This is to certify that the

thesis entitled
Comparative Effects of Divalent Cations on the

Methylmercury-Induced Alterations of Acetylcholine Release

presented by

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has been accepted towards fulfillment
of the requirements for
M.S. degree in Pharmacology/Toxicology

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Date Aug 1, 1986

COMPARATIVE EFFECTS OF DIVALENT CATIONS ON THE METHYLMERCURY-INDUCED ALTERATIONS OF ACETYLCHOLINE RELEASE

By

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A THESIS

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

MASTER OF SCIENCE

Department of Pharmacology and Toxicology

ABSTRACT

COMPARATIVE EFFECTS OF DIVALENT CATIONS ON THE METHYLMERCURY-INDUCED ALTERATIONS OF ACETYLCHOLINE RELEASE

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Methylmercury (MeHg) blocks synchronous evoked release and increases spontaneous release of acetylcholine at the murine neuromuscular junction. The overall objective of these studies was to investigate the mechanism of action of MeHg in blocking synchronous evoked release and in inducing an increase in spontaneous release. Strontium and barium substitution was used as a means of elucidating the MeHg-calcium interaction and its effects on the release process.

Experiments were conducted using the isolated phrenic nerve-hemidiaphragm preparation of rats and conventional intracellular microelectrode recording techniques. Results suggest that 1) the effect of MeHg on spontaneous release is not calcium-specific and appears to be due to MeHg-induced release of divalent cation from intracellular stores, 2) block of evoked release is due predominantly to block of impulse propagation by MeHg and is reversible by increasing the intensity of nerve stimulation.

ACKNOWLEDGEMENTS

I would like to thank Dr. William D. Atchison, my advisor, for his guidance throughout the course of this study.

I would like to express my appreciation to my graduate committee members Drs. Theodore M. Brody and Jacob Krier and especially to Dr. Susan M. Barman for her helpful advice during my graduate work.

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LIST OF ABBREVIATIONS

ACh----------acetylcholine

Ba----------barium

Ca----------calcium

EPP----------endplate potential

Fm(t)--------fractional increase in MEPP frequency

Kf----------fast rate constant

Ks----------slow rate constant

MeHg---------methylmercury

MEPP---------miniature endplate potential

MEPPf--------miniature endplate potential frequency

NMJ----------neuromuscular junction

Sr----------strontium

m----------mean quantal content

mM----------millimolar

uM----------micromolar

4-AP---------4-aminopyridine

INTRODUCTION

A. General Introduction

Mercury is a heavy metal which is utilized extensively in a variety of industrial and agricultural areas worldwide. As a result, many forms of mercury have been released either directly or indirectly into the environment. The toxic effects of mercurials were known as early as the 16th century (Chang, 1977), but the importance of mercury compounds as hazardous environmental pollutants has only recently been recognized. Methylmercury (MeHg) is a highly toxic organic form of mercury which has been implicated in at least two major outbreaks of mercury poisoning. An examination of the cellular mechanisms underlying the neurotoxic effects of MeHg in mammals is presented in this thesis.

B. Contamination

Mercury is found in the environment in three general chemical forms: elemental mercury (Hg^0), inorganic mercury (Hg+ and Hg++), and organic mercury (R-Hg+). Elemental and inorganic mercury are used in the electrical apparatus, mining, paint, chloralkali, and plastic industries; mercury is released into the

environment from these sources through waste-water discharges or atmospheric venting. Organic mercury compounds are utilized mainly for their fungicidal properties and are found in seed grain dressings, orchard sprays, foliar dusts, and preservative solutions for wood, paper pulp, and leather. Contamination occurs through surface runoff and release of wastes into rivers. Mercury poisoning can occur from inhalation of elemental mercury vapor or from absorption of organic and inorganic mercury compounds through the skin. Most commonly, mercury poisoning occurs via ingestion of mercury-contaminated food. Elemental mercury is very poorly absorbed from the gastrointestinal tract, inorganic mercury is absorbed to the extent of about 7 percent of the ingested load while organic methylmercury (MeHg) is absorbed much more efficiently (90%) (Chang, 1977). Once absorbed, mercury is transported in the plasma and red blood cells. Elemental mercury vapor is oxidized to mercuric ion (Hg++) in the blood. Inorganic mercury is distributed non-uniformly in the body, with the highest concentration found in the kidneys. Excretion occurs via the urine and feces. MeHg is distributed more evenly to various tissues, with the highest concentrations reported in the brain and blood. Excretion of MeHg is mainly in the feces.

C. Episodes of MeHg Intoxication

In recent years, ingestion of MeHg-contaminated food has caused two devastating epidemics of MeHg poisoning. The first occurred in Minamata, Japan in the 1950's when a vinyl chloride plant discharged organic and inorganic mercury-containing effluent into Minamata Bay. Microorganisms converted the inorganic mercury to MeHg and the MeHg was then taken up by plankton algae and accumulated in fish via the food chain. Chronic MeHg poisoning through consumption of contaminated fish affected approximately 1500 people and 46 deaths were reported. In addition many infants were born with severe nervous system damage from prenatal mercury intoxication. A second massive outbreak affecting 6500 people occurred in Iraq in 1972. Seed grain that had been treated with a MeHg fungicide was inadvertently used for baking bread resulting in an acute epidemic of MeHg poisoning and 450 deaths. The Iraqi episode differed from that at Minamata in that exposure to MeHg was more acute, and at higher doses. The clinical signs of MeHg poisoning in both cases included sensory and visual disturbances, ataxia, impairments of speech, hearing, and gait, and a generalized weakness of the extremities (Takeuchi et al., 1962; Bakir et al., 1973). Lesions in both the central (cerebellar cortex) and peripheral nervous system (dorsal root ganglion, nerve fibers) have also been reported. A myasthenia gravis-like weakness was reported

only in the Iraqi outbreak. This condition was treated successfully with neostigmine, an acetylcholinesterase inhibitor (Rustam et al., 1975).

D. Specific Background for Research Objectives

The molecular and cellular mechanisms responsible for the neurotoxic effects of MeHg are not known. However it is known that the pathological lesions described above occur in response to more subtle biochemical or physiological effects of MeHg on the nerve. The sensory and motor defects could be due to disruption of synaptic transmission. The neuromuscular junction (NMJ) provides a model synapse for studying the effects of MeHg on synaptic transmission. Biochemical and physiological processes involved in transmitter release at this synapse are well characterized. For example, heavy metals have been tested at the NMJ to examine the normal sequence of events underlying neurotransmitter release and to investigate neurotoxic effects as well (Cooper et al., 1984). The effects of MeHg on synaptic function are not unique to the cholinergic system (Bondy et al., 1979; Nakazato et al., 1979; Tuomisto and Komulainen, 1983) and it may well be that the mechanisms responsible for MeHg's effects at the NMJ are similar to those mechanisms underlying MeHg's effect at other chemical synapses in the peripheral and central nervous system.

1. Neurotransmitter release at the NMJ

Three different terms are used to describe neurotransmitter release at the NMJ. First, spontaneous quantal release describes the spontaneous release of a single quantum of acetylcholine (ACh) from the nerve terminal (Fatt and Katz, 1952; del Castillo and Katz, 1954). This type of release occurs randomly at a frequency of 0.3-3.0/sec (Hz) in response to changes in intracellular calcium (Ca) concentration (Llinas and Nicholson, 1975). The interaction of the released ACh with receptors on the postsynaptic membrane results in a small depolarization of the membrane, a phenomenon recorded electrophysiologically as miniature endplate potentials (MEPPs). The MEPP is thought to represent the fundamental quantum of secretion. Second, synchronous evoked release decribes the simultaneous synchronous release of many quanta of ACh from the nerve terminal. This type of release occurs in response to depolarization of the nerve terminal, usually by an action potential, and requires Ca entry into the terminal through voltage-regulated Ca channels during the presynaptic action potential (Katz and Miledi, 1967; Llinas *et al.*, 1981; Augustine *et al.*, 1985 a,b). Released ACh diffuses across the synaptic cleft; interaction of ACh with the postsynaptic receptors results in a large, graded depolarization of the membrane which can be recorded

electrophysiologically from the skeletal muscle endplate region. This depolarization is referred to as an end-plate potential (EPP) (Fatt and Katz, 1951). Under normal conditions, the EPP is so large as to reach threshold for generation of a muscle action potential. A third type of release, called asynchronous evoked release (Silinsky, 1985), occurs in response to repetitive stimulation of the nerve. The prolonged depolarization of the nerve terminal induced by repetitive stimulation causes a steady movement of Ca through Ca channels into the terminal resulting in asynchronous or phasic release of large numbers of ACh quanta. This phenomenon is recorded electrophysiologically as a transient increase in MEPP frequency (MEPPf) followed by a gradual decline to prestimulus levels (Liley and North, 1953; Miledi and Thies, 1971). The changes in MEPPf are mainly attributed to residual intraterminal Ca which accumulates during repetitive stimulation (Erulkar and Rahamimioff, 1978; Zengel and Magleby, 1981).

2. Effects of MeHg at the NMJ

Experimental studies utilizing intracellular microelectrode recording techniques have shown that MeHg exerts two effects on ACh release at the NMJ (Barrett *et al.*, 1974; Juang, 1976; Atchison and Narahashi, 1982; Atchison *et al.*, 1984). First, synchronous evoked release of ACh is blocked by MeHg. Second, spontaneous

quantal release is first stimulated and then depressed by MeHg. The first effect is observed as a complete cessation of nerve-evoked EPPs (Figure 1). The second effect is observed as a tremendous increase in MEPPf followed by a precipitous decline until MEPPs can no longer be recorded (Figure 2). Thus MeHg has a depressant effect on synchronous evoked release and a biphasic effect on spontaneous release. These two effects occur with differing time courses suggesting that they may occur by different mechanisms. However, both suppression of synchronous evoked release and stimulation and depression of spontaneous release have been attributed to presynaptic actions of MeHg (Atchison and Narahashi, 1982). The depression of spontaneous release is not due to depletion of transmitter stores since La3+, which stimulates release of any available vesicular ACh stores (Heuser and Miledi, 1973), can restore spontaneous release from MeHg-poisoned preparations (Atchison, 1986).

Further experiments (Atchison, 1986) designed to investigate in more detail the Ca-dependence of the MeHg-induced increase of spontaneous release of ACh indicate that the interaction between MeHg and Ca at the motor nerve terminal is complex. MeHg can increase spontaneous release of ACh in Ca-deficient solutions or in Ca-deprived preparations prompting the suggesting that MeHg induces release of Ca from bound intracellular stores (Atchison, 1986). Atchison et al. (1986) also found that

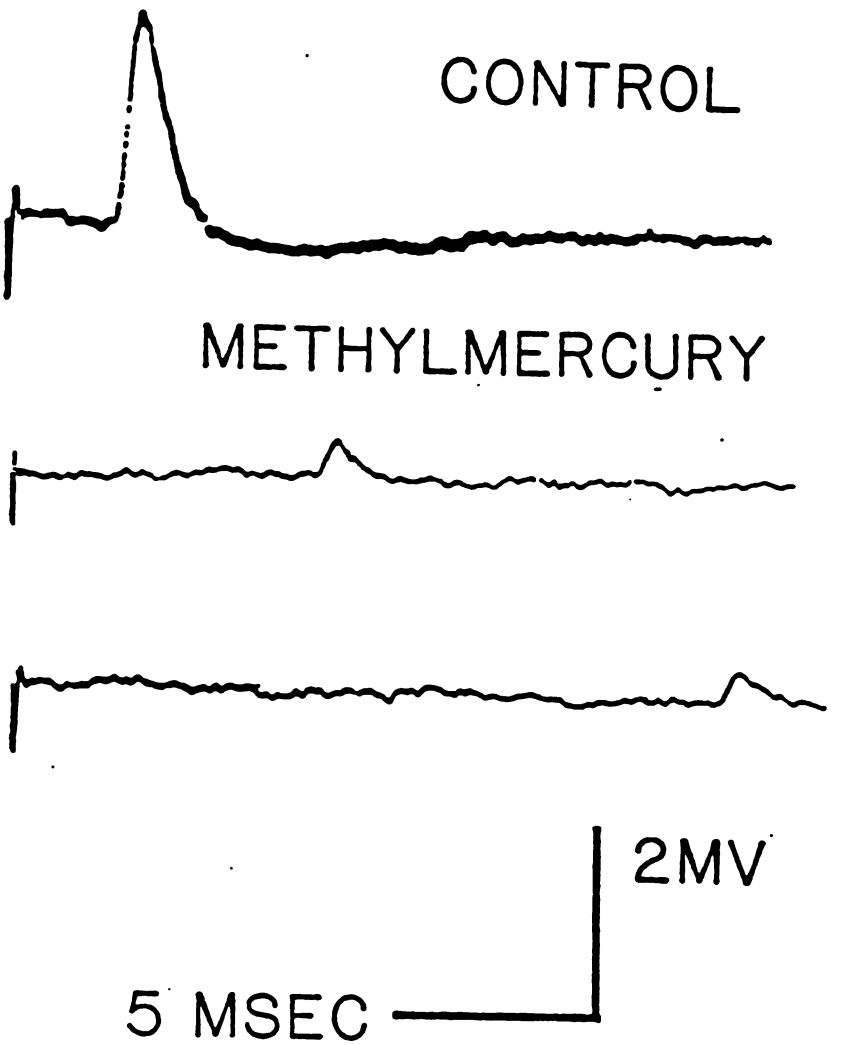

Figure 1. Block of synchronous evoked release induced by MeHg. EPP recorded before (top) and after 10 min exposure to 100uM MeHg (bottom). Note the presence of MEPPs at the time when the EPP is blocked completely. (From Atchison and Narahashi, 1982.)

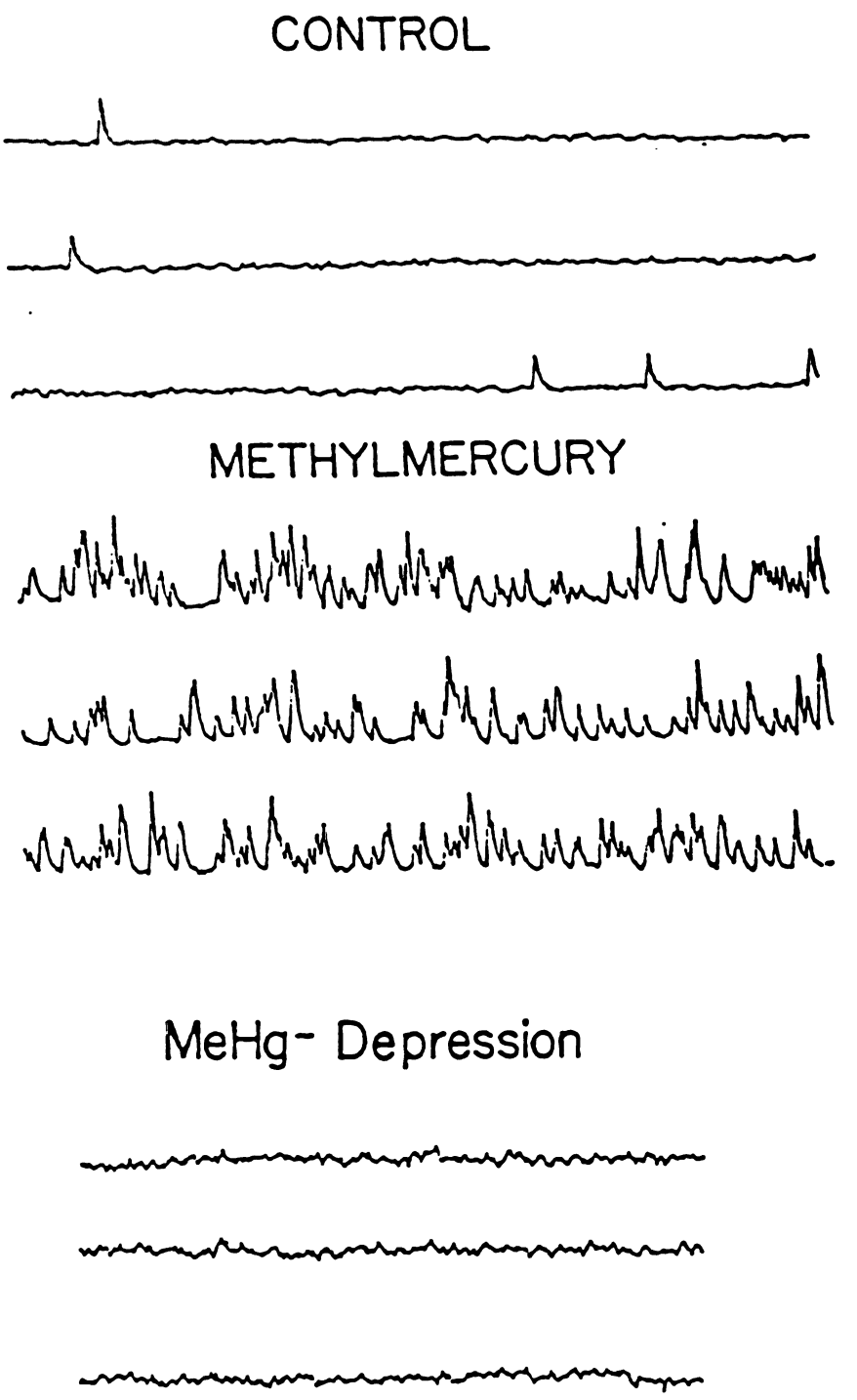

Figure 2. Stimulation and depression of spontaneous quantal release induced by MeHg. MEPPs recorded before (top), after 30 min exposure (middle), and after 50 min exposure to 40uM MeHg (bottom). (From Atchison et al., 1984.)

MeHg blocks depolarization-induced Ca influx into isolated synaptosomes of rat brain in a noncompetitive, irreversible manner. This result might explain in part the rapid, irreversible block of synchronous evoked release caused by MeHg (Juang, 1976; Atchison and Narahashi, 1982).

3. Barium and strontium and transmitter release

Other divalent cations besides Ca can modulate transmitter release; the actions of these cations have been used to elucidate mechanisms underlying transmitter release. Barium (Ba) and strontium (Sr) are divalent cations which can substitute for Ca in Ca-dependent stimulus-secretion coupling with varying degrees of effectiveness. Ba can support release of 1) catecholamines from adrenal chromaffin cells (Douglas and Rubin, 1964) 2) melanocyte stimulating hormone from pituitary melanotrophs (Douglas et al., 1983) and 3) insulin from pancreatic islet cells (Henquin, 1980). Sr can substitute for Ca in glucose-stimulated insulin release (Henquin, 1980) and in the renin secretory process (Churchill et al., 1986). Both cations can substitute for Ca in supporting histamine release from mast cells (Payne and Garland, 1978) and in the release of noradenaline from adrenergic nerve terminals in guinea pig vas deferens (Nakazato and Onoda, 1980). In the isolated superior cervical ganglion of the guinea pig

(McLachlan, 1977) both Ba and Sr augment the frequency of evoked miniature excitatory postsynaptic potentials, but only Sr supports evoked excitatory postsynaptic potentials.

Results obtained with Ba and Sr substitution at the frog NMJ are similar to those reported by McLachlan. Dodge et al. (1969) found that while spontaneous MEPP frequency was unchanged with Sr substitution, asynchronous evoked MEPP frequency was greater in Sr than in Ca. However, while Sr could replace Ca in the process of nerve-evoked synchronous ACh release (EPPs) it was less effective than Ca (fewer quanta released per stimulus). Mellow et al. (1982) reported that this selectivity of the synchronous quantal release process (EPPs) for Ca over Sr is preserved at the frog NMJ even when the Ca channel is bypassed. When liposomes prepared from equal amounts of Ca and Sr are delivered to the inside of the nerve terminal, spontaneous MEPP frequency is increased to the same level. However, when the nerve is stimulated the mean number of quanta released per impulse is significantly greater in Ca than in Sr. Mellow et al. (1982) suggested that the affinity of the intraterminal release site associated with synchronous ACh secretion is greater for Ca than for Sr. Silinsky (1978) showed that Ba was unable to support nerve-evoked synchronous release (i.e. EPPs), but repetitive stimulation in Ba elicited a larger increase in MEPP

frequency than reported for Sr or Ca.

The different effects of Sr and Ba in supporting transmitter release could be due to differences in their ability to enter the nerve terminal. Nachshen and Blaustein (1982) reported that voltage-dependent Ca channels regulate the influx of Ca, Sr, and Ba into rat brain synaptosomes with a selective permeability sequence Ca>Sr>Ba. This permeability sequence is also found to occur in barnacle muscle fiber (Hagiwara et al., 1974) and in sea urchin and tunicate eggs (Okamoto et al., 1977). Indeed Sr and Ba have been found to carry inward current in all Ca channels so far studied (Hagiwara, 1981) including those in mouse motor nerve terminal (Penner and Dreyer, 1986). However, with equimolar concentrations of the three divalent cations, the efficacy in carrying current is Ba>Ca=Sr (Nelson et al., 1984). In addition, Augustine and Eckert (1984) showed that at the squid giant synapse divalent cations support evoked synchronous transmitter release in the sequence Ca>Sr>>Ba. They concluded that the different efficacy of these cations is not due to postsynaptic alterations, presynaptic potential changes or differences in presynaptic divalent cation conductances, suggesting a cation selectivity of the release process itself.

Ca, Sr, and Ba also differ in their ability to stimulate protein phosphorylation, a process which may be linked to transmitter release (Michaelson and Avissar,

1979; DeLorenzo et al., 1979; Robinson and Dunkley, 1985). Ca accumulation by Torpedo synaptosomes has been shown to increase the phosphorylation of a specific protein and to increase ACh release. Sr and Ba can replace Ca in this process but the order of effectiveness of these ions is Ca>Sr>Ba (Michaelson and Avissar, 1979). Protein phosphorylation in rat brain synaptosomes (Robinson and Dunkley, 1985) and in synaptic particulate fractions from rat hippocampus (Hoch and Wilson, 1984) also is stimulated by divalent cations in order of effectiveness Ca>Sr>Ba. It is interesting to note that this sequence of effectiveness is the same as the sequence of effectiveness for divalent cations in supporting transmitter release at the NMJ.

In addition to their different abilities to support transmitter release, there is evidence that Ca, Sr, and Ba are buffered differently by the intraterminal organelles. Mitochondria and smooth endoplasmic reticulum (SER) are known to buffer elevations in intracellular Ca that occur during nerve terminal action potentials (Alnaes and Rahamimoff, 1975; McGraw et al., 1980). Ca uptake into mitochondria occurs via an energy-dependent uniporter which also has been shown to take up Sr (Lehninger et al., 1967). Mitochondrial efflux pathways appear to be more ion selective; Sr is unable to exit mitochondria via the same efflux pathways as Ca (Coelho and Vercesi, 1980; Saris and Bernardi, 1983). Ba

is also taken up by the mitochondrial uniporter (Vainio et al., 1970) but much more slowly than either Ca or Sr. All three divalent cations are taken up by SER (Ornberg and Reese, 1980) with an apparent affinity sequence of Sr>Ca>Ba (Rasgado-Flores et al., 1982). Exchange mechanisms in the nerve terminal also appear to be cation selective to some extent. Ca and Sr both utilize the sodium-calcium exchange mechanism effectively while Ba is much less able to do so (Gill et al., 1981; Rasgado-Flores et al., 1982). The ATP-dependent Ca exchange mechanism is more selective for Ca than Sr or Ba (Gill et al., 1981). Overall Ba appears to be buffered more poorly in the terminal than either Ca or Sr (Tillotson and Gorman, 1983; Nasi and Tillotson, 1985).

E. Research Objectives

The purpose of this research was to investigate more completely the complex interaction between MeHg and Ca at the motor nerve terminal. Given the differences in the ability of the divalent cations Ca, Sr, and Ba to support transmitter release and the way in which they are buffered in the nerve terminal, Sr and Ba substitution was used in experiments as a means of elucidating the MeHg-Ca interaction and its effects on the release process at the NMJ. The overall objectives of the experiments were to answer two basic questions;

1. How does MeHg induce an increase in spontaneous

quantal release of ACh from the motor nerve terminal?

2. How does MeHg block synchronous evoked release of ACh?

The first objective was investigated in two series of experiments. In the first series the specificity of the MeHg-induced increase in spontaneous release for Ca was tested by substitution of Sr and Ba for Ca. Analysis of the MeHg-induced increases in spontaneous release and the time course of this effect might implicate a particular site of action of MeHg in the nerve terminal. The second series of experiments was designed to test the hypothesis that MeHg increases spontaneous release by inducing release of Ca from bound-intracellular stores. These experiments were conducted by investigating the effects of MeHg on the asynchronous evoked release process with Ca and Sr and Ba substitution. Analysis of the kinetics of MEPPf decay with MeHg plus Sr, Ba, and Ca, and the MeHg-induced increases in MEPPf might yield information regarding potential alteration of nerve terminal Ca buffering with MeHg treatment.

The second objective was investigated by studying the effects of Sr substitution and increased Ca concentrations on the EPP amplitude and the latent period of the MeHg-induced block of synchronous evoked release. Reversal of the block of evoked release was attempted

using methods designed to overcome a possible MeHg-induced, Ca-dependent transmission block or nerve conduction block.

METHODS

A. Electrophysiological Procedures

Experiments were conducted using the isolated phrenic nerve-hemidiaphragm preparation (Bülbring, 1946) of male rats (190-240 g, Harlan Sprague-Dawley). The diaphragm was pinned out in a Sylgard-coated, plexiglas chamber and superfused continuously with a physiological saline solution modified from that described by Liley (1956); it contained [mM]: NaCl - 135, KCl - 5, MgCl2 -1, CaCl2 - 2, glucose - 11, and HEPES (N-2 hydroxyethyl-piperazine-N-2-ethanesulfonic acid) - 14. HEPES was used in lieu of the normal phosphate-bicarbonate buffer (Liley, 1956) to prevent precipitation of MeHg. SrCl2 (2 mM) and BaCl2 (0.5 mM) were substituted in place of CaCl2 (2 mM). In experiments in which the muscle was cut to prevent contractions (Barstad and Lilleheil, 1968), a lower KCl concentration (2.5 mM) was used in order to prevent axonal conduction block due to high K+ levels (Hubbard and Wilson, 1973). For experiments in which ionic concentrations were altered, equiosmolar substitutions of NaCl were made to maintain osmolarity and Cl- concentration constant. In experiments utilizing the cut muscle preparation, the diaphragm was equilibrated with the Ca, Sr, or Ba physiological saline in an ice bath for one hour before recording. The uncut

diaphragms used in the spontaneous release experiments were equilibrated at room temperature. Solutions were oxygenated continuously with 100% O2. All experiments were conducted at room temperature of 23-26 ℃.

Intracellular recordings were made using conventional methods (Brock et al., 1952) with borosilicate glass microelectrodes (Figure 3). The microelectrodes were made from glass capillary tubes (WP Instruments, New Haven, CT) using a vertical pipette puller (Model 700B, Kopf Intruments, Tujunga CA). The microelectrodes were filled with an electrolyte solution of 3M KCl and had resistances of 15-25 MΩ . The microelectrode was positioned with a micromanipulator (Model MP2, Narishige Scientific Instruments, Tokyo, Japan) over muscle endplate regions visualized through an upright binocular compound microscope (Microstar Series, AO Scientific Instruments, Buffalo, NY). The electrode was then introduced into the muscle cell with the micromanipulator. Miniature endplate potentials (MEPPs) and endplate potentials (EPPs) were amplified (M707A, WP Instruments, New Haven, CT), displayed on a Nicolet 4094 storage oscilloscope (Nicolet Instruments, Verona, WI) and recorded on magnetic tape (Model B, Vetter Instruments, Rebersburg, PA) and floppy disk, respectively. Resting membrane potential was monitored continuously. MEPPf was determined from Gould 2200 (Gould Inc. Cleveland, OH) chart recordings of the data

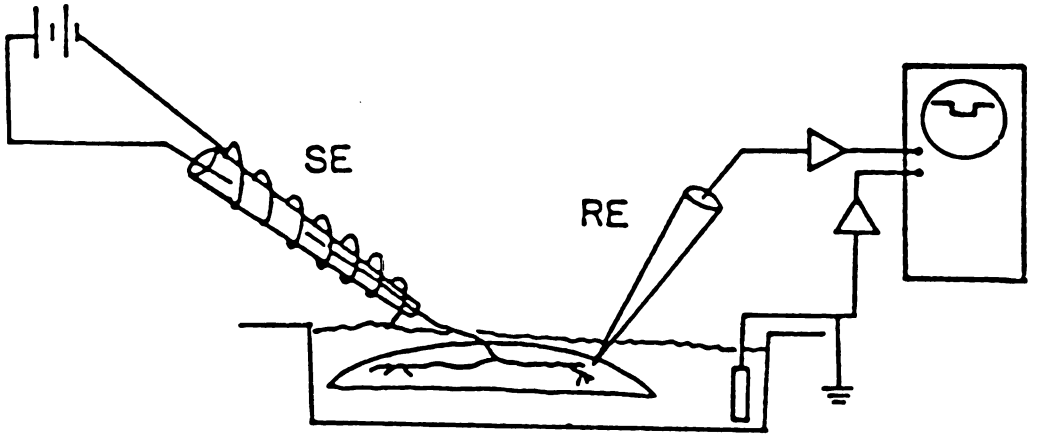

Figure 3. Diagram of conventional intracellular microelectrode recording set-up. SE = stimulating suction electrode; RE = recording electrode. The muscle is pinned out in the plexiglas chamber and the nerve aspirated up into the suction electrode. (From Atchison et al., 1984.)

stored on the magnetic tapes. EPP amplitudes were analyzed from the disks using the Nicolet 4094 digital oscilloscope. For studies of effects of MeHg on asynchronous and synchronous evoked release, the phrenic nerve was stimulated (supramaximal intensity), 50 usec duration, 0.25 or 40 Hz) with a suction electrode and a S-88 stimulator (Grass Instruments, Quincy, MA) with stimulus isolation units (SIU 5A).

B. Cut Muscle Technique

Muscle contractions evoked by nerve stimulation in the synchronous and asynchronous evoked release experiments were blocked by use of a modified "cut muscle" technique (Barstad and Lilleheil, 1968; Hubbard and Wilson, 1973). In this preparation the ends of the muscle fibers in the hemidiaphragm are cut longitudinally approximately 5 mm on each side of the main intramuscular nerve branch (Hubbard and Wilson, 1973). This procedure normally leaves a preparation some 10 mm wide. After cutting, the muscle resting potential declines below the level at which action potentials can be generated; most of the fibers have a resting potential of -30 to -50 mV at the endplate region. Muscle contractions evoked by nerve stimulation are normally blocked by depressing transmitter release with solutions containing low concentrations of Ca and high concentrations of Mg, or inducing postjunctional receptor block with

d-tubocurarine. Use of the cut muscle technique avoids two problems associated with these methods. First, the cut muscle technique permits simultaneous recordings of EPPs and MEPPs. Only EPPs can be recorded from d-tubocurarine-treated preparations. Second, the effects of MeHg on transmitter release can be examined at normal quantal contents and without the complicating interaction of the substituted divalent cations with the increased Mg used with the high [Mg]/low [Ca] treatment. In addition, the cutting procedure does not produce significant changes in the muscle cable properties nor in normal transmitter release (Glavinović, 1979; Lambert et al., 1981). Resting potentials were usually stable for up to 3 hours after the muscle was equilibrated for a one hour period in an ice bath with a low potassium (KCl=2.5mM) physiological saline. The reduced KCl content was chosen to avoid nerve conduction block (Hubbard and Wilson, 1973) due to release of K+ from the cut fibers (Glavinović, 1979).

Three general types of experiments were conducted. Separate preparations were used for each cation and concentration tested and each experimental paradigm. Experiments were replicated at least 4 times, with each preparation serving as its own control.

C. Spontaneous Release Experiments

Initial experiments were performed with different

concentrations of Sr and Ba in order to obtain similar control MEPP frequencies as observed in 2 mM Ca. MEPPs were recorded continuously from cells for one hour in 2, 4, or 8 mM Sr and 0.5, 1, or 2 mM Ba. MEPP frequencies were determined over 5 min intervals in each condition.

In subsequent experiments, MEPPs were recorded continuously from a single cell during a 20 min control period in 2 mM Ca, 2 mM Sr, or 0.5 mM Ba and then subsequently in the cation plus 100 uM MeHg until the characteristic MeHg-induced increase and then decrease of spontaneous release was observed. In preparations in which single cell impalements could not be maintained during treatment with MeHg, other cells were sampled until a stable cell impalement was achieved. MEPP frequency was determined over 3 min intervals during control and MeHg treatment. The maximum MEPP frequency and the time in MeHg at which it occurred were used in the data analysis.

D. Synchronous Evoked Release Experiments

EPPs were elicited continuously by single shocks applied to the nerve at a frequency of 0.25 Hz during a 5-min control period in 2, 4, or 8 mM Ca, or 2 or 4 mM Sr and then subsequently in the cation plus 100 uM MeHg until the EPP was abolished completely. In most experiments the value of the intensity of the stimulus applied to the nerve was adjusted to be just sufficient

for generation of the EPP. This value was considered the "threshold voltage". However, in 9 experiments with 2, 4, or 8 mM Ca or 2 or 4 mM Sr the nerve was stimulated at 2 or 3 times the threshold voltage in order to determine the effect of this stimulus intensity on the latency to block of the EPP. EPP amplitudes were corrected for nonlinear summation (McLachlan and Martin, 1981) using the formula $Vl = V/[(1-0.8V)/E]$ where Vl is the corrected EPP amplitude, V is the uncorrected EPP amplitude, and E is the resting membrane potential. The reversal potential for ACh release at the endplate of the rat diaphragm is estimated to range from -7 to 0 mV (Glavinovic, 1979). Since in earlier studies (Alema et al., 1981; McLachlan and Martin, 1981) the reversal potential was estimated to be 0 mV, this value was used for our estimate as well. All EPP amplitudes were measured and an average EPP amplitude was calculated for the 5 min control period. Average EPP amplitudes were calculated at 1 min intervals in MeHg. Latency to block of the EPP was determined as the time in MeHg at which complete failure of the EPP occurred.

Six additional experiments were performed using the above protocol with the exception that the MeHg concentration was reduced to 20 uM. Average EPP amplitudes were calculated at 5 min intervals in MeHg in 3 experiments with 2 mM Ca plus 20 uM MeHg and 3 experiments with 0.5 mM Ca plus 20 uM MeHg.

E. Asynchronous Evoked Release Experiments

MEPPs and EPPs were evoked at 1 min intervals by 3 sec trains of stimuli at a frequency of 40 Hz during a 4-min control period in 2 mM Ca; 2, 4, or 8 mM, Sr; or 0.5 mM Ba and then subsequently in the cation plus 100 uM MeHg until EPPs and stimulated increases in MEPPf were abolished. In each experiment, MEPPf was determined immediately following cessation of stimulation at 1 sec intervals for the 1 min period between stimulus trains. Average values for each second were determined in control and then in MeHg until block of asynchronous evoked release occurred. Lines were fit by the method of least squares to the data from the two phases of MEPP frequency decay. The cut-off point for the fast component of MEPP frequency decay was determined by eye. Each line is described by the general equation $Fm(t) = e^{-kt}$, where $Fm(t)$ is the fractional increase in MEPPf over control, t is the time after stimulation, and k is a rate constant, measured as the slope of the line. Rate constants for the phases of MEPP frequency decay were determined from the slopes of the lines. The MEPP amplitude distribution was determined for the control period and for each 1 min period between stimulus trains in MeHg. In some experiments in which the resting membrane potential was low, the smaller MEPPs tended to be lost in the noise of the recording system, resulting in a skewed MEPP

amplitude distribution. To avoid an overestimate of the average MEPP amplitude, the mode of the amplitude distribution was used in all experiments for quantal release determination (Wilson, 1982). Average EPP amplitude for each train in control and MeHg with Ca and Sr was obtained from the fifth to the last EPP in the train. Since facilitation of EPP amplitudes occurs during repetitive stimulation (Hubbard and Wilson, 1973), the first four EPP amplitudes were not included in the analysis to avoid an overestimate of the average EPP amplitude.

EPP amplitudes were corrected for nonlinear summation as before (McLachlan and Martin, 1981). An additional correction factor was applied to all EPP amplitudes to compensate for differences in resting membrane potential in MeHg. EPP amplitudes were normalized to a resting membrane potential of -50mV using the formula EPPn = EPPc x 50/Em where EPPn is the normalized EPP amplitude, EPPc is the EPP amplitude corrected for non linear summation, and Em is the muscle membrane potential. The statistical release parameter **m** (mean quantal content) was determined in the control period and at 1 min intervals in MeHg using the mean value method (del Castillo and Katz, 1954) in which **m** = EPPn/MEPPm where EPPn is the normalized EPP amplitude and MEPPm is the mode of the MEPP amplitude distribution.

F. Reversal Attempts

In an attempt to reverse the MeHg-induced block of the EPP, the MeHg was washed out using a) control solutions, b) 4 mM Ca or Sr, or c) 4-aminopyridine (4-AP) (50-100 uM) for periods of up to 30 min. 4-AP facilitates transmitter release (Molgo et al., 1977) by prolonging the action potential through decreased K+ conductance (Llinas et al., 1976). In addition, after the EPP was abolished, the intensity and duration of the stimulus applied to the nerve were increased both before and after washout of MeHg in an attempt to overcome the block of synchronous and asynchronous evoked release.

G. Chemicals

Methylmercuric acetate (Pfaltz-Bauer Chemical Co., Stamford, CT) was dissolved in 4% (v/v) glacial acetic acid to yield a stock solution of 2 mM. Dilutions were made of this stock for test solutions; pH was adjusted to 7.4. Final concentration of MeHg used in all but 6 experiments was 100 uM. This concentration of MeHg is approximately 5 times the concentration reported (after appropriate conversions) in MeHg-poisoned patients exhibiting generalized weakness of the extremities progressing to ataxia (Bakir et al., 1973). However, MeHg exerts identical effects on spontaneous and synchronous evoked release of ACh at bath concentrations of 20 uM (Atchison and Narahashi, 1982; Atchison et al.,

1984). The 100 uM concentration of MeHg was chosen to shorten the latency preceding the increase in spontaneous release in order to facilitate the experiments as impalements are difficult to maintain for long periods in Ba or Sr (Silinsky, 1978). BaCl2 and SrCl2 were obtained from Mallinckrodt In. (Paris, KY). HEPES was obtained from the United States Biochemical Corporation (Cleveland, OH). 4-Aminopyridine was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI).

H. Statistical Analyses

1. Spontaneous release experiments

Peak MEPP frequency and time to peak MEPP frequency in MeHg with Ca, Sr, and Ba were compared using a one way analysis of variance (1 way ANOVA) (Steel and Torrie, 1980).

2. Synchronous evoked release experiments

EPP amplitudes from control and MeHg treatment were compared using the split-plot analysis of variance. The latency to block of the EPP in Ca and Sr was compared using a 1 way ANOVA. Latency to block of EPP with 20 and 100 uM MeHg was compared using a unpaired Student's t-test.

3. Asynchronous evoked release experiments

A test for parallelism was used to determine whether significant differences occurred between rate contants obtained from MEPP frequency decay in control

and MeHg conditions. Fractional increases in MEPP frequency (Fm(t)) were compared using the paired Student's t-test. Mean quantal content, EPP amplitudes, and MEPP amplitudes from control and MeHg treatment were compared using a 1 way ANOVA. The latency to block of asynchronous and synchronous evoked release were compared using an unpaired Student's t-test.

Bonferroni's test for multiple comparisons was used when applicable to compare means for statistical significance (Steel and Torrie, 1980). Significance was set at $p \leq 0.05$ for all experiments.

RESULTS

A. Spontaneous Release Experiments

1. Effects of Ba and Sr on MEPPf

Ba and Sr support spontaneous quantal release of ACh at the rat neuromuscular junction in the absence of Ca (Elmqvist and Feldman, 1965; Anwyl et al., 1982). Ba supports spontaneous release to a greater extent than Ca (Elmqvist and Feldman, 1965), while Sr has been shown to be both equieffective (Anwyl et al., 1982) and less effective (Elmqvist and Feldman, 1965) than Ca in supporting spontaneous quantal release. Results from initial experiments indicated that with different concentrations of Ba and Sr, 2 mM Ba elicited MEPPs at such high frequencies that they were virtually uncountable. Reducing the Ba concentration to 1 mM resulted in MEPP frequencies of approximately 33 MEPPs/sec (Hz). MEPPf in 0.5 mM Ba was similar (0.3-1.8 Hz) to that observed with 2 mM Ca (0.5-2.0 Hz). MEPP frequencies elicited in 4 and 8 mM Sr ranged from 2.4 to 4.0 Hz. MEPPf in 2 mM Sr was similar (0.4-2.0 Hz) to that observed with 2 mM Ca (0.5-2.0 Hz). Therefore 2 mM Sr and 0.5 mM Ba were substituted for 2 mM Ca in the subsequent experiments.

2. Effects of MeHg with Sr and Ba substitution

Under normal conditions (i.e. [Ca]o = 2 mM) MeHg

in concentrations of 20-100 uM, produces a biphasic effect on spontaneous release of ACh as measured by MEPPf: an initial stimulation followed by inhibition (Atchison and Narahashi, 1982; Atchison et al., 1984; Atchison, 1986). To test the specificity of the MeHg-induced biphasic effect on MEPPf for Ca, 2 mM Sr and 0.5 mM Ba were substituted for Ca in these experiments.

Figure 4A depicts the effects of 2 mM Sr and 0.5 mM Ba substitution on the time to peak increase in MEPPf produced by MeHg. With substitution of 2 mM Sr the time to peak effect was 21 $\pm$ 6 min, (mean $\pm$ SEM, n=5) compared to Ca (37 $\pm$ 5 min, n=9); however, this difference was not significant. Ba substitution resulted in a peak MEPPf of 44 $\pm$ 12 min, (n=6), which also was not significantly different from Ca.

In solutions containing 2 mM Ca, 100 uM MeHg increases MEPPf from an average pre-MeHg value of 1.3 MEPPs/sec (Hz) to an average of 65 Hz (Atchison, 1986). Substituting 0.5 mM Ba for Ca had no dramatic effect on the maximal frequency of MEPPs evoked by MeHg (Figure 4B). However, peak MEPPf induced by MeHg in 2 mM Sr was significantly lower than in Ca.

In most experiments, single cell impalements could not be maintained through the entire MeHg perfusion period thus the time course of the biphasic stimulation followed by suppression of spontaneous release could not be quantified further. However, in two experiments each

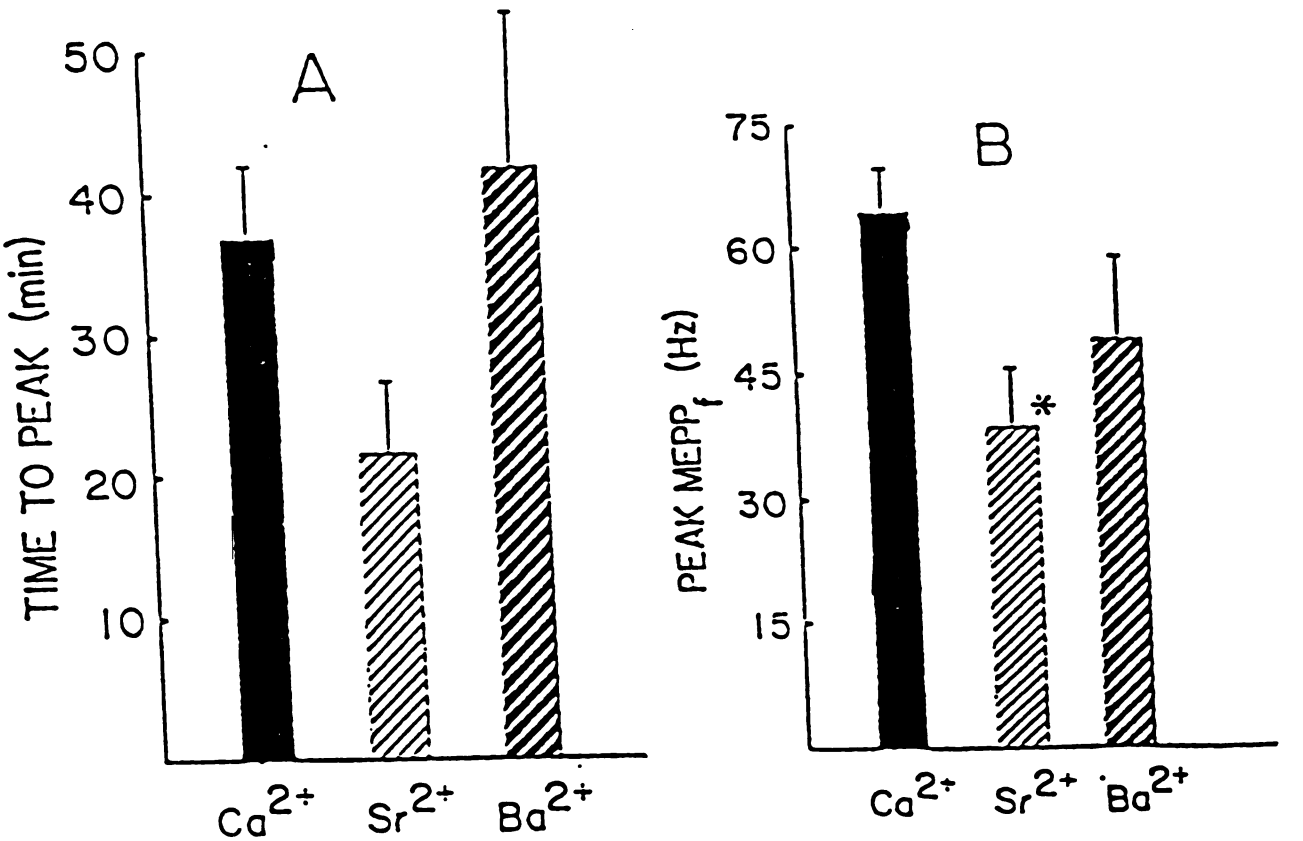

Figure 4. A) Effects of Sr and Ba substitution on the time to peak increase in MEPP frequency induced by MeHg (100 uM). Uncut preparations were treated with solutions containing MeHg in either 2mM Ca, 2mM Sr or 0.5mM Ba. Each preparation was treated with only one divalent cation. B) Effects of Sr and Ba substitution on the peak MEPPf induced by MeHg. Values represent the maximal frequency induced by MeHg. Control frequencies were obtained in the absence of MeHg and were similar for all preparations (0.5-2.0 Hz). Values are the mean (± SEM) of 5-9 preparations. The asterisk (*) indicates results significantly different from those of Ca.

in Sr and Ba in which continuous single cell impalements were maintained, the MeHg-induced stimulation of MEPPf was followed by a gradual inhibition. The inhibition of MEPPf was not reversed by washout of the MeHg with control solutions. Thus, MeHg appears to exert a biphasic effect on spontaneous quantal ACh release in Sr and Ba as well as Ca solutions.

B. Synchronous Evoked Release Experiments

1. Effects of MeHg with increasing [Ca] and [Sr]

MeHg is known to block synchronous evoked release of ACh by presynaptic mechanisms (Atchison and Narahashi, 1982; Atchison et al., 1986) which may include block of Ca influx into the nerve terminal (Atchison et al., 1986).

Sr is capable of supporting synchronous evoked release at the neuromuscular junction, but is less efficacious than Ca (Dodge et al., 1969). Sr mediates synchronous release by entering the nerve terminal through the same channel normally traversed by Ca (Hagiwara and Byerly, 1981; Nachshen and Blaustein, 1982; Augustine and Eckert, 1984). If MeHg blocks evoked release by blocking Ca influx through voltage-sensitive Ca channels (Atchison et al., 1986), then EPPs elicited in Sr should also be blocked by MeHg. In addition, MeHg may influence either Ca or Sr selectively since, as full and partial agonists of synchronous evoked release,

(Silinsky, 1985) Ca and Sr may exert different properties at nerve terminal receptors or binding sites (Michaelson and Avissar, 1979).

As a comparative test of the effects of MeHg on evoked release with Ca and Sr, I repeated part of the study by Atchison et al. (1986). I determined the effects of MeHg with increasing Ca concentrations on the latency to block of the EPP and the EPP amplitude in the cut muscle preparation in the absence of d-tubocurarine. Figure 5 depicts the effects of increasing [Ca]o from 2 to 4 or 8 mM on the latency to block of the EPP. The latency to block in 4 and 8 mM Ca was not significantly different from that in 2 mM Ca, in agreement with results of Atchison et al. (1986). In each case, the EPP was abolished after 8-9 minutes of exposure to MeHg. This latency to block was shorter than that reported by Atchison et al. (1986). MeHg also blocked synchronous release when 2 or 4 mM Sr was substituted for Ca. The latency to block in 2 or 4 mM Sr was not different from Ca; the Sr-supported EPP also was abolished by MeHg in 8-9 minutes (Figure 5).

In 9 of these experiments with Ca and Sr, EPPs were elicited by stimulation of the nerve at 2 or 3 times the threshold voltage. The mean latency to block of the EPP in these experiments (8.3 $\pm$ 0.6 min, n=9) was not significantly different from the latency to block in experiments in which the nerve was stimulated at

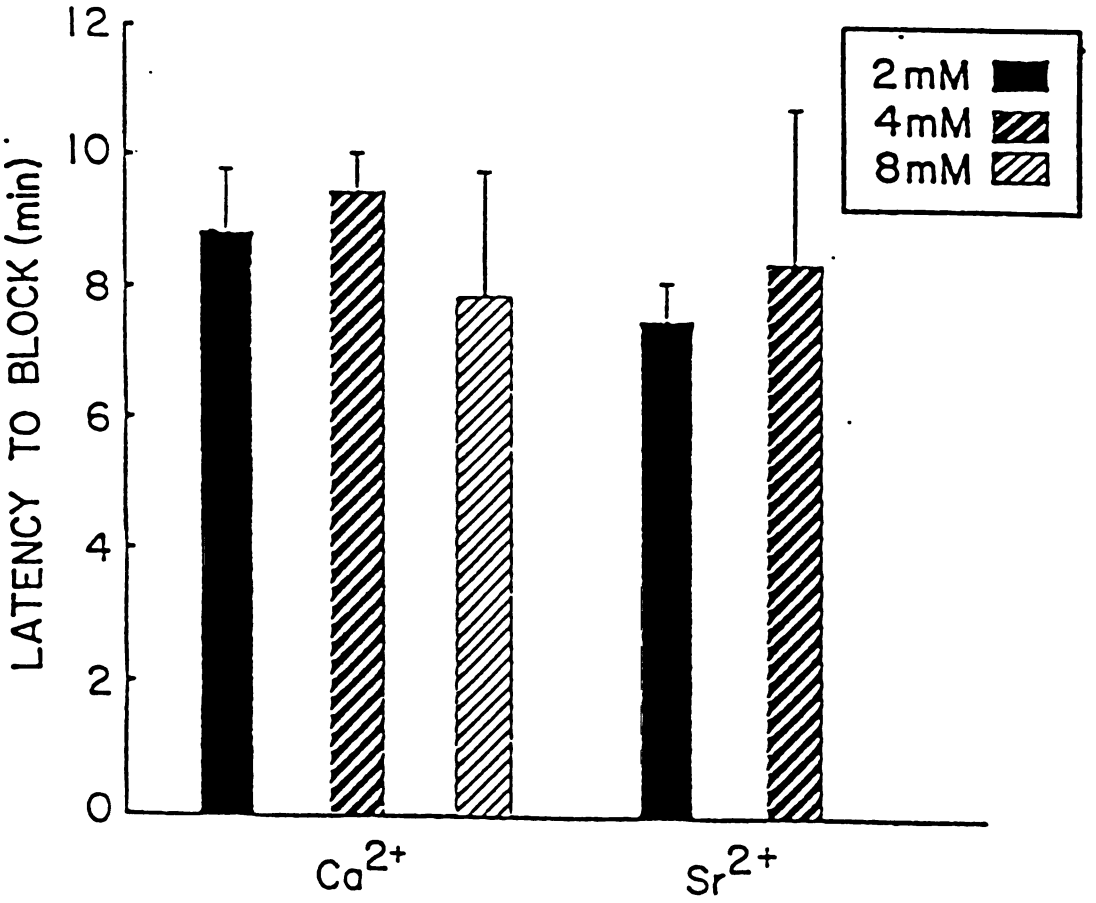

Figure 5. Effects of increased [Ca] amd Sr substitution on the latency to block of the EPP by MeHg. EPPs were recorded continuously (0.25 Hz) in [Ca] of 2, 4, or 8mM or in [Sr] of 2 or 4mM before and during application of MeHg (100 uM) which commenced at time "zero". Values are the mean (± SEM) of 4-9 preparations.

threshold voltage (8.4 ± 0.5 min, n=23).

Atchison et al., (1984, 1986) also reported that the EPP was noticeably reduced in amplitude before complete block occurred in MeHg in curarized preparations. However, in cut muscle preparations, with 2, 4, and 8 mM Ca and 2 and 4 mM Sr, EPP amplitude as a function of time in MeHg did not change significantly from control values. This effect is illustrated in Figure 6 by the pooled data from these experiments. Under all conditions, EPP amplitude remained virtually unchanged from control values until complete block occurred after 8-9 minutes exposure to MeHg.

The latency to block of the EPP with 2 mM Ca plus 20 uM MeHg (48.7 ± 15.1 min, n=3) was not significantly different from that in 0.5 mM Ca plus 20 uM MeHg (43.7 ± 16.1 min, n=3). The mean latency to block in 20 uM MeHg (46.2 ± 11.1 min, n=6) was significantly longer than the mean latency to block in 100 uM MeHg (8.4 ± 0.5 min, n=41) as expected (Atchison et al., 1984). However, as in the previous experiments, the EPP amplitude did not change significantly from control values until complete block occurred after 46 min of exposure to MeHg.

C. Asynchronous Evoked Release Experiments

1. Effects of MeHg on MEPPf decay

Brief, repetitive stimulation produces a

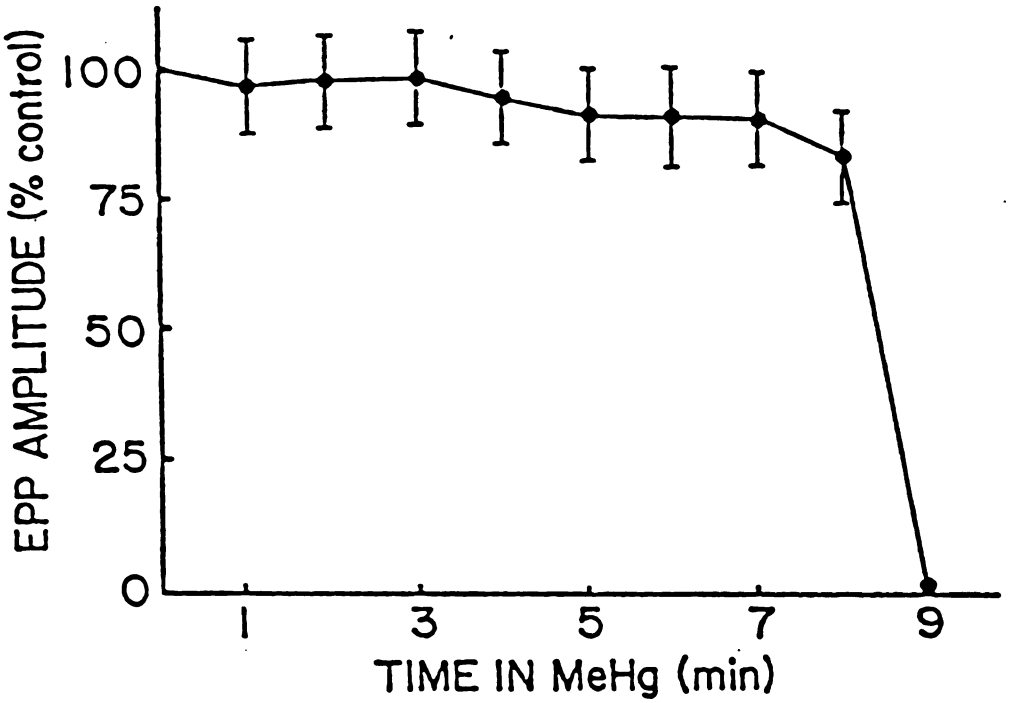

Figure 6. Time course of EPP amplitude in MeHg. In experiments with 2, 4, and 8mM Ca and 2 and 4mM Sr, EPP amplitude as a function of time in MeHg did not change significantly from control values. Data from the experiments were normalized to percent of control amplitude and pooled. Average values from 1 min intervals in MeHg are plotted. Each value is the mean (± SEM) from 31 preparations.

progressive increase in MEPPf (Liley and North, 1953; Miledi and Thies, 1971; Erulkar and Rahamimoff, 1978) which decays gradually with multiple components back to control levels after stimulation (Zengel and Magleby, 1981). The term asynchronous evoked release is used to describe the evoked increases in MEPP frequency following the stimulus train (Silinsky, 1985). The extracellular divalent cation potency sequence for evoked asynchronous release in the frog is Ba> Sr> Ca (Silinsky and Mellow, 1981). Asynchronous evoked release has not been studied systematically at the mammalian NMJ. Clearance of the cations by nerve terminal organelles or membrane processes is generally thought to be responsible for the decay of MEPPf following stimulation (Magleby and Zengel, 1982; Silinsky, 1985) although other Ca-activated factors may be involved as well (Zengel and Magleby, 1982). If MeHg were acting on the intraterminal Ca stores (Atchison, 1986; Levesque and Atchison, 1986) or the Ca channel (Atchison et al., 1986) we might expect to see changes in the decay of MEPPf following repetitive stimulation.

A representative experiment in 0.5 mM Ba showing effects of MeHg on the decay of MEPPf as a function of time after repetitive stimulation is shown in Figure 7. The decay of MEPPf is expressed as Fm(t), the fractional increase in MEPPf over the control; Fm(t)=(fm(t)/fmo) -1 where fm(t) is the MEPPf at time t and fmo is the control

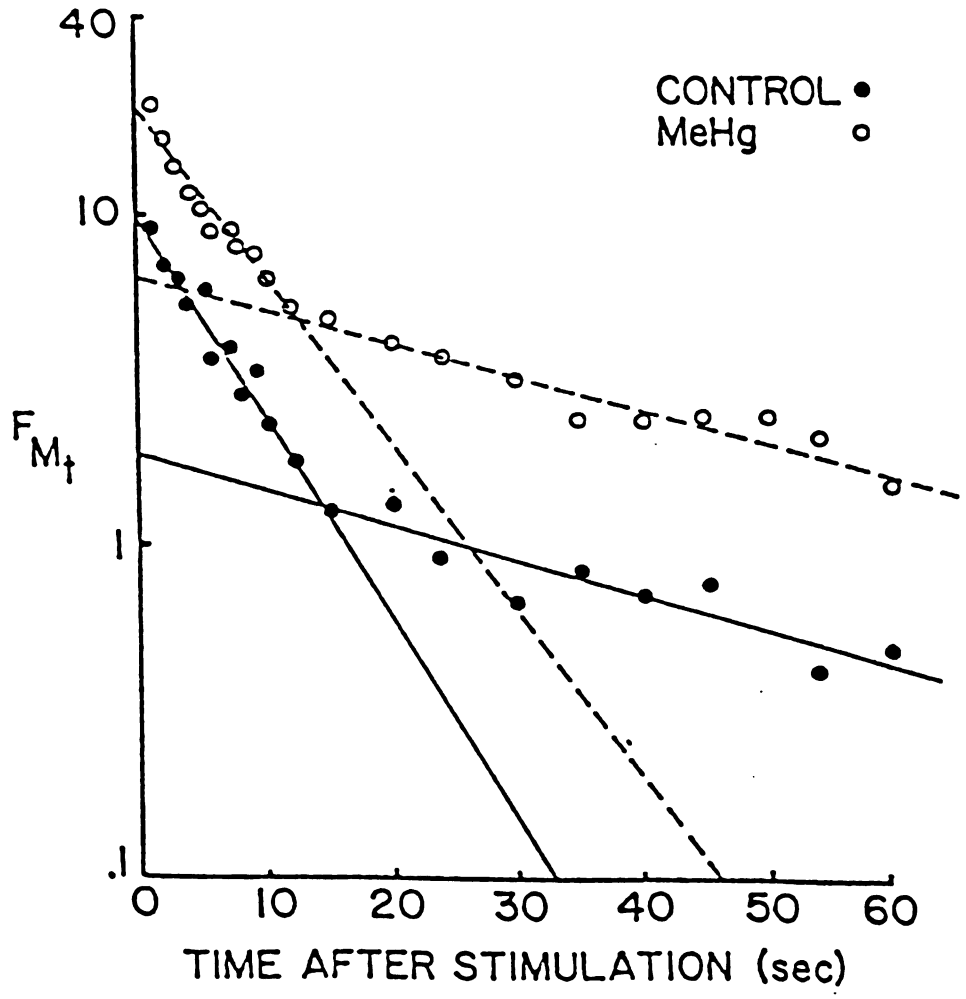

Figure 7. Decay of the fractional increase in MEPPf, Fm(t), after repetitive stimulation from a representative experiment in 0.5mM Ba. Each line is described by the general equation $Fm(t) = e$. Lines were fit by the method of least squares. Each control value is the average of 4 measurements and each MeHg value is the average of 10 measurements from the same cell.

MEPPf (Zengel and Magleby, 1981). Fm(t) was then plotted semilogarithmically against time after stimulation (Figure 7). When the decay of MEPPf is determined in 1 sec intervals two distinct components become apparent. The lines in this figure represent both a fast and slow component of MEPPf decay. In both control and MeHg conditions using 0.5 mM Ba, MEPPf was maximal immediately following stimulation, declined rapidly in the next 15 sec, and then declined more slowly over the next 45 sec. MEPPf in Sr declined rapidly in the first 6 sec after stimulation and then slowly thereafter. Table 1 lists the rate constants of MEPPf decay determined from the slopes of the lines fit by the method of least squares for the fast (Kf) and slow (Ks) phases of decay in both control and MeHg conditions. There were no significant differences between control and MeHg with either the fast or the slow rate constants in either Sr or Ba solutions. Rate constants of MEPPf decay were determined for fast and slow components in only two experiments each in 4 and 8 mM Sr due to the low stability of the preparation with the increased Sr concentrations. In these 4 experiments there were no significant differences between control and MeHg with either the fast or the slow rate constants.

Although the rate constants of MEPPf decay were not significantly different in MeHg, the fractional increases in MEPPf were elevated in MeHg. Figure 8A illustrates a significantly greater fractional increase in MEPPf

TABLE 1

RATE CONSTANTS OF MEPP FREQUENCY DECAY

		Kf	Ks	n
Sr	Control	-0.402 ±0.061	-0.032 ±0.007	5
	MeHg	-0.312 ±0.033	-0.022 ±0.005	
Ba	Control	-0.181 ±0.021	-0.029 ±0.006	6
	MeHg	-0.146 ±0.019	-0.017 ±0.002	

Rate constants were determined from the slopes of the lines fit by the method of least squares to the data from the fast and slow phases of MEPPf decay. Significance was determined using a test for parallelism.

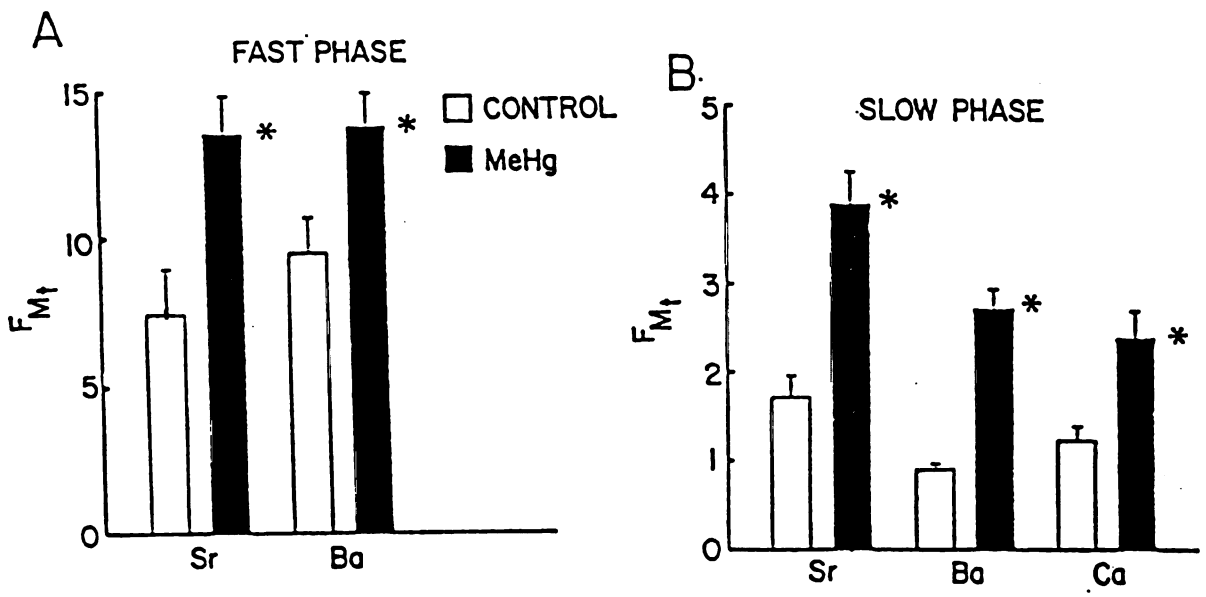

Figure 8. Fractional increases in MEPPf. A) The fractional increase in MEPPf (Fm(t)) during the fast phase of MEPPf decay in Sr and Ba. Values in control and MeHg represent the mean (± SEM) of the average Fm(t) from the first 15 sec after stimulation in 6 preparations with 0.5mM Ba, and average Fm(t) of the first 6 sec after stimulation in 5 preparations with 2mM Sr. B) Fm(t) during the slow phase of MEPPf decay in Sr, Ba, and Ca. Values in control and MeHg represent the mean (± SEM) of the average Fm(t) after the first 15 sec of MEPPf decay in Ba and after the first 6 sec of decay in Sr. Values for control and MeHg in Ca represent the mean (± SEM) of the Fm(t) for the entire 60 sec period after stimulation in 4 preparations. The asterisk (*) indicates results significantly greater than control.

(Fm(t)) with MeHg treatment in both Sr and Ba during the fast phase of MEPPf decay. While Fm(t) during the slow phase of MEPPf decay was much lower than in the fast phase, Fm(t) was still significantly greater in MeHg than in control in both Sr and Ba solutions (Figure 8B). Repetitive stimulation at 40 Hz for 3 sec in Ca, while increasing MEPPf above control levels, failed to produce a consistent pattern of MEPPf decay. Therefore, we were unable to determine rate constants for MEPPf decay in Ca. However, Fm(t) was elevated significantly in MeHg throughout the entire 60 sec period after stimulation (Figure 8B).

2. Mean quantal content determination

Figure 9 depicts mean quantal content (m) as a function of time in MeHg in 2 mM Ca and 2 and 4 mM Sr. Although m decreases over time in MeHg, it does not become significantly different from control until after approximately 8 min of exposure to MeHg. EPP amplitude does not change significantly from control over time in MeHg with either 2 mM Ca or 2, 4, or 8 mM Sr. However, MEPP amplitude increases significantly over control as a function of time in MeHg (Table 2). m is determined using the equation m = EPPn/MEPPm where EPPn and MEPPm represent the mean EPP and MEPP amplitudes respectively. Since the mean EPP amplitude does not change significantly in MeHg, the decreases in m are mainly due to the significant increases in mean MEPP amplitude in

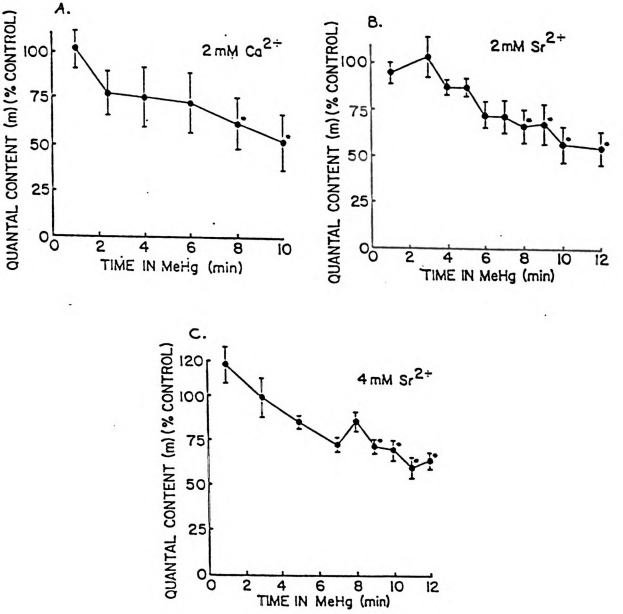

Figure 9. Mean quantal content in MeHg. Effects of increasing time of exposure to MeHg on mean quantal content (m) in 2mM Ca (A), 2mM Sr (B), and 4mM Sr (C). m values were determined using the mean value method. Data from the experiments were normalized to percent of control m value. Average values from 1 or 2 min intervals in MeHg are plotted. Values are the mean (±SEM) of 4-6 preparations. The asterisk (*) indicates results significantly different from control.

Table 2

MEPP AMPLITUDES

	2mM Ca	2mM Sr	4mM Sr
Control	0.240 ± 0.045	0.179 ± 0.022	0.121 ± 0.032
MeHg	0.211 ± 0.049	0.203 ± 0.031	0.102 ± 0.031
	0.226 ± 0.041	0.183 ± 0.012	0.118 ± 0.021
	0.250 ± 0.039	0.199 ± 0.023	0.137 ± 0.040
	0.267 ± 0.023	0.193 ± 0.019	0.156 ± 0.048
	0.296 ± 0.033	0.208 ± 0.029	0.154 ± 0.044
	0.332 ± 0.041*	0.201 ± 0.030	0.161 ± 0.046
	0.326 ± 0.035*	0.221 ± 0.034	0.164 ± 0.046*
		0.234 ± 0.034*	0.193 ± 0.056*
		0.279 ± 0.044*	0.194 ± 0.059*
		0.286 ± 0.055*	

MEPP amplitudes (mV) from asynchronous evoked release experiments in 2mM Ca (n=4), 2mM Sr (n=6), and 4mM Sr (n=5). Values represent the mean (± SEM) of the average MEPP amplitudes from all experiments determined after repetitive stimulation from four 1 min intervals in control and from each 1 min interval in MeHg.

MeHg.

3. Block of asynchronous evoked release

As was the case with block of the EPP in the synchronous evoked release experiments, asynchronous evoked release was also eventually blocked by MeHg. This block was evidenced by complete cessation of the repetitively-evoked EPPs in Ca and Sr, and failure of the repetitive stimulation to elicit an increase in MEPPf in Ba as well as in Ca and Sr solutions. With all three cations, block of both the EPP and asynchronous evoked MEPPs occurred after 12 min of exposure to MeHg. The mean latency to block of asynchronous evoked release was significantly longer (12.0 $\pm$ 0.8 min, n=18) than the mean latency to block (8.4 $\pm$ 0.5 min, n=41) of synchronous evoked release.

When EPPs are evoked in Ca or Sr plus MeHg at a frequency of 0.25 Hz intermittent failures do not occur; EPPs are elicited faithfully following every stimulus until sudden and complete failure occurs after 8-9 min in MeHg. In contrast, during the asynchronous evoked release experiments (40 Hz, 3 sec) intermittent failures appeared during stimulation in MeHg before complete block occurred after 12 min (Figure 10) while no EPP failure occurred during the control trains in Ca or Sr. Intermittent failures occurred in MeHg in 9 of the 10 experiments using either Ca or Sr. In 5 of these experiments there was a trend toward increasing numbers

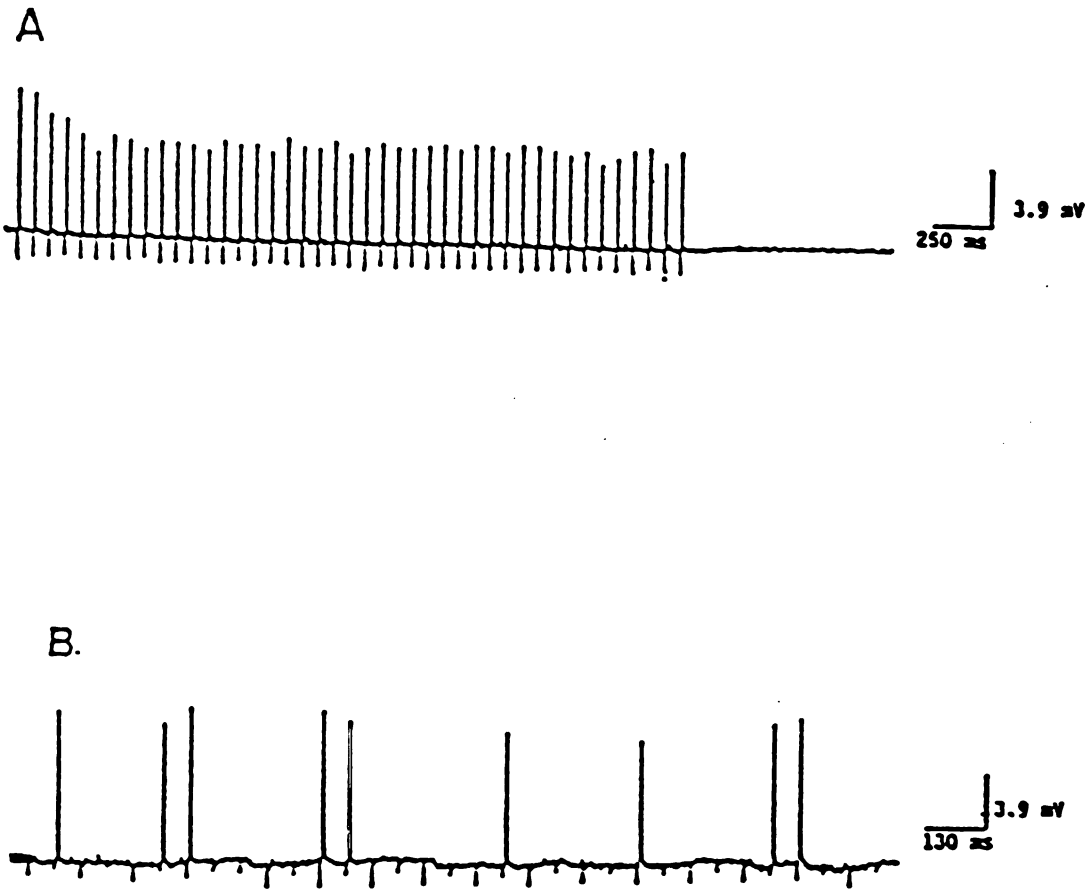

Figure 10. EPPs evoked from a cut muscle fiber during repetitive stimulation at 40 Hz. A) EPPs with 2mM Ca in the absence of MeHg. B) EPPs and failures with 2mM Ca plus 100uM MeHg.

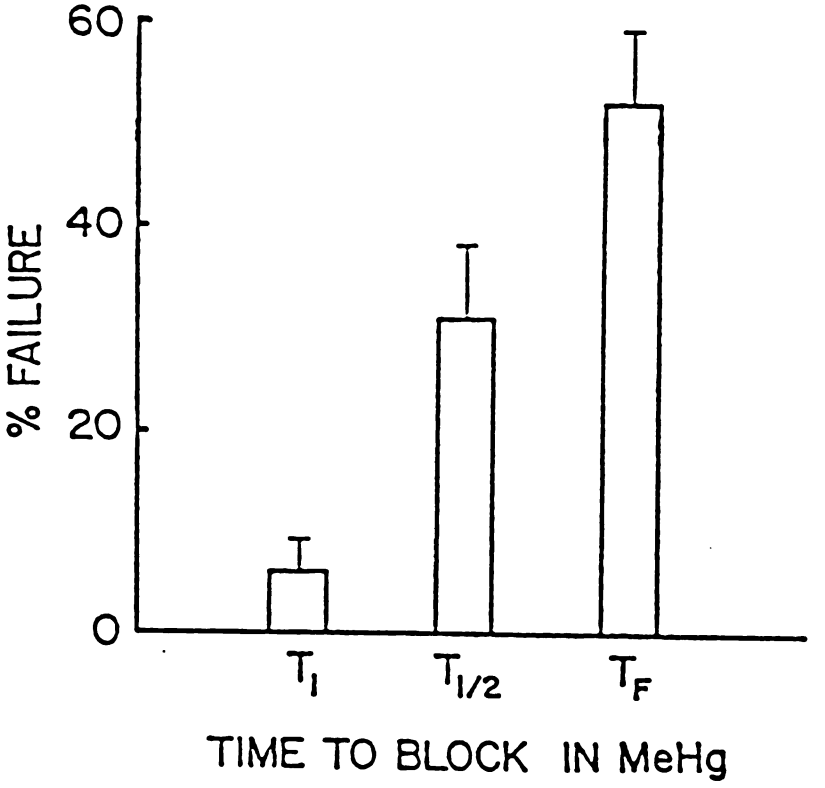

Figure 11. Effects of increasing time of exposure to MeHg on the number of EPP failures during repetitive stimulation. Each value is the mean (± SEM) of the percent of failures per total stimuli from 5 experiments. T1 = the first minute of exposure to MeHg; T1/2 = the time in MeHg halfway to complete block; Tf = the time immediately preceding complete block of asynchronous release by MeHg.

of failures with increasing time of exposure to MeHg (Figure 11). This trend was not due to the effect of repeated stimulation over time; preparations stimulated with 40 Hz, 3 sec trains at 1 min intervals in the absence of MeHg did not exhibit failures even after 30 min of stimulation.

D. Reversal Attempts

Block of synchronous evoked release by MeHg cannot be reversed by washing preparations with MeHg-free solutions (Atchison, 1982; Atchison et al., 1986). As a comparative test with the cut muscle, reversal of EPP block was attempted by washout of MeHg with control solutions. In addition, MeHg was washed out with increased concentrations of Ca and Sr and with 4-AP in an attempt to overcome a possible MeHg-induced block of Ca influx. Washout of MeHg using control solutions (n=5), 4 mM Ca or Sr (n=6), or 50 (n=5) and 100 uM (n=5) 4-AP failed to reverse the block of synchronous evoked release in 20 out of 21 experiments (Figure 12). However, increasing the intensity and duration of the stimulus applied to the nerve at the time of EPP block was successful in reversing MeHg-induced block. After washout of MeHg with 4 mM Ca or Sr, or 4-AP, increasing the intensity and duration of stimulation reversed the block in 70% of the preparations tested (7/10) with 4 mM Ca or Sr washout and in 36% of the preparations tested

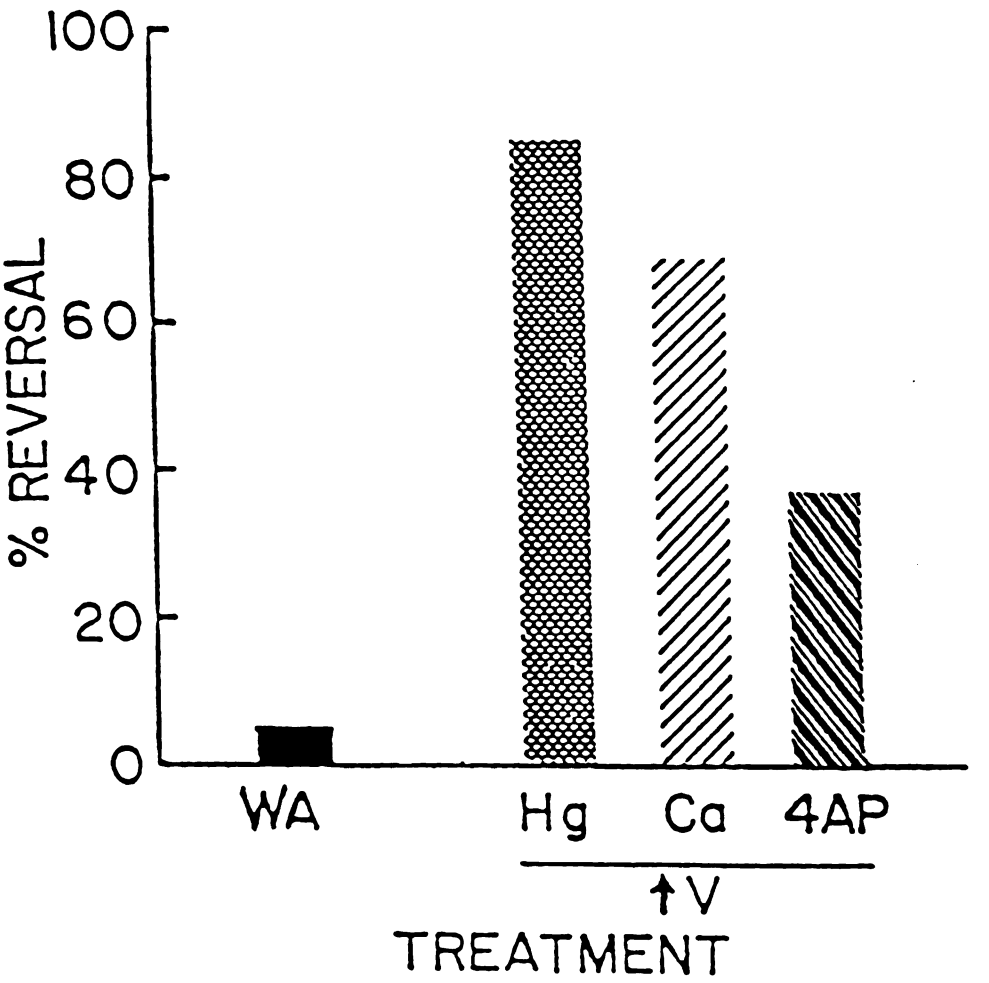

Figure 12. Reversal of MeHg-induced block. Each value represents the percent of reversals per total number of attempts under each treatment condition. WA = washout of MeHg with control solutions, 4 mM Ca or Sr, or 4-aminopyridine. Increasing intensity and duration of nerve stimulation following EPP block in MeHg (Hg) and in 4 mM Ca of Sr (Ca) and 4-aminopyridine (4AP) after washout of MeHg.

(4/11) with 4-AP washout (Figure 12). In six of these experiments in which block of the EPP was reversed by increasing the stimulus intensity and duration, a subsequent second exposure to MeHg resulted in block of the EPP within approximately 3 minutes. Interestingly, block of the EPP at this point was reversed in 2 out of 2 experiments in which MeHg was washed out with 4 mM Ca and in 2 out of 4 experiments in which MeHg was washed out with 100 uM 4-AP. In 3 experiments, washout with Sr was not successful in reversing the EPP block after a second MeHg exposure. When MeHg was not washed out, in 85% of the preparations tested (11/13), EPPs could again be elicited from MeHg-blocked preparations merely by increasing the intensity and duration of stimulation, despite the continued presence of MeHg (Figure 12). Following reversal of transmission block, continued exposure to MeHg resulted in a subsequent second block of the EPP within approximately 3 min.

DISCUSSION

Results of the present studies suggest four main conclusions regarding the effects of MeHg on transmitter release at the neuromuscular junction. First, the MeHg-induced biphasic effect on spontaneous release of ACh is not Ca-specific; MeHg produces almost identical effects when Sr or Ba are substituted for Ca. Second, the mechanism of block of synchronous and asynchronous evoked release of ACh observed with MeHg may be due to block of impulse propagation or noncompetitive, irreversible block of divalent cation entry. Third, in the time course of these experiments, the processes involved in the decay of MEPP frequency following repetitive stimulation are not altered by MeHg. Fourth, MeHg-induced block of evoked transmitter release can be overcome by increasing the intensity of nerve stimulation.

A. Effect of MeHg on Spontaneous Release

In agreement with previous studies (Elmqvist and Feldman, 1965; Anwyl et al., 1982), Ba and Sr were found to be capable of supporting spontaneous quantal release of ACh at the rat NMJ in the absence of Ca. The MEPP frequencies obtained in my experiments with 1 or 2mM Ba substitution were much higher than those reported by

Elmqvist and Feldman (1965). One explanation for this difference may be that the preparations used by Elmqvist and Feldman were pretreated for 3 hours in Ca-free solutions containing EDTA, a divalent cation chelator, before recording in Ba. In my studies, muscle membrane potentials fell rapidly with 1 or 2 mM Ba substitution making stable cell impalements difficult. This depolarization was likely due to a direct effect of Ba to decrease the potassium conductance of the muscle fiber (Silinsky, 1978). Reducing the Ba concentration to 0.5mM resulted in MEPP frequencies comparable to those obtained with 2mM Ca. In addition, the preparations bathed in 0.5 mM Ba exhibited stable muscle resting potentials. Thus single cell impalements could be maintained for long periods. Obtaining similar control MEPP frequencies in Sr, Ba, and Ca allowed for comparison of peak MEPP frequencies obtained in Sr, Ba, and Ca with MeHg treatment.

Normal spontaneous release of transmitter, recorded electrophysiologically as MEPPs, presumably occurs as a result of transient increases in free intracellular Ca (Llinas and Nicholson, 1975). In the absence of nerve impulses, the rise in free intraterminal Ca is presumed to be due to release of bound Ca from intracellular stores such as the mitochondria and smooth endoplasmic reticulum (SER) (Blaustein et al., 1978; 1980). Sr and Ba can also support spontaneous release of ACh in the

absence of Ca at the neuromuscular junction, presumably via the same mechanism as Ca (Elmqvist and Feldman, 1965; Meiri and Rahamimoff, 1971). Substitution of Sr or Ba for Ca indicates that the MeHg-induced increase in spontaneous quantal release of ACh occurs irrespective of the cation used. Moreover, the time to peak increases in spontaneous release does not differ among the cations. Both Sr and Ba can be taken up and sequestered by intraterminal organelles such as the mitochondrion and SER (Lehninger et al., 1970; Vainio et al., 1970; Ornberg and Reese, 1980; Rasgado-Flores et al., 1982), and also may bind to cytoplasmic Ca-binding proteins in the terminal which normally bind Ca (Blaustein et al., 1978). The increase in spontaneous release produced at the NMJ by MeHg does not require the presence of extracellular Ca (Atchison, 1986). The implication of this is that MeHg increases intracellular Ca by inducing its release from bound intracellular stores or can itself support release. The same mechanism of action of MeHg may be responsible for the results obtained in the experiments with Sr and Ba substitution. A rise in free intracellular divalent cation via MeHg-induced release of divalent from bound intracellular stores could result in the increases in spontaneous release of ACh observed in these experiments.

Since control MEPP frequencies in 2 mM Sr were similar to those observed with 2 mM Ca I expected to see

identical peak MEPP frequencies in Sr and Ca with MeHg treatment. Indeed, an apparent non-selectivity of the spontaneous release process has been demonstrated at the frog NMJ. When liposomes prepared from equal concentrations of Ca snd Sr are delivered to the nerve terminal cytoplasm they increase MEPPf to similar levels (Mellow et al., 1982). However, I found that peak MEPPf in MeHg with Sr was significantly lower than in MeHg plus Ca. It is possible that less Sr is released from intracellular stores by MeHg. Coelho and Vercesi (1980) and Saris and Bernardi (1983) have shown that while Sr is readily transported into mitochondria by the Ca uniporter, Sr is unable to exit the mitochondria through the phosphate-induced Ca efflux pathway. In addition, Sr may inhibit the efflux of Ca through this pathway as well. If MeHg were acting to induce release of divalent cations from mitochondria, lower peak MEPPf in Sr plus MeHg would be expected. Other investigators (Beatrice et al., 1980; Palmer and Pfeiffer, 1981) have shown that certain agents can induce release of Ca from mitochondria by a mechanism which requires the activity of an intramitochondrial phospholipase A2. The affinity of phospholipase A2 is higher for Ca than Sr. Lower peak MEPPf in Sr plus MeHg might also occur if MeHg were acting via a phospholipase mechanism.

Alternatively, the lower peak MEPPf induced by MeHg with Sr may be due to a smaller bound store of Sr,

although this possibility is unlikely (Rasgado-Flores et al., 1982; Ornberg and Reese, 1980). The range of peak MEPP frequencies induced by MeHg in conjunction with Sr does include values equal to those observed with the combination of MeHg and Ca. Thus the significance of the difference between Sr and Ca in inducing peak MEPPf with MeHg is unclear.

A second action of MeHg on spontaneous release is complete cessation of spontaneous transmitter release which follows the initial stimulation. This suppression also appeared to occur in my experiments with Sr and Ba. Although the mechanism responsible for this depression is unknown it has been shown that it is not due to depletion of releasable transmitter stores nor to decreased sensitivity of the postjunctional receptor to ACh (Atchison and Narahashi, 1982; Atchison et al., 1984; Atchison, 1986). Thus, the general pattern of effects, time course, and for the most part, the maximal effect of MeHg occurs irrespective of the divalent cation used to support release.

Results from the studies of asynchronous evoked release support the hypothesis that MeHg releases Ca from bound intracellular stores as opposed to merely preventing its uptake via buffering systems. Repetitive electrical stimulation in Ba and Sr results in elevated MEPP frequencies which decay slowly after the cessation of nerve stimulation (Silinsky and Mellow, 1981). MEPP

frequencies recorded after repetitive stimulation in Ba are greater than those recorded in equimolar Sr or Ca and the decay of MEPPf in Ba is slower than in Sr and Ca. The slower decay of MEPPf in Ba has been mainly attributed to a slower clearance of the cation from the regions associated with release (Silinsky, 1978). Ba is reported to be less well buffered in the axon terminal than either Sr or Ca (Tillotson and Gorman, 1983; Rasgado-Flores, et al., 1982). However, in my experiments the one min interval between stimuli appeared to be a sufficient amount of time for the 0.5 mM Ba to be buffered since MEPPf decayed to near control levels near the end of this period. If MeHg were altering one of the divalent cation buffering mechanisms then the rate constants for the decay of MEPPf should have been altered. However, there were no significant differences between the control and MeHg rate constants indicating that the processes responsible for the decay of asynchronous evoked release (clearance of the cations by mitochondria, SER, or membrane exchangers, etc. Silinsky, 1985) were not altered by MeHg. Since we were limited in these experiments by the time restriction imposed by the MeHg-induced block of asynchronous release, it is possible that MeHg does block one or more of the buffering processes at a later time, but we were not able to observe this effect. However, this seems unlikely since Fm(t) was already significantly elevated

in MeHg before block of asynchronous evoked release occurred. During this period of elevated Fm(t) the rate constants were not different, suggesting that MeHg was causing release of cation but not blocking its uptake. Perhaps more importantly, in six experiments the peak MEPPf induced by MeHg occurred before block of asynchronous evoked release. The rate constants obtained in MeHg in these experiments also were not significantly different from control.

B. Effects of MeHg on Synchronous and Asynchronous Evoked Release

Synchronous and asynchronous evoked release could be blocked by action of MeHg at a number of sites at the NMJ. Possible mechanisms of action of MeHg to block evoked release include 1) a postsynaptic blocking effect, 2) block of the release process itself, 3) block of Ca influx into the terminal, 4) depolarization of the nerve terminal membrane and 5) axonal conduction block.

1. Postsynaptic effects

Sulfhydryl groups and disulfide bonds are thought to be involved in cholinergic receptor activation (del Castillo et al., 1971; Ben-Haim et al., 1973; Karlin, 1980) and in the activity of transmitter-activated ionic channels found in the postsynaptic endplate regions (del Castillo et al., 1971). MeHg exhibits a high affinity

for sulfhydryl groups (Huneeus-Cox et al., 1966). Indeed MeHg has been shown to decrease the binding of cholinergic agonists to nicotinic receptors (Eldefrawi et al., 1977). Thus it is conceivable that MeHg would exert postsynaptic effects. However, results from previous studies (Atchison, 1982; Atchison et al., 1984) indicate that it is unlikely that postjunctional actions contribute significantly to the MeHg-induced block of evoked release. Response of the muscle endplates to iontophoretically-applied ACh at the time of EPP block was not different from the pre-MeHg response. Also MEPPs could still be observed at the time of EPP block, and their amplitude was unchanged from control values (Atchison et al., 1984). MEPPs were also observed at the time of EPP block in my experiments. These results suggest that during the time course of my experiments the postjunctional sensitivity to ACh was not altered significantly by MeHg.

2. Effects on the release process

Results from the mean quantal content determination would seem to indicate that MeHg is acting on the release process itself. The value of **m**, or the number of quanta liberated in response to a nerve impulse, is reduced significantly immediately before complete block of asynchronous evoked release. The values of **m** were derived by using the mean value method

(del Castillo and Katz, 1954), dividing the mean EPP amplitude by the mean MEPP amplitude. The MEPP is equal to the basic quantum of ACh, and an increase in MEPP amplitude might indicate an increase in the postsynaptic sensitivity to ACh or an increase in the amount of ACh in a quantum. Since the EPP is made up of quantal units and assuming that each quantum contributes equally to the EPP amplitude, an increase in the postsynaptic sensitivity to ACh or an increased quantal size should be reflected as an increased EPP amplitude as well. In my experiments, however, EPP amplitude did not change significantly in MeHg while MEPP amplitude was increased immediately before block of the EPP. It is possible that the mean MEPP amplitude was artificially elevated. MEPP frequency in a number of experiments was very high before complete block occurred resulting in the occurrence of many summed or multiquantal responses, recorded electrophysiologically as large amplitude MEPPs. Thus m values determined in these experiments may not be an accurate indicator of MeHg's effect on the release process.

MeHg could also block the release process through an effect on protein phosphorylation, which has been linked to transmitter release (Michaelson and Avissar, 1979). If MeHg were acting to block protein phosphorylation then the latency to block of asynchronous evoked release should have been different in Ca, Sr, and Ba since Sr is

less effective than Ca in the protein phosphorylation process (Michaelson and Avissar, 1979) while Ba is a very weak or ineffective stimulator of this process (Hoch and Wilson, 1984). However, latency to block was the same in Ca, Sr, and Ba in my experiments suggesting that a direct effect of MeHg on protein phosphorylation was not responsible for the MeHg -induced block of evoked release. The release process, however, does involve more than just simple protein phosphorylation. The possibility that MeHg may be acting to depress transmitter release at another site in the complex release process cannot be ruled out.

3. Block of Ca influx

Synchronous evoked release of neurotransmitter requires the influx of Ca from the extracellular milieu following the invasion of the presynaptic nerve terminal by an action potential (Katz and Miledi, 1967). Recent radiotracer flux analyses (Atchison et al., 1986) suggested that MeHg caused an irreversible, noncompetitive block of Ca influx at central nervous system nerve terminals. Electrophysiological data obtained from the mammalian NMJ provided a tenable functional correlate for this irreversible block of Ca influx (Atchison et al., 1986). The results with the cut muscle preparation on latency to block of the EPP with increasing [Ca] and [Sr] substitution would appear to

support the hypothesis that MeHg blocks synchronous evoked release by irreversibly and noncompetitively blocking Ca influx. The latency to block of asynchronous evoked release by MeHg was not different in Ca, Sr, or Ba. This result suggests a common mechanism of action of MeHg to block asynchronous evoked release in Ca, Sr, and Ba such as block of divalent cation influx into the nerve terminal. Given the longer period of stimulation in the asynchronous evoked release experiments, if MeHg were acting to block influx of divalent cation by irreversibly binding to voltage-activated Ca channels then it is reasonable to assume that block of asynchronous evoked release would occur in a shorter period of time than block of synchronous evoked release. If the action of MeHg were not dependent on binding to the Ca channel in the open or activated state then the latency to block of asynchronous and synchronous evoked release should be the same. However, it is puzzling that the mean latency to the block of asynchronous evoked release was actually longer than latency to the block of synchronous evoked release.

Block of evoked release appears to be irreversible and noncompetitive since washout of MeHg with increased concentrations of Ca or Sr, which might overcome a competitive block of divalent cation influx, failed to reverse the MeHg-induced block of the EPP. Similarly 4-AP, which has been shown to increase evoked transmitter

release in Ca or Sr (Molgo et al., 1982) and which has been used to antagonize block of neuromuscular transmission following Botulinum toxin poisoning (Lundh, et al., 1977) also failed to reverse the EPP block. 4-AP blocks the potassium conductance in the nerve terminal and thereby enhances the influx of Sr or Ca into the nerve terminal by prolonging the depolarization phase of the action potential. Moreover, 4-AP has been suggested to increase Ca influx by direct enhancement of nerve terminal Ca permeability (Kus and Glavinovic, 1984). Thus failure of 4-AP and increased Ca and Sr solutions to reverse EPP block indicates that MeHg-induced block of evoked release is not reversible by methods designed to overcome a competitive block of cation influx by MeHg.

4. Nerve terminal depolarization

Other explanations for the results from synchronous and asynchronous evoked release experiments are possible. MeHg has been shown to cause changes in membrane permeability (Passow and Rothstein, 1960) and leakage conductance (Shrivastav et al., 1976) effects which could lead to changes in resting membrane potential. The effects of MeHg on evoked release are consistent with the hypothesis that MeHg acts to depolarize the presynaptic nerve terminal. While the latency to block in the synchronous evoked release experiments was the same under all conditions, the mean

latency to block in the experiments was shorter than that reported previously (Atchison et al., 1986). In the cut muscle preparation the muscle fibers are partially depolarized from normal values of -75 mV to approximately -45 mV. Given the greater surface area to volume ratio of the nerve terminal compared to the postjunctional muscle cell, it is reasonable to assume that the terminal is partially depolarized as well. A depolarizing action of MeHg coupled with an initially depolarized nerve terminal could explain the shorter time to block of the EPP observed in this study. A gradual depolarization of the nerve terminal, however, should be reflected in a parallel gradual decrease in EPP amplitude. This effect on EPP amplitude did not occur in the synchronous or asynchronous evoked release experiments. In addition, MeHg-induced nerve terminal depolarization might be expected to cause a decrease in MEPP frequency following repetitive stimulation with increasing time of exposure to MeHg, and perhaps a shorter latency to block of asynchronous evoked release. However, neither of these results were observed suggesting that depolarization of the nerve terminal was not occuring.

5. Conduction block

The intermittent failures of the EPP during the stimulus train in the asynchronous experiments also suggest an alternative mechanism of action of MeHg. The

failures occurred equally in Sr and Ca yet other EPPs evoked in the same train were not reduced in amplitude. This effect and the sudden block of the EPP with no amplitude reduction in the synchronous evoked release experiments would be more consistent with conduction block of the nerve as opposed to block of Ca influx into the nerve terminal or nerve terminal depolarization (Krnjevic and Miledi, 1958; 1959; Heggli and Röed, 1981). It has been shown that a conformational change involving axonal membrane proteins and their sulfhydryl groups takes place during propagation of the nerve impulse (Huneeus-Cox et al., 1966; Marquis and Mautner, 1974). Sulfhydryl reagents, including mercury compounds, can cause conduction block in a variety of preparations such as squid giant axons (Marquis and Mautner, 1974; Shrivastav et al., 1976), frog nerve fibers (Smith, 1958), and N1E-115 neuroblastoma cells (Quandt et al., 1983). Block of evoked release by MeHg could be due to interaction of MeHg with sulfhydryl groups in the phrenic nerve axonal membrane resulting in conduction block. MeHg could also be causing conduction block by inducing changes in permeability at the nerve terminal. Indeed block of impulse propagation has been shown to occur at nonmyelinated nerve endings at which the safety factor for conduction may be inherently low making this area more susceptible to block (Hatt and Smith, 1976). N-ethylmaleimide (NEM), a sulfhydryl group blocking agent,

has been shown to exert effects similar to MeHg on synaptic transmission at the rat NMJ (Röed, 1974). NEM causes a sudden block of evoked EPPs and a marked increase in spontaneous MEPPf. Block of the EPP was attributed to axonal conduction block due to a NEM-induced decrease in excitability of the nerve. The block could be antagonized by increasing the stimulating voltage applied to the phrenic nerve. Thus reversal of MeHg-induced block with increased nerve stimulation (both stimulus duration and intensity) suggests that block could be caused by a MeHg-induced decrease in excitability of the nerve terminal.

Reversal of the EPP block by washing with Ca and 4-AP after a second MeHg exposure suggests that there may be a more complex action of MeHg involving numerous sites at the presynaptic nerve terminal. While this suggestion is plausible the evidence supports MeHg-induced conduction block as an initial mechanism of action in MeHg-induced block of evoked release.

C. Summary

While these results do not illustrate a definitive mechanism of action of MeHg, they do provide new and useful information supporting existing hypotheses regarding the action of MeHg.

In conclusion, in studying the effects of MeHg on spontaneous release, when Sr and Ba are substituted for

Ca the general pattern of effects of MeHg to stimulate and then inhibit spontaneous release, the time to peak increase, and for the most part, the maximal effect of MeHg on spontaneous release occurs irrespective of the divalent cation used to support spontaneous release. Thus the effects of MeHg on spontaneous release are not Ca-specific. In addition, since the rate constants of MEPPf decay after repetitive stimulation were not altered by MeHg, the MeHg-induced increase in spontaneous release is more consistent with MeHg-induced release of divalent cation from bound intracellular stores as opposed to block of cation uptake by intraterminal buffering systems.

Block of synchronous and asynchronous evoked release by MeHg occurs with both Ca and Sr serving as the divalent cation supporting release. Block of evoked release occurs suddenly and without decrement of EPP amplitude, leading to the conclusion that the predominant mechanism of action of MeHg appears to be MeHg-induced block of impulse propagation into the nerve terminal. However, other mechanisms of action cannot be definitively ruled out. The MeHg-induced block of evoked release has been shown to be reversible by increasing the intensity and duration of nerve stimulation.

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