Lett. **49**, 33-37.

HENKART, M., LANDIS, D. M., and REESE, T. S. (1976). Similarity of junctions between plasma membranes and endoplasmic reticulum in muscle and neurons. J. Cell Biol. **70**, 338-347.

HEUSER, J., and MILEDI, R. (1971). Effects of lanthanum ions on function and structure of frog neuromuscular junctions. Proc. Roy. Soc. London Ser. B. **179**, 247-260.

HEUSER, J., AND LENNON, A. M. (1973). Morphological evidence for exocytosis of acetylcholine during formation of synaptosomes from torpedo electric organ. J. Physiol. **223**,39-41P.

HOFMANN, W. W. (1969). Caffeine effects on transmitter depletion and mobilization at motor nerve terminals. Am. J. Physiol. **216**, 621-629.

HOFSTETTER, W., MUHLEBACH, T., LOTSCHER, H., WINTERHALTER, K. H., and RICHTER, C. (1981). ATP prevents both hydroperoxide-induced hydrolysis of pyridine nucleotides and release of calcium in rat liver mitochondria. Eur. J. Biochem. **117**, 361-367.

HUBBARD, J. I. and WILSON, D. F. (1972). Neuromuscular transmission in a mammalian preparation in the absence of blocking drugs and the effect of D-tubocurarine. J. Physiol. (Lond.) **228**, 307-325.

HUNTER, D., BOMFORD, R., and RUSSELL, D. S. (1940). Poisoning by methylmercury compounds. Quart. J. Med. **9**, 193-241.

JENG, A. Y., and SHAMMOO, A. E. (1980). Isolation of a Ca^{2+} carrier from calf heart inner mitochondrial membrane. J. Biol. Chem. **14**, 6897-6903.

JOHNSON, M. K., and WHITTAKER, V. P. (1963). Lactate dehydrogenase as a cytoplasmic marker in brain. Biochem. J. **88**, 404-409.

JUANG, M. S. (1976). An electrophysiological study of the action of methylmercuric chloride and mercuric chloride on the sciatic nerve-sartorius muscle preparation of the frog. Toxicol. Appl. Pharmacol. **37**, 339-348.

JURKOWITZ, M. S., GEISBUHLER, T., JUNG, D. W., and BRIERLEY G. P. (1983). Ruthenium red-sensitive and -insensitive release of Ca^{2+} from uncoupled heart mitochondria. Arch. Biochem. Biophys. **223,** 120-128.

KATZ, B., and MILEDI, R. (1967). The timing of calcium action during neuromuscular transmission. J. Physiol. (Lond.) **189,** 535-544.

KATZ, B., and MILEDI, R. (1968). The role of calcium in neurotransmitter facilitation. J. Physiol. (Lond.) **195,** 481-492.

KAUPPINEN, R. A., KOMULAINEN, H., AND TAIPALE, H. (1989). Cellular mechanisms underlying the increase in cytosolic free calcium concentration induced by methylmercury in cerebrocortical synaptosomes from guinea pig. J. Pharmacol. Exper. Ther. **248,** 1248-1254.

KAUPPINEN, R. A., McMAHON, H., AND NICHOLLS, D. G. (1988). Ca^{2+}-dependent and Ca^{2+}-independent glutamate release, energy status and cytosolic free Ca^{2+} concentration in isolated nerve terminals following metabolic inhibition: Possible relevance to hypoglycemia and anoxia. Neuroscience **27,** 175-182.

KENDRICK, N. C., BLAUSTEIN, M. P., FRIED, R. C., and RATZLAFF, R. W. (1977). ATP-dependent calcium storage in presynaptic nerve terminals. Nature **265,** 246-248.

KITA, H., and VAN DER KLOOT, W. (1976). Effects of the ionophore X-537A on acetylcholine release at the frog neuromuscular junction. J. Physiol. (Lond.) **259,** 177-198.

KOBAYASHI, H., YUYAMA, A., MATSUSAKA, N., TAKENO, K., and YANAGIYA, I. (1979). Effect of methylmercury chloride on various cholinergic parameters **in vitro**. J. Toxicol. Sci. **4,** 351-362.

KOBAYASHI, H., YUYAMA, A., MATSUSAKA, N., TAKENO, K., and YANAGIYA, I. (1980). Effect of methylmercury on brain acetylcholine concentration and turnover in mice. Toxicol. Appl. Pharmacol. **54,** 1-8.

KOJIMA, I., SHIBATA, H., and OGATA, E. (1986). Action of TMB-8(8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate) on cytoplasmic free calcium in adrenal glomerulosa cell. Biochim.Biophys. Acta **888,** 25-29.

KOMULAINEN, H., and BONDY, C. (1987a). Increased free intrasynaptosomal Ca^{2+} by neurotoxic organometals: Distinctive mechanisms. Toxicol. Appl. Pharmacol. **88,** 77-86.

KOMULAINEN, H., and BONDY, C. (1987b). The estimation of free Ca^{2+} within synaptosomes and mitochondria with Fura-2; comparison to quin-2. Neurochem.Int. **10**, 55-64.

KOMULAINEN, H., and TUOMISTO, J. (1981). Interference of methyl mercury with monoamine uptake and release in rat brain synaptosomes. Acta Pharmacol. Toxicol. **48**, 214-222.

KOMULAINEN, H., and TUOMISTO, J. (1982). Effects of heavy metals on monoamine uptake and release in brain synaptosomes and blood platelets. Neurobehav. Toxicol. Teratol. **4**, 647-649.

KRAATZ, H. G., and TRAUTWEIN, W. (1957). Die Wirkung von 2,4-Dinitrophenol auf die neuromuskulare Erregungsubertragung. Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmak. **231**, 419-439.

KUHAR, M. J., SETHY, V. H., ROTH, R. V., and AGHAJANIAN, G. C. (1973). Choline: selective accumulation by central cholinergic neurons. J. Neurochem. **20**, 581-593.

LAKOWICZ, J. R., and ANDERSON, C. J. (1980). Permeability of lipid bilayers to methylmercuric chloride: Quantification by fluorescence quenching of a carbazole-labeled phospholipid. Chem. Biol. Interact. **30**, 309-323.

LEBLANC, R. M., JOLIY, L. P., and PAIEMENT, J. (1984). pH-dependent interaction between methylmercury chloride and some membrane phospholipids. Chem. Biol. Interact. **48**, 237-241.

LEHNINGER, A. L. (1970). Mitochondria and calcium ion transport. Biochem. J. **119**, 129-138.

LEHNINGER, A. L. and CARAFOLI, E. (1969). In: Biochemistry of the Phagocytic Process (Schultz, J., ed.), pp. 922-931. North-Holland, Amsterdam.

LEHNINGER, A. L., CARAFOLI, E., and ROSSI, C. S. (1967). Energy-linked ion movements in mitochondrial systems. Adv. Enzymol. **29**, 259-320.

LEHNINGER, A. L., ROSSI, C. S., GREENAWALT, J. W. (1963). Respiration-dependent accumulation of inorganic phosphate and calcium ions by rat liver mitochondria. Biochem. Biophys. Res. Commun. **10**, 444-448.

LEVESQUE, P. C., and ATCHISON, W. D. (1987). Interactions of mitochondrial inhibitors with methylmercury on spontaneous quantal release of acetylcholine. Toxicol. Appl. Pharmacol. **87**, 315-324.

LEVESQUE, P. C., and ATCHISON, W. D. (1988). Effect of alteration of nerve terminal Ca^{2+} regulation on increased spontaneous quantal release of acetylcholine by methylmercury. Toxicol. Appl. Pharmacol. **94**, 55-65.

LILEY, A. W. (1956a). An investigation of spontaneous activity at the neuromuscular junction of the rat. J. Physiol. (Lond.) **132**, 650-666.

LILEY, A. W. (1956b). The effect of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. J. Physiol. (Lond.) **134**, 427-443.

LLINAS, R., and NICHOLSON, C. (1975). Calcium role in depolarization-secretion coupling: An aequorin study in squid giant synapse. Proc. Natl. Acad. Sci. USA **72**, 187-190.

LLINAS, R., STEINBERG, I. Z., and WALTON, K. (1981). Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. Biophys. J. **33**, 323-352.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951). Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275.

LUFT, J. H. (1971). Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscope and mechanism of action. Anat. Rec. **171**, 347-368.

LUTHRA, R., and OLSON, M. S. (1977). The inhibition of calcium uptake and release by rat liver mitochondria by ruthenium red. FEBS Lett. **81**, 142-146.

MAGNAVAL, R., BATTI, R., and THIESSAND, J. (1975). Methylmercury effect on rat liver mitochondrial dehydrogenases. Experientia **31**, 406-407.

MALAGODI, M. H., and CHIOU, C. Y. (1974). Pharmacological evaluation of a new Ca^{2+} antagonist, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8): Studies in smooth muscles. Eur. J. Pharmacol. **27**, 25-33.

MANALIS, R. S., and COOPER, G. P. (1975). Evoked transmitter release increased by inorganic mercury at frog neuromuscular junction. Nature (Lond.) **257**, 690-691.

MANTHORPE, C. M. NETTLETON, D. O., and WILSON, J. E. (1976). Purification of rat brain mitochondria: A new procedure. J. Neurochem. **27**, 1547-1549.

MARSHALL, I. G., and PARSONS, R. L. (1975). The effects of tetraphenylboron on spontaneous transmitter release at the frog neuromuscular junction. Br. J. Pharmacol. **54**, 333-338.

MARTIN, R., and MILEDI, R. (1978). A structural study of the squid synapse after intraaxonal injection of Ca^{2+}. Proc. R. Soc. Lond. **201**, 317-333.

MARTINOSI, A., and FERETOS, R. (1964). Sarcoplasmic reticulum. I. The uptake of calcium by sarcoplasmic reticulum fragments. J. Biol. Chem. **239**, 648-658.

MCGRAW, C. F., SOMLYO, A. V., and BLAUSTEIN, M. P. (1980). Localization of calcium in presynaptic nerve terminals. An ultrastructural and electron microprobe analysis. J. Cell Biol. **85**, 228-241.

MEISTER, A., and ANDERSON, M. E. (1983). Glutathione. Ann. Rev. Biochem. **52**, 711-760.

MELA, L. (1968). Interactions of La^{3+} and local anesthetic drugs with mitochondrial Ca^{2+} and Mn^{2+} uptake. Arch. Biochem. **123**, 286-293.

MELA, L. (1969). Inhibition and activation of Ca^{2+} transport in mitochondria. Effect of lanthanides and local anesthetic drugs. Biochemistry **8**, 2481-2486.

MILEDI, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. Proc. Roy. Soc. B. **183**, 421-425.

MILEDI, R., MOLENAAR, P. C., and POLAK, R. L. (1980). The effect of lanthanum ions on acetylcholine in frog muscle. J. Phys. **309**, 199-214.

MINNEMA, D. J., COOPER, G. P., and GREENLAND, R. D. (1989). Effects of methylmercury on neurotransmitter release from rat brain synaptosomes. Toxicol. Appl. Pharmacol. **99**, 510-521.

MITCHELL, J. F. and SILVER, A. (1963). The spontaneous release of acetylcholine from the denervated hemidiaphragm of the rat. J. Physiol. **165**, 117-129.

MITCHELL, P., and MOYLE, J. (1969). Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. Eur. J. Biochem. **7**, 471-484.

MIYAMOTO, M. D. (1983). Hg^{2+} causes neurotoxicity at an intracellular site following entry through Na^{+} and Ca^{2+} channels. Brain Res. **267**, 375-379.

MOLENAAR, P.C., NICKOLSON, V. J., and POLAK, R. L. (1973). Preferential release of newly synthesized [^{3}H]acetylcholine from rat cerebral cortex **in vitro**. Br. J. Pharmacol. **47**, 97-108.

MOORE, C. L. (1971). Specific inhibition of mitochondrial Ca^{2+} transport by ruthenium red. Biochem. Biophys. Res. Commun. **42,** 298-305.

MOORE, C., and PRESSMAN, B. L. (1964). Mechanism of action of valinomycin on mitochondria. Biochem. Biophys. Res. Commun. **15,** 548-562.

MURRIN, L. C., DEHAVEN, R. N., and KUHAR, M. J. (1977). On the relationship between [^{3}H]choline uptake activation and [^{3}H]acetylcholine release. J. Neurochem. **29,** 681-687.

NAKAZATO, Y., ASANO, T., and OHGA, A. (1979). The in vitro effect of mercury compounds on noradrenaline output from guinea pig vas deferens. Toxicol. Appl. Pharmacol. **48,** 171-177.

NICHOLLS, D. G. (1974). The influence of respiration and ATP hydrolysis on the protonelectrochemical gradient across the inner membrane of rat liver mitochondria is determined by ion distribution. Eur. J. Biochem. **50,** 305-315.

NICHOLLS, D. G. (1978). Calcium transport and proton electrochemical potential gradient in mitochondria from guinea-pig cerebral cortex and rat heart. Biochem. J. **170,** 511-522.

NICHOLLS, D. G. (1982). Bioenergetics: An introduction to the chemiosmotic theory, Academic Press, Inc, New York.

NICHOLLS, D. G. (1986). Intracellular calcium homeostasis. Brit. Med. Bull. **42,** 353-358.

NICHOLLS, D., and AKERMAN, K. (1982). Mitochondrial Ca^{2+} transport. Biochim. Biophys. Acta **683,** 57-88.

NICHOLLS, D. G., and CROMPTON, M. (1980). Mitochondrial calcium transport. FEBS Lett. **111,** 261-268.

NICHOLLS, D. G., and SCOTT, I. D. (1980). The regulation of brain mitochondrial calcium-ion transport. The role of ATP in the discrimination between kinetic and membrane-potential-dependent calcium-ion efflux mechanisms. Biochem. J. **186,** 833-839.

NORDBERG, G. F., BERLIN, M. H., and GRANT, C. A. (1970). Methylmercury in the monkey -Autoradiographical distribution and neurotoxicity. Proceedings, 16th International Congress on Occupational Health, Tokyo.

NORSETH, T. (1969). Studies of intracellular distribution of mercury. In: Chemical Fallout, Current Research on Persistent Pesticides (M. W. Miller and G. G. Berg, eds.), pp. 408-427. Charles Thomas, Springfield, IL.

NORSETH, T., and BRENDEFORD, M. (1971). Intracellular distribution of inorganic and organic mercury in rat liver after exposure to methylmercury salts. Biochem. Pharmacol. **20,** 1101-1107.

O'KUSKY, J. (1983). Methylmercury poisoning of the developing nervous system: Morphological changes in neuronal mitochondria. Acta Neuropathol. (Berl.) **61,** 116-122.

ONODERA, K. (1973). Effect of caffeine on the neuromuscular junction of the frog and its relation to external calcium concentration. Jap. J. Physiol. **23,** 587-597.

ORRENIUS, S., McCONKEY, D. J., JONES, D. P. and NICOTERA, P. (1988). Ca^{2+}-activated mechanisms in toxicity and programmed cell death. ISI Atlas Sci: Pharmacology **2**:319-324.

OZAWA, K., SETA, K., TAKEDA, H., ANDO, K., HANDA, H. and ARAKI, C. (1966). On the isolation of mitochondria with high respiratory control from rat brain. J. Biochem. **59,** 501-510.

PALMER, J. W., and PFEIFFER, D. R. (1981). The control of Ca^{2+} release from heart mitochondria. J. Biol. Chem. **256,** 6742-6750.

PATERSON, R., and USHER, D. (1971). Acute toxicity of methylmercury in glycolytic intermediates and adenine nucleotides of rat brain. Life Sci. **10,** 121-128.

PFEIFFER, D. R., PALMER, J. W., BEATRICE, M. C., and STIERS, D. L. (1983). The mechanism and regulation of Ca^{2+} efflux from mitochondria. Elsevier Science Publishing Co., Inc. (D. L. F. Lennon, F. W. Stratman, and R. N. Zahlten, eds.) pp. 67-80.

PFEIFFER, D. R., SCHMID, P. C., BEATRICE, M. C., and SCHMID, H. H. O. (1979). Intramitochondrial phospholipase activity and the effects of Ca^{2+} plus N-ethylmaleimide on mitochondrial function. J. Biol. Chem. **254,** 11485-11494.

POLAK, R. L., SELLIN, L. C., and THESLEFF, S. (1981). Acetylcholine content and release denervation on botox poisoned rat skeletal muscle. J. Physiol. (Lond.) **319,** 253-259.

POUNDS, J. G., and ROSEN, J. F. (1988). Cellular Ca^{2+} homeostasis and Ca^{2+}-mediated cell processes as critical targets for toxicant action: Conceptual and methodological pitfalls. Toxicol. Appl. Pharmacol. **98,** 001-0011.

POZZAN, T., BRAGADIN, M. and AZZONE, G. F. (1977). Disequilibrium between steady-state Ca^{2+} accumulation ratio and membrane potential in mitochondria. Pathway and role of Ca^{2+} efflux. Biochemistry **16,** 5618-5624.

PRESSMAN, B. C. (1973). Properties of ionophores with broad range cation selectivity. Fed. Proc. **32,** 1698-1703.

PUSKIN, J. S., GUNTER, T. E., and GUNTER, K. K. (1976). Evidence for more than one Ca^{2+} transport mechanism in mitochondria. Biochemistry **15,** 3834-3842.

PUTNEY, J. W., and BIANCHI, P. C. (1974). Site of action of dantrolene in frog sartorius muscle. J. Pharmacol. Exp. Ther. **189,** 202-212.

RAFALOWSKA, U., ERECINSKA, M., and WILSON, D. F. (1980). Energy metabolism in rat brain synaptosomes from nembutal-anesthetized and nonanesthetized animals. J. Neurochem. **34,** 1380-1386.

RAMACHANDRAN, C., and BYGRAVE, F. L. (1978). Calcium ion cycling in rat liver mitochondria. Biochem. J. **174,** 613-620.

RAHAMIMOFF, R., and ALNAES, E. (1973). Inhibitory action of ruthenium red on neuromuscular transmission. Proc. Natl. Acad. Sci. **70,** 3613-3616.

RECKNAGEL, R. O., and GLENDE, E. A. (1973). Carbon tetrachloride hepatotoxicity: An example of lethal cleavage. CRC Crit. Rev. Toxicol. **2,** 263-297.

REED, K. C., and BYGRAVE, F. L. (1974). The inhibition of mitochondrial Ca^{2+} transport by lanthanides and ruthenium red. Biochem. J. **140,** 143-155.

RIGONI, F., MATHIEN-SHIRE, Y., and DEANA, R. (1980). Effect of ruthenium red on calcium efflux from rat liver mitochondria. FEBS Lett. **120,** 255-258.

RILEY, W. W., and PFEIFFER, D. R. (1986). Rapid and extensive release of Ca^{2+} from energized mitochondria induced by EGTA. J. Biol. Chem. **261,** 28-31.

ROBERTSON, B., and WANN, K. T. (1987). On the action of ruthenium red and neuraminidase at the frog neuromuscular junction. J. Physiol. **382,** 411-423.

ROED, A. (1982). Caffeine-induced blockade of neuromuscular transmission and its reversal by dantrolene sodium. Eur. J. Pharmacol. **83,** 83-90.

ROSSI, C. S., and LEHNINGER, A. L. (1963). Stoichiometric relationships between accumulation of ions by mitochondria, and the energy-coupling sites in the respiratory chain. Biochem. Zeit. **338**, 698-713.

ROSSI, C. S., and LEHNINGER, A. L. (1964). Stoichiometry of respiratory stimulation, accumulation of Ca^{2+} and phosphate, and oxidative phosphorylation in rat liver mitochondria. J. Biol. Chem. **239**, 3971-3980.

ROSSI, C. S., VASINGTON, F. D., and CARAFOLI, E. (1973). The effect of ruthenium red on the uptake and release of Ca^{2+} by mitochondria. Biochem. Biophys. Res. Commun. **50**, 846-852.

ROTTENBERG, H. (1975). The measurement of transmembrane electrochemical proton gradients. J. Bioenerg. **7**, 61-74.

ROTTENBERG, H. and MARBACH, M. (1989). Adenine nucleotides regulate Ca^{2+} transport in brain mitochondria. FEBS Lett. **247**, 483-486.

ROTTENBERG, H., and SCARPA, A. (1974). Calcium uptake and membrane potential in mitochondria. Biochemistry **13**, 4811-4817.

ROWELL, P. P., and DUNCAN, G. E. (1981). The subsynaptosomal distribution and release of [^{3}H]acetylcholine synthesized by rat cerebral cortical synaptosomes. Neurochem. Res. **6**, 1265-1281.

RUSTAM, H., VON BURG, R., AMIN-ZAKI, L., and HASSANI, S. (1975). Evidence for a neuromuscular disorder in methylmercury poisoning. Arch. Environ. Hlth. **30**, 190-195.

SAIJOH, K., INOUE, Y., and SUMINO, K. (1987). Stimulating effect of methylmercury chloride on [^{3}H]acetylcholine release from guinea-pig striatal slices. Toxic. in Vitro **1**, 233-237.

SCARPA, A., and AZZONE, G. F. (1970). The mechanism of ion translocation in mitochondria. 4. Coupling of K^+ efflux with Ca^{2+} uptake. Europ. J. Biochem. **12**, 328-335.

SCHNAITMAN, C., ERWIN, V. G., and GREENAWALT, J. W. (1967). The submitochondrial localization of monoamine oxidase, an enzymatic marker for the outer membrane of rat liver mitochondria. J. Cell. Biol. **32**, 719-735.

SCOTT, I. D., ÅKERMAN, K. E. O., and NICHOLLS, D. G. (1980). Calcium-ion transport by intact synaptosomes. Intrasynaptosomal compartmentation and the role of the mitochondrial membrane potential. Biochem. J. **192**, 873-880.

SCOTT, K. M., KNIGHT, V. A., SETTLEMIRE, C. T., and BRIERLEY, G. P. (1970). Differential effects of mercurial reagents on membrane thiols and on the permeability of the heart mitochondrion. Biochemistry **9,** 714-723.

SETTLEMIRE, C. T., HUNTER, G. R., and BRIERLEY, G. P. (1968). Ion transport in heart mitochondria. 8. The effect of ethylenedtylenediaminetertraacetate on monovalent ion uptake. Biochim. Biophys. Acta **162,** 487-499.

SIESJO, B. K. and BENGTSSON, F. (1989). Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: A unifying hypothesis. J. Cereb. Blood Flow Metab. **9**: 127-140.

SILINSKY, E. M. (1985). The biophysical pharmacology of calcium-dependent acetylcholine secretion. Pharmacol. Rev. **37,** 81-132.

SHAFER, T. J., and ATCHISON, W. D. (1989). Block of ^{45}Ca uptake into synaptosomes by methylmercury: Ca^{2+}- and Na^{+}-dependence. J. Pharmacol Exp. Ther. **248,** 696-702.

SIMON, J. R., ATWEH, S., and KUHAR, M. J. (1976). Sodium-dependent high affinity choline uptake: A regulatory step in the synthesis of acetylcholine. J. Neurochem. **26,** 909-922.

SINGER, N., KRISHNAN, N., and FYFE, D. A. (1972). Penetration of ruthenium red into peripheral nerve fibers. Anat. Rec. **173,** 375-390.

SONE, N., LARSSTUVOLD, M. K., and KAGAWA, Y. (1977). Effect of methylmercury on phosphorylation, transport, and oxidation in mammalian mitochondria. J. Biochem. **82,** 859-868.

SOTTOCASA, G. L., SANDRI, G., PANFILI, E., DE BERNARD, B., GAZZOTTI, P., VASINGTON, F. D., and CARAFOLI, E. (1972). Isolation of a soluble Ca^{2+} binding glycoprotein from ox liver mitochondria. Biochem. Biophys. Res. Commun. **47,** 808-813.

SOUTHARD, J. H., BLONDIN, G. A., and GREEN, D. E. (1974a). Induction of transmembrane proton transfer by mercurials in mitochondria. II. Release of a Na^{+}/K^{+} ionophore. J. Biol. Chem. **249,** 678-681.

SOUTHARD, J., NITISEWOJO, P., and GREEN, D. E. (1974b). Mercurial toxicity and the perturbation of the mitochondrial control system. Fed. Proc. **33,** 2147-2153.

SOUTHARD, J. H., PENNISTON, J. V., and GREEN, D. E. (1973). Induction of transmembrane proton transfer by mercurials in mitochondria. I. Ion movements accompanying transmembrane proton transfer. J. Biol. Chem. **218,** 3546-3550.

STATHAM, H. E. and DUNCAN, C. J. (1976). Dantrolene and the neuromuscular junction: Evidence for intracellular calcium stores. Eur. J. Pharmacol. **39,** 143-152.

STEEL, R. G. D., and TORRIE, J. H. (1960). Principles and Procedures of Statistics, McGraw-Hill, New York.

STEFANI, E., and CHIARANDINI, D. J. (1973). Skeletal muscle: Dependence of potassium contractures on extracellular calcium. Pflugers Arch. **343,** 143-150.

SUSZKIW, J. B., and O'LEARY, M. E. (1983). Temporal characteristics of potassium-stimulated acetylcholine release and inactivation of calcium influx in rat brain synaptosomes. J. Neurochem. **41,** 868-873.

SUSZKIW, J., TOTH, G., MURAWSKY, M., and COOPER, G. P. (1984). Effects of Pb^{2+} and Cd^{2+} on acetylcholine release and Ca^{2+} movements in synaptosomes and subcellular fractions from rat brain and Torpedo electric organ. Brain Res. **323,** 31-46.

TAIPALE, H. T., KAUPPINEN, R. A., and KOMULAINEN, H. (1989). Ruthenium red inhibits the voltage-dependent increase in cytosolic free calcium in cortical synaptosomes from guinea-pig. Biochem. Pharmacol. **38,** 1109-1113.

TAKEUCHI, T., MATSUMOTO, H., SASKI, M., KAMBARA, T., SHIRAISHI, Y., HIRATA, Y., NOBUKIRO, M., and STOH, H. (1968). Pathology of Minamata disease. Kumamoto Med. J. **34,** 521-524.

TAKEUCHI, T., MORIKAWA, N., MATSUMOTO, H., and SHIRAISHI, Y. (1962). A pathological study of Minamata disease in Japan. Acta Neuropathol. **2,** 40-57.

TJIOE, S., BIANCHI, C. P. and HAUGAARD, N. (1970). The function of ATP in Ca^{2+} uptake by rat brain mitochondria. Biochim. Biophys. Acta **216,** 270-273.

TRAXINGER, D. L., and ATCHISON, W. D. (1987a). Comparative effects of divalent cations on the methylmercury-induced alterations of acetylcholine release. J. Pharmacol. Exp. Ther. **240,** 451-459.

TRAXINGER, D. L. and ATCHISON, W. D. (1987b). Reversal of methylmercury-induced block of nerve-evoked release of acetylcholine at the neuromuscular junction. Toxicol. Appl. Pharmacol. **90,** 23-33.

TRUMP, B. F., and ARSTILA, A. V. (1971). Cell injury and cell death. In: Principles of Pathobiology (M. F. LaVia, R. B. Hill, eds.), pp. 9-95. Oxford University Press, New York.

TRUMP, B. F. and BEREZESKY, I. K. (1985). Cellular ion regulation and disease: A hypothesis. Curr. Top. Membr. Transp. **25**: 279-319.

TRUMP, B. F., BEREZESKY, I. K., and OSORNIO-VARGAS, A. R. (1981). Cell death and the disease process. The role of calcium. In: Cell Death In Biology and Pathology. (J. D. Bower and R. A. Lockshin, eds), Chapman and Hall, London and New York, pp. 209-242.

TSIEN, R. Y., POZZAN, T., and RINK, T. J. (1982). Calcium homeostasis in intact lymphocytes: Cytoplasmic free calcium monitored with a new intracellularly trapped fluorescent indicator. J. Cell. Biol. **94,** 325-334.

TUOMISTO, J., and KOMULAINEN, H. (1983). Release and inhibition of uptake of 5-hydroxytryptamine in blood platelets in vitro by copper and methylmercury. Acta Pharmacol. Toxicol. **52,** 292-297.

VAN WINKLE, W. B. (1976). Calcium release from skeletal muscle sarcoplasmic reticulum: Site of action of dantrolene sodium? Science **193,** 1130-1131.

VASINGTON, F. D., GAZZOTTI, P., TIOZZO, R., and CARAFOLI, E. (1972). The effect of ruthenium red on Ca^{2+} transport and respiration in rat liver mitochondria. Biochim. Biophys. Acta **256,** 43-54.

VASINGTON, F. D., and MURPHY, J. V. (1962). Ca^{2+} ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. J. Biol. Chem. **237,** 2670-2677, 1962.

VERCESI, A. E. (1984). Possible participation of membrane thiol groups on the mechanism of $NAD(P)^{+}$-stimulated Ca^{2+} efflux from mitochondria. Biochem. Biophys. Res. Commun. **119,** 305-310.

VERITY, M. A., BROWN, W. J., and CHEUNG, M. (1975). Organic mercurial encephalopathy: In vivo and in vitro effects of methylmercury on synaptosomal respiration. J. Neurochem. **25,** 759-766.

VYSKOCIL, F., ZEMKOVA, H., and EDWARDS, C. (1989). Non-quantal acetylcholine release. In Neuromuscular Junction (L. Sellin, R. Libelius and S. Thesleff, eds.), pp. 197-205. Elsevier, Amsterdam.

VON BURG, R., LIJOI, A., and SMITH, C. (1979). Oxygen consumption of rat tissue slices exposed to methylmercury in vitro. Neurosci. Letters **14,** 309-314.

WHERLE, J. P., JURKOWITZ, M., SCOTT, K. M., and BRIERLEY, G. P. (1976). Mg^{2+} and the permeability of heart mitochondria to monovalent cations. Arch. Biochem. Biophys. **174,** 312-323.

WHITTAKER, V. P. (1984). The Synaptosome. in Handbook of Neurochemistry, (ed. A. Lajtha) 2nd ed. Vol. 7, pp. 1-39, Plenum Press, New York.

WIKSTROM, M. K., and SAARI, H. T. (1976). Conformational changes in cytochrome aa3 and ATP synthetase of the mitochondrial membrane and their role in mitochondrial energy transduction. Mol. Cell. Biochem. **11,** 17-33.

YAMAMURA, H. I., and SNYDER, S. H. (1973). Choline: High-affinity uptake by rat brain synaptosomes. Science **178,** 626-628.

YOSHINO, Y., MOZAI, T., and NAKAO, K. (1966a). Distribution of mercury in the brain and its subcellular units in experimental organic mercury poisoning. J. Neurochem. **13,** 397-406.

YOSHINO, Y., MOZAI, T., and NAKAO, K. (1966b). Biochemical changes in the brain in rats poisoned with an alkylmercury compound with special reference to the inhibition of protein synthesis in brain cortex slices. J. Neurochem. **13,** 1223-1230.