RELATIONSHIP BETWEEN METHYLMERCURY-INDUCED DISRUPTION OF INTRACELLULAR CALCIUM AND NEURONAL DEATH

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ABSTRACT

RELATIONSHIP BETWEEN METHYLMERCURY-INDUCED DISRUPTION OF INTRACELLULAR CALCIUM AND NEURONAL DEATH

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The mechanism by which the environmental neurotoxicant methylmercury (MeHg) causes elevations of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and subsequent neuronal death was examined using single cell microfluorimetry of rat cerebellar granule neurons in primary culture. In granule cells loaded with fura-2 to monitor changes in $[Ca^{2+}]_i$, 0.2-1.0 μM MeHg causes early release of Ca^{2+} from at least one intracellular source; the possible sources examined were the smooth endoplasmic reticulum (SER) and the mitochondria. The non-specific muscarinic acetylcholine (ACh) receptor antagonist atropine delayed MeHg-induced elevations of $[Ca^{2+}]_i$, while down-regulation of the muscarinic receptors and the inositol-1,4,5-triphosphate (IP_3) receptor with 24 hr application of the muscarinic agonist bethanechol decreased the amplitude of the MeHg-induced release of $Ca^{2+}{}_i$ by approximately 30-40%. Depletion of SER Ca^{2+} stores with thapsigargin also reduced the amplitude of the MeHg-induced release of $Ca^{2+}{}_i$ by approximately 30-40%. Inhibition of Ca^{2+} release through the ryanodine receptors had minimal effect. Removal of mitochondrial Ca^{2+} ($Ca^{2+}{}_m$) content prior to MeHg exposure using carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and oligomycin decreased the amplitude of the MeHg-induced release of $Ca^{2+}{}_i$ by approximately 70%. Additionally, inhibition of the mitochondrial permeability transition pore (MTP) using cyclosporin A (CsA) delayed the increase in $[Ca^{2+}]_i$. In granule cells loaded with tetramethylrhodamine ethyl ester

(TMRE) to monitor changes in mitochondrial membrane potential, CsA delayed the irreversible loss of membrane potential caused by 0.5 μM MeHg. In granule cells loaded with rhod-2 to monitor changes in $[Ca^{2+}]_m$, MeHg caused an early increase in $[Ca^{2+}]_m$, followed several minutes later by release of dye from the mitochondria into the cytosol. The initial increase in $[Ca^{2+}]_m$ occurred independently of extracellular Ca^{2+}, while the release of dye from the mitochondria was delayed by prior application of thapsigargin or CsA. Use of a calcein AM-ethidium homodimer cell viability assay revealed that increasing concentrations of MeHg (0.2-1.0 μM) caused a corresponding increase in cell death at 24 hr post-exposure. Atropine and ryanodine did not protect against MeHg-induced cell death, while 24 hr BCh pretreatment significantly protected against cell killing. The BCh-mediated protection was reversed by atropine and the M3 muscarinic ACh receptor antagonist 4-diphenylacetoxyl-N-methylpiperidine methiodide (4-DAMP) but not by the M2 receptor antagonist methoctramine or the nicotinic ACh receptor antagonist dihydro-β-erythroidine hydrobromide (DHE). Thapsigargin itself was toxic, highlighting the sensitivity of granule cells to disruption of SER Ca^{2+}. CsA also provided significant protection against cell death at 24 hr post-MeHg exposure. These results suggest that MeHg: 1) acts at M3 muscarinic ACh receptors to cause production of IP_3, which releases Ca^{2+} from the SER and 2) causes Ca^{2+} uptake into mitochondria which is then released into the cytosol *via* opening of the MTP. Additionally, these disruptions of $[Ca^{2+}]_i$ contribute to MeHg-induced neuronal death, and may underlie the specific neurotoxicity of MeHg within granule neurons of the cerebellar cortex.

For the family and friends who never gave up hope, and passed that hope to me.

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PREFACE

Some of the data contained in this dissertation has been published previously. Portions of Chapter 5 appeared as Limke and Atchison (2002).

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LIST OF SYMBOLS AND ABBREVIATIONS

Ψ_m – inner mitochondrial membrane potential

ACh – acetylcholine

AMPA - alpha-amino-3-hydroxy-5-methyl-5-isoxazole propionate

ANOVA – analysis of variance

Ara-C – cytosine-β-arabinofuranoside

ATP – adenosine 5'-triphosphate

BAPTA – 1,2- bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl)ester

BCh – carbamyl-β-methylcholine chloride (bethanechol)

Calcein AM – calcein acetoxymethylester

$[Ca^{2+}]_e$ – extracellular Ca^{2+} concentration

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

$Ca^{2+}{}_m$ – Ca^{2+} contained within the mitochondrial lumen

CCh – carbamylcholine

CCCP - carbonyl cyanide m-chlorophenylhydrazone

CICR – Ca^{2+}-induced Ca^{2+} release

CMF-HBSS – Ca^{2+}-Mg^{2+} free Hank's Buffered Saline Solution

CN – calcineurin (protein phosphatase 2B)

CNS – central nervous system

CsA – cyclosporin A

4-DAMP – 4-diphenylacetoxyl-N-methylpiperidine methiodide

DHE – dihydro-β-erythroidine hydrobromide

DMEM – Dulbecco's Modified Eagle Medium

DNase I – deoxyribonuclease I

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

EtOH – ethanol

FBS – fetal bovine serum

Fura-2 AM – fura-2 acetoxymethylester

GABA – gamma-aminobutyric acid

GSH – glutathione

HBS – HEPES-buffered saline solution

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

hr – hour(s)

IP_3 – inositol-1,4,5-triphosphaste

MeHg – methylmercury

min – minute(s)

MTP – mitochondrial permeability transition pore

NMDA – N-methyl-D-aspartate

PhAsO – phenylarsine oxide

PLA_2 – phospholipase A_2

PLC – phospholipase C

PNS – peripheral nervous system

ROS – reactive oxygen species

SEM – standard error of the mean

SER – smooth endoplasmic reticulum

SERCA – smooth endoplasmic reticulum Ca^{2+} ATPase

SOCC – store-operated Ca^{2+} channel

SOCE – store-operated Ca^{2+} entry

TMRE – tetramethylrhodamine ethyl ester

TPEN – N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine

W7 – N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide

XeC – xestospongin C

CHAPTER ONE

INTRODUCTION

A) GENERAL INTRODUCTION

Methylmercury (MeHg) is a highly lipophilic environmental contaminant which easily crosses both the blood-brain barrier and placental barrier, resulting in neurotoxicity in adults and fetuses alike (Friberg and Mottet, 1989; Aschner and Aschner, 1990; Myers and Davidson, 1998). Despite its ability to bind thiols indiscriminately with high affinity (Roberts *et al.*, 1980), making any protein or peptide with a cysteine group a potential target, MeHg causes a surprisingly specific pattern of neuronal damage in both the central and peripheral nervous systems (CNS and PNS, respectively). Over the past few decades, there has been considerable effort to elucidate the specific mechanism(s) by which MeHg causes neurotoxicity within discrete areas of the CNS by focusing on specific molecular targets of the compound. By identifying the molecular targets of MeHg within the CNS, particularly proximate targets in cells which exhibit heightened sensitivity, it is hoped that more reliable risk assessment can be performed in which results from *in vitro* assays can be correlated with *in vivo* effects observed at environmentally relevant exposure levels.

The primary pathological result of both acute and chronic MeHg poisoning is neurotoxicity, with clinical signs ranging from ataxia and visual disturbances to paralysis and death (Rustam *et al.*, 1975; Chang, 1980). Several areas of the brain exhibit marked pathological damage upon MeHg exposure, including the cerebral and cerebellar cortices (Hunter and Russell, 1954; Leyshon-Sorland *et al.*, 1994). In both humans and rats, the granule cell layer of the cerebellum is particularly sensitive to MeHg-induced cell death (Hunter and Russell, 1954) through an as yet undefined mechanism. In contrast, the neighboring Purkinje cells are less sensitive to MeHg-induced cell death despite

accumulating equal or greater MeHg concentrations (Hunter and Russell, 1954). The mechanism underlying the heightened susceptibility of these neurons is not yet known, but is hypothesized to be due to interactions with specific molecular targets which have not yet been identified (Castoldi *et al.*, 1996; Robertson and Orrenius, 2000; Castoldi *et al.*, 2001).

Given the large number of potential molecular targets containing cysteine moieties, it is not surprising that MeHg has a number of effects which could contribute to the observed toxicity. At the cellular level, MeHg interacts with a number of sites to cause disruption of divalent cation homeostasis, particularly Ca^{2+} and Zn^{2+} (Denny *et al.*, 1993; Hare *et al.*, 1993), elevation of spontaneous release of neurotransmitter (Juang, 1976; Atchison and Narahashi, 1982; Atchison, 1986), generation of reactive oxygen species (Sarafian and Verity, 1991; Yee and Choi, 1994) and cell death (Kunimoto, 1994; Marty and Atchison, 1998). The disruption of intracellular Ca^{2+} (Ca^{2+}_i) has received much attention in recent years, with both plasma membrane Ca^{2+} channels and intracellular Ca^{2+} stores being investigated as potentially critical targets during MeHg exposure (Tan *et al.*, 1993; Oyama *et al.*, 1994; Sakamoto *et al.*, 1996; Marty and Atchison, 1997; Marty and Atchison, 1998; Sirois and Atchison, 2000). Cell viability assays in which Ca^{2+}_i was buffered by the Ca^{2+}_i chelator 1,2- bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl)ester (BAPTA) indicated that removal of Ca^{2+}_i provides significant protection against neuronal death at 3 hr post-exposure, suggesting an important role for loss of Ca^{2+} homeostasis in MeHg-induced neurotoxicity (Marty and Atchison, 1998).

In the highly susceptible cerebellar granule cell, MeHg affects multiple subtypes of voltage-gated Ca^{2+} channels on the plasma membrane (Marty and Atchison, 1997; Sirois and Atchison, 2000). However, very little is known about the intracellular Ca^{2+} targets in this cell type. Previous studies demonstrated that MeHg disrupts intracellular Ca^{2+} (Ca^{2+}_i) homeostasis in cultures of rat cerebellar granule cells, resulting in a biphasic increase in $[Ca^{2+}]_i$: the first phase results from release of Ca^{2+} from intracellular pools, and the second phase results from influx of extracellular Ca^{2+} (Ca^{2+}_e) (Hare *et al.*, 1993; Marty and Atchison, 1997) (Figure 1.1). The initial increase in $[Ca^{2+}]_i$ is intracellular in origin, as removal of Ca^{2+}_e abolishes the secondary fura-2 fluorescence elevation (Hare *et al.*, 1993; Marty and Atchison, 1997) (Figure 1.2). In cerebellar granule cells, the MeHg-induced influx of Ca^{2+}_e occurs through nifedipine- and ω-conotoxin-MVIIC-sensitive pathways, suggesting that L, N and/or P/Q-type voltage-gated Ca^{2+} channels participate in MeHg-induced Ca^{2+} entry; however, the intracellular Ca^{2+} source has not yet been identified (Marty and Atchison, 1997). Two intracellular Ca^{2+} stores could contribute to the early-onset elevation of $[Ca^{2+}]_i$ during MeHg exposure: the smooth endoplasmic reticulum (SER) and the mitochondria. The SER provides high-affinity, low-capacity Ca^{2+} storage while the mitochondria are generally considered to be a low-affinity, high-capacity pool (Fohrman *et al.*, 1993; Gunter and Gunter, 1994; Simpson *et al.*, 1995; Budd and Nicholls, 1996b; Masgrau *et al.*, 2000). The SER contains ryanodine- and IP_3-sensitive Ca^{2+} pools which can release sequestered Ca^{2+} into the cytosol (Irving *et al.*, 1992). In NG108-15 cells, part of the MeHg-induced first-phase is due to release of Ca^{2+} from the IP_3-sensitive pool in the SER; however, emptying

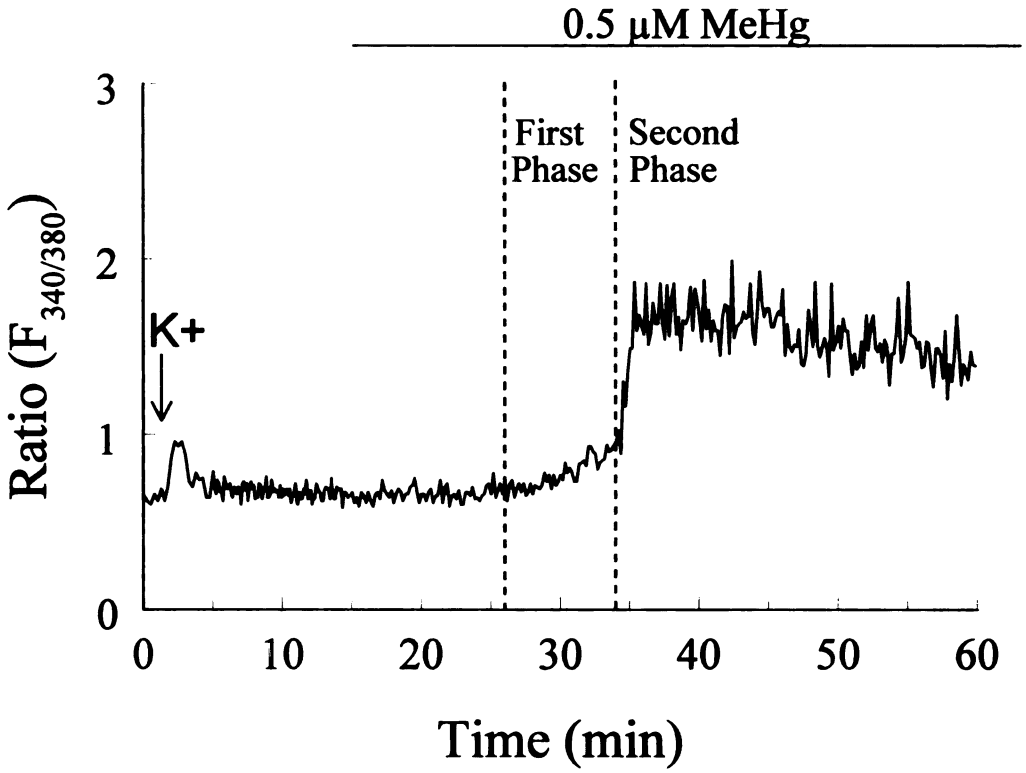

Figure 1.1. MeHg causes a biphasic increase in $[Ca^{2+}]_i$ in cerebellar granule cells in primary culture. Representative tracing of the fura-2 fluorescence from a cerebellar granule cell exposed to 40 mM K^+ for 1 min (as a viability test), followed by HBS washout and 0.5 μM MeHg. The ratio of fluorescence emitted at excitation wavelengths of 340 nm and 380 nm gives the approximate level of $[Ca^{2+}]_i$, which increases in a biphasic manner. Time-to-onset of fluorescence increases is measured from the beginning of MeHg treatment.

Modified from: Marty and Atchison, *Toxicol. Appl. Pharmacol.* **147**:319-330 (1997).

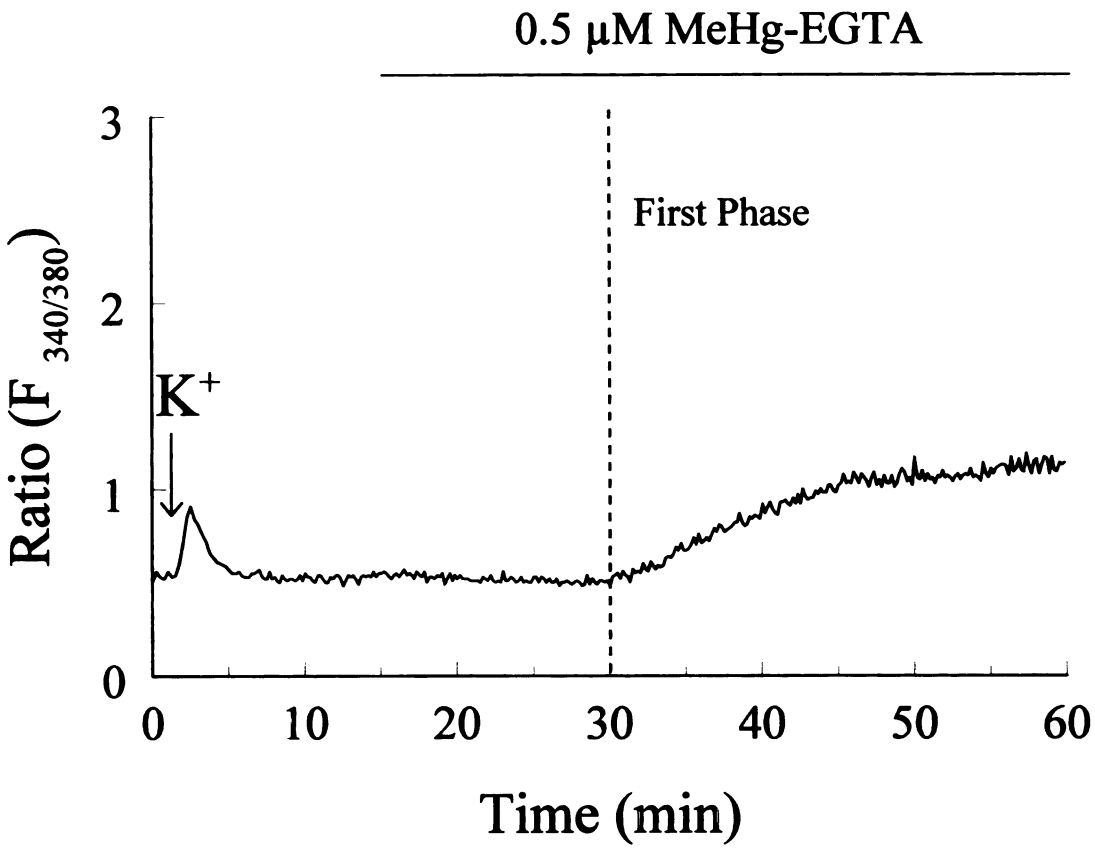

Figure 1.2. Elevations of fura-2 fluorescence in a cerebellar granule cell in culture exposed to 0.5 μM MeHg in Ca^{2+}-free buffer. Under these conditions, the first $[Ca^{2+}]_i$ phase is evident while the second phase (due to influx of $[Ca^{2+}]_e$) is absent.

Modified from: Marty and Atchison, *Toxicol. Appl. Pharmacol.* **147**:319-330 (1997).

this pool does not eliminate the first phase, suggesting that at least one additional source contributes to the first phase Ca^{2+} increase (Hare and Atchison, 1995). Mitochondria also store and release Ca^{2+}, and are also affected by MeHg. Ca^{2+} release can occur *via* reversal of the uniporter, activation of the Na^{+}/Ca^{2+} exchanger, or opening of the mitochondrial permeability transition pore (MTP). MeHg has a number of effects on mitochondria, including decreased Ca^{2+} uptake and increased Ca^{2+} efflux from preloaded rat brain mitochondria, as well as inhibition of respiration, increased permeability of the inner membrane, and depolarization of the inner mitochondrial membrane (Sone *et al.*, 1977; Levesque and Atchison, 1991; Hare and Atchison, 1992), any of which could contribute to the first phase Ca^{2+} elevation *via* disruption of mitochondrial Ca^{2+} buffering.

Several lines of evidence suggest a critical relationship between intracellular Ca^{2+} pools and cell death, thus necessitating inquiry in this area to gain full understanding of neuronal death induced by MeHg. As such, the goal of this dissertation is to identify the intracellular Ca^{2+} pools targeted by MeHg in cerebellar granule neurons and determine their relative importance in MeHg-induced cytotoxicity of cerebellar granule neurons. This dissertation reports results of experiments designed to investigate the hypothesis that MeHg initiates Ca^{2+} release from the SER which is buffered by mitochondria; however, MeHg impairs the ability of mitochondria to hold Ca^{2+} (by causing loss of mitochondrial membrane potential and inhibiting respiration), leading to opening of the mitochondrial permeability transition pore and cell death (Figure 1.3).

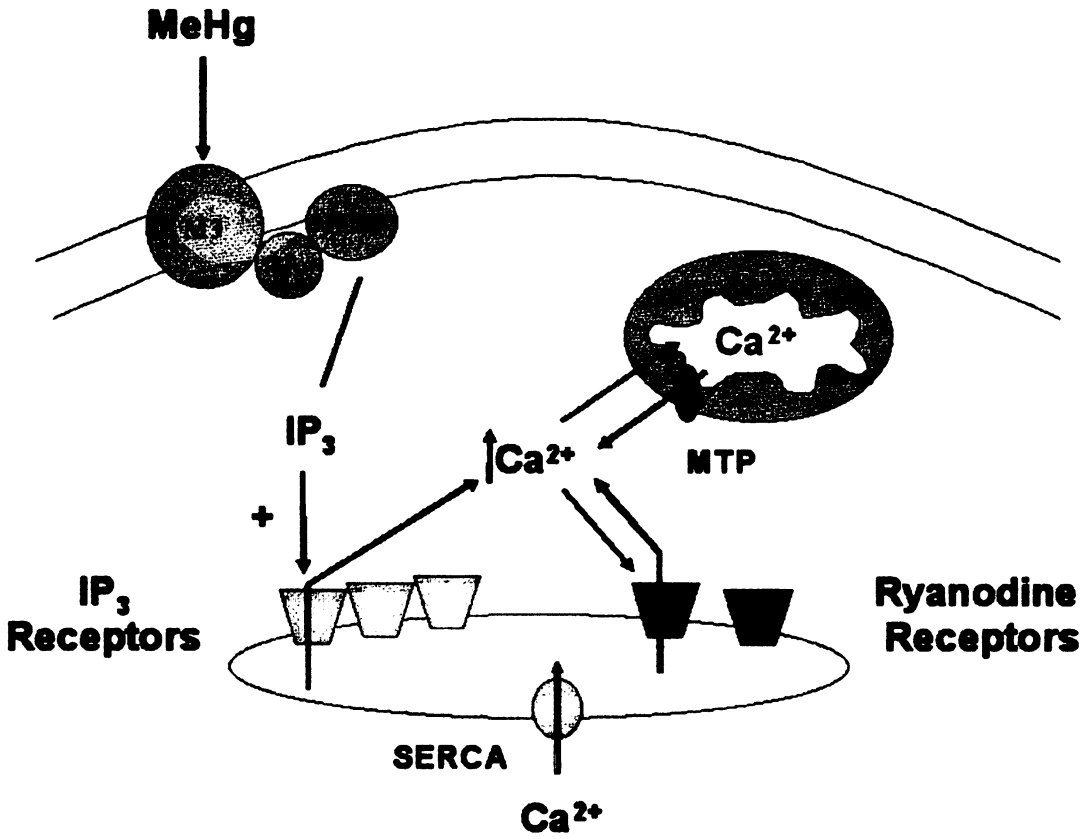

Figure 1.3. Hypothesis regarding MeHg-induced elevations of $[Ca^{2+}]_i$ in rat cerebellar granule neurons. MeHg binds M3 muscarinic ACh receptors, causing production of IP_3 and release of Ca^{2+} through the IP_3 receptors. This elevation of $[Ca^{2+}]_i$ triggers Ca^{2+}-induced Ca^{2+} release through the ryanodine receptors. Ca^{2+} released from the SER is buffered by mitochondria. Excess uptake of Ca^{2+} into the mitochondria, combined with direct effects of MeHg on the mitochondrial respiratory chain, leads to opening of the mitochondrial permeability transition pore (MTP) and promotion of cell death.

B) BACKGROUND

a) Humans and MeHg: Routes and Biological Consequences of Exposure

MeHg is an organic form of mercury which enters the environment through two primary routes: release of MeHg itself as a by-product of human activities, and conversion of inorganic mercury to MeHg by biological organisms. Organic mercury has been used as a fungicide and in preservative solutions for wood, paper pulp and leather. Additionally, inorganic mercury has been used as a catalyst in several industrial processes, allowing for the release of both inorganic and organic mercury (as a by-product of the reaction) into the environment through discharge of untreated waste into the water and air (Kurland, 1973; Gerstner and Huff, 1977). Organic mercury is also produced by methylation of mercury by aquatic microorganisms in the sediment of river and lake beds (Choi and Bartha, 1994; Baldi, 1997; Guimaraes *et al.*, 2000). MeHg is a highly lipophilic substance which, once in the environment, readily accumulates in the food chain (Rabenstein and Evans, 1978). The most common mode of MeHg intoxication for humans is through ingestion of contaminated foodstuffs (Hansen, 1990; Renzoni *et al.*, 1998). Once ingested, MeHg readily enters the blood and is transported in the plasma throughout the body, where it easily crosses the blood-brain barrier (Steinwall and Klatzo, 1966; Chang and Hartmann, 1972; Chang and Hartmann, 1972; Aschner and Aschner, 1990). In rats exposed to radioactively labeled MeHg, the greatest accumulation of the compound occurs in the spinal dorsal root ganglia, with the cerebral cortex and cerebellum also exhibiting high concentrations following exposure (Somjen *et al.*, 1973). MeHg is excreted from the body at a slow rate, with a half-life of

approximately 70 days in humans (Clarkson, 1972; Neathery and Miller, 1975). While the rate of MeHg elimination *via* metabolism and excretion is sufficient that accumulation within the body is limited (Clarkson, 1972), there is evidence suggesting that the brain half-life is considerably longer than that of the whole body during chronic exposure (Rice, 1989; Carrier *et al.*, 2001).

MeHg poisoning was first examined in the 1950s following the study of four patients poisoned *via* inhalation of MeHg from fungicidal dusts in a cereal factory. The predominant symptoms in these patients were neurological, including generalized gross ataxia, impaired speech, and severely constricted visual fields. Histological analysis of one of the four patients in autopsy revealed gross atrophy of the cerebrum as well as marked degeneration of the granule cell layer of the cerebellum and neuronal destruction in the visual area of the calcarine (visual) cortex (Hunter and Russell, 1954). The authors noted that the observed ataxia was probably due to gross alterations in the cerebellar cortex because there was no damage to the cerebellar motor pathways other than the loss of granule cells. The cerebellar damage itself was very selective, with loss of granule cells occurring in the absence of damage to nearby Purkinje cells.

The two most widely known incidents of MeHg poisoning occurred in Japan in the 1950s and 1960s (Takeuchi *et al.*, 1962; Harada, 1995) and Iraq in 1972 (Bakir *et al.*, 1973; Clarkson *et al.*, 1976). There were epidemics in Japan in both Minamata (from 1953 to 1960) and Niigata (from 1960 to 1965) as a result of eating contaminated seafood (Chang *et al.*, 1977). In Minamata, both organic and inorganic mercury were part of a chemical plant's waste effluent discharged into Minamata Bay. Once in the waterway, MeHg accumulated in the food chain, resulting in 46 deaths and

approximately 2200 people poisoned through consumption of contaminated seafood (Harada, 1995). In addition, prenatal exposure to MeHg resulted in many infants being born with nervous system damage. This fetal neurological syndrome is now referred to as "Minamata syndrome." During the Iraqi poisoning incident, approximately 6500 hospitalizations and 450 deaths were reported as a result of MeHg poisoning from ingestion of contaminated seed grain in bread (Clarkson *et al.*, 1976). In both the Minimata and Iraqi incidents, the primary sign of exposure was neurological dysfunction as characterized by cerebellar ataxia, generalized extremity weakness and sensory disturbances including impairments of speech, vision and hearing. Correlations between body burden of MeHg and frequency of adverse symptoms (ataxia, paresthesia, etc.) in adult victims of the Iraqi poisoning indicated that the threshold for adverse effects was at a body burden level of 25 to 40 mg of mercury (Bakir *et al.*, 1973). Additionally, Iraqi children exposed *in utero*, through breastfeeding, or both, exhibited marked developmental deficiencies, including delayed onset of speech and impaired motor, sensory and autonomic function (Bakir *et al.*, 1980). In the more severe cases, children were born blind and deaf (Bakir *et al.*, 1980). Dose-response analysis suggests that fetal effects are observed when maternal hair mercury concentrations exceed 10 ppm (Crump *et al.*, 2000).

In adults, MeHg causes a very specific pattern of damage within the PNS and CNS that correlates with the observed symptoms of poisoning (Chang and Hartmann, 1972; Chang *et al.*, 1977). In the PNS, there is axonal degeneration characterized by secondary loss of the myelin sheath of the sensory branch (Hunter and Russell, 1954; Takeuchi, 1982). The disruption of motor and sensory nerve function correlates with the

observed extremity weakness and impairment of sensory function (Chang, 1980). Early studies focused on effects of MeHg at the neuromuscular junction, in part due to the observed increased incidence of neuromuscular weakness during the Iraqi incident (Rustam *et al.*, 1975) and in part because the ACh release process is well characterized both biochemically and physiologically. At the neuromuscular junction, the primary effect is to decrease nerve-evoked release of acetylcholine (ACh) and alter its spontaneous quantal release by causing it to first increase, then decrease (Juang, 1976; Atchison and Narahashi, 1982). Interestingly, the elevated spontaneous release of neurotransmitter may be due to increased $[Ca^{2+}]_i$ because MeHg increases $[Ca^{2+}]_i$ in the absence of Ca^{2+}_e in isolated nerve terminals (Komulainen and Bondy, 1987). Further, MeHg increases the frequency of spontaneous ACh release in the absence of Ca^{2+}_e in the neuromuscular junction (Atchison, 1986).

While MeHg can damage the PNS, its effects within the CNS are of greater concern, especially in the developing organism. During chronic MeHg exposure, MeHg accumulates in the cerebellum, with the highest concentration in the Purkinje and Golgi cells of the granular layer, and a slightly lower concentration in the granule cells, stellate cells, and basket cells (Chang *et al.*, 1977; Moller-Madsen, 1990; Moller-Madsen, 1991) (Leyshon-Sorland *et al.*, 1994) (Figure 1.4). However, despite the lower accumulation of MeHg in the granule cell layer, these neurons are most affected during both acute and chronic MeHg poisoning, resulting in a characteristic loss of the granule cell layer (Hunter and Russell, 1954; Chang *et al.*, 1977; Leyshon and Morgan, 1991). The resultant cerebellar ataxia observed in both acute and chronic MeHg poisoning is attributed to the loss of these neurons. Additionally, MeHg causes specific loss of

neurons from the second through fourth layers of the visual area of the calcarine cortex, resulting in constriction of the visual field (Hunter and Russell, 1954; Takeuchi, 1982).

The fetal CNS exhibits greater sensitivity to MeHg than does the adult CNS, as children exposed *in utero* may be affected in the absence of maternal toxicity (Bakir *et al.*, 1980; Takeuchi, 1982). Unlike the toxicity in adults, fetal poisoning produces a less specific pattern of damage that is dependent on the concentration and duration of exposure, as well as the stage of development during exposure (Rodier, 1995). Prenatal exposure interferes with neuron migration (Choi *et al.*, 1978) and decreases the mitotic activity of CNS neurons (Rodier *et al.*, 1984). Following the Iraqi incident, infants born to highly exposed mothers demonstrated permanent damage to the CNS, with symptoms including delayed achievement of developmental milestones, seizures and abnormal reflexes (Marsh *et al.*, 1987). In several cases involving Japanese children, there was a marked decrease in the number of neurons in all lobes of the cerebral cortex, as well as widespread disorganization of the cerebral cortex architecture resulting from neuronal degeneration (Choi, 1989). Similar to the adult symptoms, severely affected children exhibit severe atrophy of the cerebellar cortex such that the granule cell layer degenerates and virtually disappears (Choi, 1989). Unlike the adult cases, in which the remaining neurons of the cerebellar cortex are relatively unaffected, children exhibit a narrowing of the cortical molecular layer and a reduction in the number of Purkinje neurons (Choi, 1989).

More recent studies have focused on the more subtle neurological effects resulting from low-concentration exposure to MeHg in the developing organism. Currently, the most common source of MeHg exposure is consumption of contaminated seafood

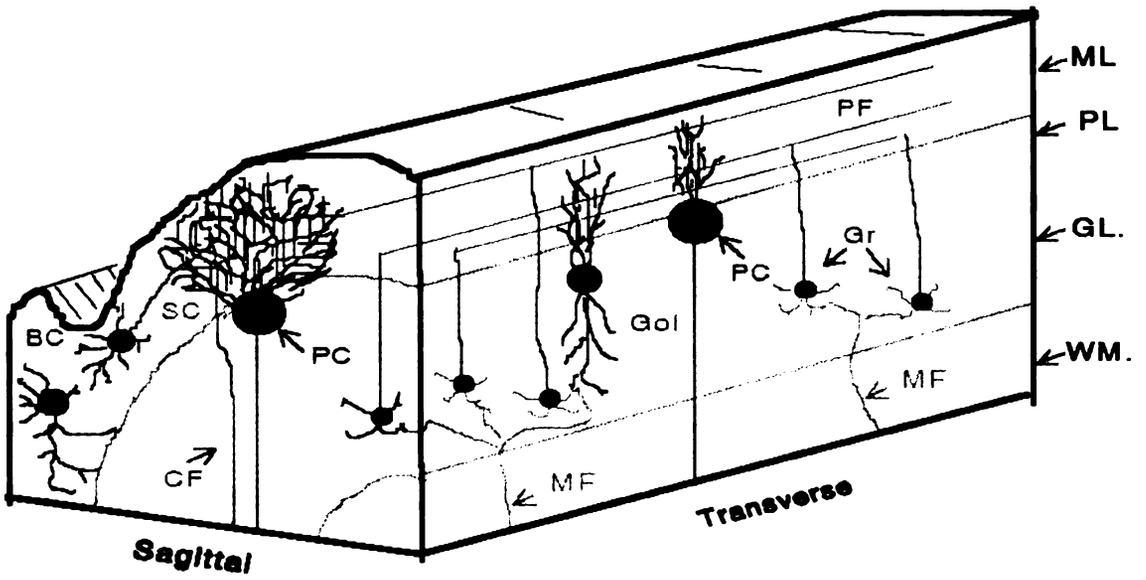

Figure 1.4. Schematic diagram of cerebellar cortex. The cerebellar cortex is divided into three distinct layers which lie outside the white matter (WM); these layers are the outermost molecular layer (ML), the Purkinje layer (PL), and the innermost granular layer (GL). The granular layer contains not only the cell bodies of granule cells (Gr) but also contains Golgi cell (Gol) bodies, axons and dendrites, mossy fiber (MF) terminals, and the axons of Purkinje cells with their accompanying climbing fibers (CF). The Purkinje layer contains only the cell bodies of Purkinje cells (PC). The molecular layer consists of the stellate cells (SC) and basket cells (BC), as well as the dendritic arbors of Purkinje cells and Golgi cells, and the parallel fiber (PF) branches of granule cell axons. *Diagram courtesy of Dr. Yukun Yuan.*

(Hansen, 1990; Renzoni *et al.*, 1998); thus, MeHg exposure is of concern in regions of the world where indigenous populations heavily consume fish as part of their diet. At-risk populations include those in the Seychelles (Myers *et al.*, 1995; Myers *et al.*, 1997; Palumbo *et al.*, 2000) and Faroe Islands (Weihe *et al.*, 1996; Grandjean *et al.*, 1999), as well as Greenland's Inuits (Hansen, 1990) and certain tribes of North American Native Americans (Dellinger *et al.*, 1996). While there were no adverse effects observed in a longitudinal study of children in the Seychelles Islands (Davidson *et al.*, 2000), studies in the Faroe Islands (Grandjean *et al.*, 1999; Steuerwald *et al.*, 2000) and Brazil (Grandjean *et al.*, 1999) found that long-term exposure to low concentrations of MeHg was correlated with deficiencies in motor function, attention, memory, and language development. The growing recognition that the greatest threat is to the developing organism is reflected in the recent lowering of the US EPA reference dose from 0.3 to 0.1 μg/kg per day (Rice *et al.*, 2000). The lower reference dose is derived from a study of children exposed *in utero* during the Iraqi poisoning, in which increased maternal MeHg exposure was associated with delayed neurodevelopment in the child (Marsh *et al.*, 1987). However, there is still considerable debate over the lowest exposure needed to produce adverse effects in the fetus.

b) Cellular and Molecular Effects of Methylmercury

One of the issues complicating the risk assessment of MeHg is the large number of potential molecular targets within the body. Because MeHg binds thiol groups with high affinity, any protein or peptide containing a cysteine is a potential target. Given the wide range of possible targets during MeHg exposure, the question arises whether there is

a specific target, or set of targets, which make specific areas of the CNS vulnerable to MeHg-induced disruption of neuronal development and viability. This dissertation will address the question of whether MeHg targets specific receptors and ion channels involved in Ca^{2+} signaling in the CNS, including plasma membrane cholinergic receptors and intracellular Ca^{2+} channels associated with Ca^{2+}-sequestering organelles, which may be involved in the pathogenesis of MeHg.

i) MeHg-Induced Cell Death: Apoptosis or necrosis?

To understand whether MeHg has specific targets during neurotoxicity, it is critical to understand the mechanisms by which MeHg causes cell death. The cell death process can be divided into two morphologically and biochemically distinguishable categories: necrosis and apoptosis. Necrosis is generally characterized by cell swelling, organelle damage, loss of plasma membrane integrity and cell lysis, as well as rapid adenosine 5'-triphophate (ATP) depletion and severe alteration of mitochondrial ultrastructure, such that an inflammatory response is initiated. Apoptosis, on the other hand, is characterized by cell shrinkage, chromatin aggregation, plasma membrane blebbing, and DNA condensation accompanied by maintenance of ATP levels, activation of caspases, and loss of plasma membrane asymmetry (Kroemer, 1997; Mattson, 2000). However, the distinction between apoptosis and necrosis is not clear-cut, as both share several critical characteristics. For example, both modes of cell death are accompanied by elevated $[Ca^{2+}]_i$ levels, and each can result from exposure to the same compound depending on the severity of the insult (Kruman and Mattson, 1999). Removal of $Ca^{2+}{}_i$ with buffering agents (such as BAPTA) can protect cells from cell death in several

different systems, including neurons (Marty and Atchison, 1998). Both mechanisms also involve plasma membrane blebbing and mitochondrial damage, making the distinction between necrosis and apoptosis difficult in some cases. There is also evidence that the antiapoptotic gene *bcl-2* can protect cells from both apoptosis and necrosis (Kane *et al.*, 1995; Kroemer, 1997). Indeed, although necrosis and apoptosis are considered to be different forms of cell death, there is now debate over whether they are actually part of a continuum of overlapping mechanisms of cell death (Raffray and Cohen, 1997).

In MeHg neurotoxicity, there is increasing evidence both *in vivo* and *in vitro* that MeHg causes apoptosis at lower concentrations and necrosis at higher concentrations (Nakada and Imura, 1983; Miura and Imura, 1987; Kunimoto, 1994; Nagashima *et al.*, 1996). When studied *in vivo*, the cerebellar damage tends to be apoptotic, as assessed using conventional techniques such as the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, DNA gel electrophoresis and ultrastructural examination (Nagashima *et al.*, 1996). When studied *in vitro*, cerebellar granule cells undergo apoptosis following 18 hr of exposure to lower MeHg concentrations (0.5-1.0 μM), and necrosis within 1 hr of exposure to higher concentrations (5 and 10 μM) (Castoldi *et al.*, 2000). When granule cells in primary culture are studied after 72 hr of exposure, apoptosis occurs at 0.1-0.3 μM, with the switch to necrosis occurring at higher MeHg concentrations (Kunimoto, 1994). Hence, the mode of cell death *in vitro* depends on both the duration and intensity of exposure. However, there is no clear-cut threshold for which death pathway is followed, as both modes of cell death can be observed in granule cells in primary culture in response to 1

μM MeHg within an hour of exposure, as determined using a combination of fluorescent microscopy and morphometric analysis, and the TUNEL assay (Castoldi *et al.*, 2000).

ii) Calcium and Cell Death

The central role of Ca^{2+} in the cell death process is a common thread between a wide range of neurotoxic insults, including the neurotoxicity of lead and organic mercury, as well as glutamate excitotoxicity (White and Reynolds, 1996). Neurons contain a variety of mechanisms for transporting Ca^{2+} across both internal and external membranes. The concentration of Ca^{2+}_i is tightly regulated to maintain a relatively low $[Ca^{2+}]_i$ (in the 100-200 nm range) compared to the $[Ca^{2+}]_e$ (in the 1-2 mM range) (Kass and Orrenius, 1999). When the $[Ca^{2+}]_i$ becomes elevated for sustained periods of time, the physiological signal becomes pathological as Ca^{2+} begins to promote cell death. Disruption of Ca^{2+}_i homeostasis results in activation of multiple enzymes which are involved in the catabolism of proteins, phospholipids and nucleic acids, thereby leading to degradation of molecules needed for cell survival (Kass and Orrenius, 1999). For example, calpains are Ca^{2+}-activated cytosolic proteases which, when activated, are capable of promoting cell death, as indicated by the effectiveness of calpain inhibitors in preventing Ca^{2+}-mediated cell death (Chan and Mattson, 1999). Several phosholipases, including PLC and phospholipase A_2 (PLA_2) are also activated by Ca^{2+} either directly (PLC) or indirectly through Ca^{2+}-calmodulin (PLA_2). PLC generates IP_3 which releases Ca^{2+}_i from the SER, while PLA_2 breaks down phospholipids to form arachidonic acid (Exton, 1990). Sustained elevations of Ca^{2+}_i can lead to sustained activation of PLA_2, thereby leading to extensive membrane degradation and the generation of toxic

metabolites (Exton, 1990). Further, there are Ca^{2+}-activated endonucleases which mediate DNA strand cleavage at internucelosomal linker regions to produce approximately 200 base pair long fragments (Arends *et al.*, 1990). Although influx of Ca^{2+}_e is often associated with apoptosis, it is not necessarily an essential requirement as elevated Ca^{2+} originating from intracellular stores can be sufficient to induce cell death (Wei and Perry, 1996; Wei *et al.*, 1998; Szalai *et al.*, 1999). However, there is also evidence for a lack of correlation between changes in $[Ca^{2+}]_i$ and apoptosis caused by specific insults, most notably in thymocytes and lymphocytes (Alnemri and Litwack, 1990; Beaver and Waring, 1994).

MeHg causes elevated $[Ca^{2+}]_i$ in a number of cell types and preparations, including neuroblastoma-glioma cells (Hare *et al.*, 1993), human lymphocytes and monocytes (Shenker *et al.*, 1992; Shenker *et al.*, 1993; Tan *et al.*, 1993), mouse thymocytes (Oyama *et al.*, 1995), rat forebrain synaptosomes (Denny *et al.*, 1993) and rat cerebellar neurons (Sarafian, 1993; Oyama *et al.*, 1994; Marty and Atchison, 1997; Mundy and Freudenrich, 2000). In cerebellar granule cells in primary culture, inhibition of MeHg-induced Ca^{2+}_e influx through voltage-operated Ca^{2+} channels with the Ca^{2+} channel blockers ω-conotoxin MVIIC and nifedipine reduced the number of dead neurons at 3.5 hr post-exposure (Marty and Atchison, 1998). Additionally, chelating Ca^{2+}_i with BAPTA protected against cell death at 3.5 hr post-exposure to 0.5-1.0 μM MeHg; however, BAPTA was not protective at 24.5 hr post-exposure (Marty and Atchison, 1998). In granule cells *in vitro*, MeHg-induced cell death is not due to excitotoxic insult, as inhibitors of excitatory amino acid channels do not prevent MeHg-induced elevations of $[Ca^{2+}]_i$ (Marty and Atchison, 1997) or MeHg-induced cell death

(Marty and Atchison, 1998; Castoldi *et al.*, 2000). *In vivo* studies support the *in vitro* experiments, as voltage operated Ca^{2+} channel blockers prevented the appearance of neurological disorders and mortality in rats treated with MeHg (Sakamoto *et al.*, 1996). Thus, MeHg neurotoxicity has a Ca^{2+}-dependent component which, when inhibited, prevents some of the toxic effects of the compounds, including cell death.

MeHg potentially causes elevations of $[Ca^{2+}]_i$ through non-specific damage to lipid membranes on intracellular organelles and/or the plasma membrane, or through specific interactions with receptors and/or ion channels associated with Ca^{2+} signaling. MeHg causes non-specific membrane damage through lipoperoxidation in cerebellar granule cells in suspension (Sarafian and Verity, 1991); however, this membrane damage does not appear to contribute to MeHg-induced cell death, as prevention of lipoperoxidation is not neuroprotective (Sarafian and Verity, 1991). Alternatively, there is substantial evidence that interactions between MeHg and specific Ca^{2+} signaling receptors and ion channels may be important in MeHg-induced neurotoxicity, as outlined in the following sections.

iii) MeHg and Cholinergic Neurotransmission

MeHg alters cholinergic transmission both pre- and post-synaptically within the CNS and PNS alike. One of the early experimental observations regarding MeHg neurotoxicity was its disruption of neurotransmitter release at the neuromuscular junction, which was studied in part because of the observed increased incidence of neuromuscular weakness during the Iraqi incident (Rustam *et al.*, 1975) and in part because the ACh release process is well characterized both biochemically and

physiologically. At the neuromuscular junction, the primary presynaptic effect is to decrease nerve-evoked release of ACh and alter its spontaneous quantal release in a mechanism apparently dependent on disruption of $[Ca^{2+}]_i$ (Juang, 1976; Atchison and Narahashi, 1982). However, it is the post-synaptic effects of MeHg on cholinergic transmission which are of interest in the study of MeHg-induced neuronal death in the CNS.

MeHg binds to both nicotinic and muscarinic cholinergic receptors in a variety of neuronal preparations. In *Torpedo*, MeHg inhibits binding of ACh to the muscle-type nicotinic ACh receptors (Shamoo *et al.*, 1976; Eldefrawi *et al.*, 1977). *In vitro*, 10-30 μM MeHg inhibits the fast depolarizing response of nicotinic receptors in N1E-115 neuroblastoma cells (Quandt *et al.*, 1982). Both muscarinic and nicotinic receptors contain a conserved pair of extracellular cysteine residues that are predicted to from a disulfide bond between two of the extracellular loops of the receptor (Sato *et al.*, 1976; Zeng *et al.*, 1999). These thiol groups are critical in agonist and antagonist binding by muscarinic receptors (Hedlund and Bartfai, 1979), and agents which modify these thiol groups alter muscarinic receptor activity (Sato *et al.*, 1976; Berstein *et al.*, 1988). Given that MeHg binds thiol groups with high affinity, it is not surprising that MeHg interacts with several subtypes of muscarinic receptors both *in vivo* and *in vitro*. MeHg inhibits agonist binding to both M1 and M2 muscarinic receptors in rat brain cortical membranes, although its affinity for M1 receptors is much higher than for M2 receptors (IC_{50} values of 3.4 μM and 149 μM for M1 and M2, respectively) (Castoldi *et al.*, 1996). In this preparation, MeHg inhibited agonist binding to the receptors in a competitive manner and decreased the affinity of the agonist carbachol for the receptor, suggesting that MeHg

prevents agonist binding to the receptors, thereby disrupting neurotransmission post-synaptically (Castoldi *et al.*, 1996).

MeHg also affects muscarinic receptors *in vivo*. In rats treated with 0.5 or 2 mg/kg/day MeHg for 16 days, there was a significant increase in the density of muscarinic receptors in the cerebellum and hippocampus with no effect on receptor affinity (Coccini *et al.*, 2000). This effect was region-specific, as there was no observable change in muscarinic receptor density in the cerebral cortex. Further, the increased receptor density was not observed until 2 weeks after the cessation of treatment, indicating a delayed up-regulation in the number of muscarinic receptors in these brain regions (Coccini *et al.*, 2000).

The specific location of muscarinic receptors within the CNS may explain, in part, the specific pattern of neurotoxicity observed following MeHg exposure. For example, cerebellar granule cells express a higher density of muscarinic receptors than do any other neurons within the cerebellar cortex as assessed by [3H]]quinuclidinyl benzilate binding (Neustadt *et al.*, 1988). The up-regulation of muscarinic receptors in the cerebellum and hippocampus is of particular interest, as MeHg damages cells and disrupts neurotransmission in these two regions (Yuan and Atchison, 1993; Yuan and Atchison, 1994; Yuan and Atchison, 1995; Yuan and Atchison, 1997; Coccini *et al.*, 2000; Gasso *et al.*, 2000). In cerebellar granule cells, muscarinic receptors modulate cell death induced by several toxicants, ranging from non-depolarizing growth medium to the wasp-venom peptide mastoporan (Galli *et al.*, 1995; Yan *et al.*, 1995; de Luca *et al.*, 1996; Lin *et al.*, 1997). Apoptosis caused by mastoparan is mediated by the M3 muscarinic receptor and not the M2 or nicotinic receptor, suggesting that activation of a

specific, PLC-linked muscarinic receptor can be important in toxicant-induced death of cerebellar granule cells (Yan *et al.*, 1995).

iv) Intracellular Calcium Stores

Given the importance of maintaining low cytosolic levels of $[Ca^{2+}]_i$ and the role of Ca^{2+} as an intracellular signal , it is not surprising that in addition to mechanisms for Ca^{2+} removal from the cell, there are also mechanisms for the intracellular storage of Ca^{2+}. Within neurons, the two primary organelles which store Ca^{2+} are the mitochondria and the SER. Mitochondria represent a low-affinity, high-capacity Ca^{2+} store, whereas the SER is a high-affinity, low-capacity Ca^{2+} pool (Somlyo *et al.*, 1985). While the SER will move Ca^{2+} from the cytosol into the SER lumen at low cytosolic $[Ca^{2+}]_i$, the mitochondria require a more powerful stimulus, with a local extra-mitochondrial Ca^{2+} concentration of approximately 0.5 μM needed for neuronal mitochondria (Nicholls, 1978; Nicholls and Scott, 1980). Disruption of Ca^{2+} regulation in either of these stores can pose a serious challenge to neuronal survival. As discussed below, mitochondrial effects are crucial in Ca^{2+}-mediated cell death, particularly apoptosis. In fact, there is substantial evidence to support the hypothesis that mitochondrial Ca^{2+} uptake is crucial for both apoptotic and necrotic neuronal death (Ichas and Mazat, 1998; Lemasters *et al.*, 1998; Andreyev and Fiskum, 1999; Kruman and Mattson, 1999; Duchen, 2000). Additionally, disruption of SER Ca^{2+} content can be lethal to neurons (Kass and Orrenius, 1999; Mattson *et al.*, 2000), as will also be discussed below.

Mitochondria accumulate Ca^{2+} based on the extremely negative membrane potential within the inner mitochondrial membrane (140-180 mV, negative inside)

(Gunter and Gunter, 1994), making Ca^{2+} buffering an energetically favorable process. Uptake occurs *via* the ruthenium red-sensitive uniporter as well as by the so-called rapid uptake mode (Gunter and Gunter, 1994; Sparagna *et al.*, 1995; Buntinas *et al.*, 2001). However, Ca^{2+} buffering comes at a price: uptake of a large amount of Ca^{2+} occurs at the expense of the proton motive force, and can thus cause uncoupling of ATP synthesis from respiration. Under physiological conditions, mitochondrial Ca^{2+} buffering is important in regulating cytosolic $[Ca^{2+}]$ elevations which result from either influx of Ca^{2+}_e or release of Ca^{2+} from the SER, and may play a role in modulating cellular Ca^{2+} oscillations (Simpson and Russell, 1996; Rizzuto *et al.*, 1998). At low concentrations, mitochondrial matrix Ca^{2+} (Ca^{2+}_m) can activate several mitochondrial enzymes associated with the tricarboxylic acid (TCA) cycle, as well as stimulate the adenine nucleotide translocase to cause increased movement of ATP from the mitochondria into the cell. Further, mitochondrial buffering of physiological Ca^{2+} transients results in parallel depolarization of the inner membrane, regardless of whether the Ca^{2+} source is intracellular or extracellular (Loew *et al.*, 1994). However, high $[Ca^{2+}]_m$ can become hazardous to the cell *via* inhibition of the TCA cycle, the electron transport chain, the mitochondrial ATPase, and dissipation of the proton gradient, all of which can contribute to loss of mitochondrial ATP synthesis and cell death (Simpson and Russell, 1998b).

Mitochondria are capable of releasing matrix Ca^{2+} by three different mechanisms (see Gunter and Pfeiffer, 1990, Gunter and Gunter, 1994, and Bernardi, 1996, for reviews of these mechanisms). The first is reversal of the uniporter, which normally extrudes cytosolic Ca^{2+} into the mitochondrial matrix. The second is a Na^+-Ca^{2+} exchanger, which is the primary mechanism for mitochondrial Ca^{2+} efflux in mitochondria isolated from

brain (Simpson and Russell, 1998b). The third is opening of the mitochondrial permeability transition pore (MTP). The MTP is a megapore formed on the inner mitochondrial membrane in response to adverse conditions including oxidative stress, high mitochondrial matrix Ca^{2+} and anoxia (Dubinsky and Rothman, 1991; Petronilli *et al.*, 1994; Chernyak *et al.*, 1995; Byrne *et al.*, 1999). When open, the MTP allows the passage of any molecule less than 1500 Da, including Ca^{2+}, Zn^{2+} and the pro-apoptotic cytochrome c, across the usually impermeable inner mitochondrial membrane (Szabo and Zoratti, 1992; Chernyak and Bernardi, 1996; Pastorino *et al.*, 1999; Szalai *et al.*, 1999; He *et al.*, 2000; Petronilli *et al.*, 2001). Because opening of the MTP can dissipate the proton gradient within the inner membrane, a corresponding loss of ψ_m (depolarization) is often observed and can be used as a diagnostic tool for observing a permeability transition (White and Reynolds, 1996; Trost and Lemasters, 1997). Induction of the MTP has been correlated with conditions such as ischemia, stroke and apoptosis, although its precise physiological role is not yet understood (see Bernardi and Petronilli, 1996 for review). Sustained opening of the MTP has also been linked to cell death caused by a variety of compounds and conditions, including glutamate excitotoxicity in cerebellar granule cells (Nieminen *et al.*, 1996; Schinder *et al.*, 1996; White and Reynolds, 1996). Opening of the MTP can be inhibited by the immunosuppressant cyclosporin A (CsA), which acts to keep the pore closed or delay its opening in the presence of inducing agents (Broekemeier *et al.*, 1989; Bernardi *et al.*, 1993; Nieminen *et al.*, 1996). Because opening of the MTP can release significant amounts of Ca^{2+} into the cytosol, MTP induction is a possible mechanism by which MeHg causes elevated $[Ca^{2+}]_i$ during the early stages of MeHg exposure.

In addition to mitochondrial Ca^{2+}, neurons can sequester Ca^{2+} within the SER. The SER contains ryanodine and IP_3-sensitive Ca^{2+} pools which are thought to be distinct in cerebellar granule cells (Irving *et al.*, 1992). The ryanodine receptors are tetrameric complexes formed from 550-565 kDa monomers, forming one of the largest proteins present within cells (Coronado *et al.*, 1994). Ryanodine receptors are characterized by the ability of the plant alkaloid ryanodine to bind with nanomolar affinity, causing Ca^{2+} release at low concentrations and inhibition of Ca^{2+} release at higher concentrations. Ca^{2+} release from the SER ryanodine-sensitive Ca^{2+} pool is elicited by a variety of agonists, including caffeine, low concentrations of ryanodine, Ca^{2+}, and cyclic ADP-ribose, and can be blocked by antagonists such as high concentrations of ryanodine, dantrolene, and ruthenium red (Coronado *et al.*, 1994). While originally identified in skeletal and cardiac muscle as a Ca^{2+} release mechanism during depolarization-mediated contraction, ryanodine receptors have now been identified in many tissues including neurons. Ryanodine receptors are involved in the process of Ca^{2+}-induced Ca^{2+}-release (CICR), in which cytosolic Ca^{2+} activates ryanodine receptors to cause Ca^{2+} release from the SER. CICR has been identified in several neuronal preparations as participating in the observed increase in Ca^{2+}_i following application of a depolarizing stimulus, and in cerebellar granule cells has been shown to be a major component of total Ca^{2+} signal detected following entry of Ca^{2+}_e (Simpson *et al.*, 1993). Immunocytochemistry in rat cerebellar granule cells indicates the presence of Type II ryanodine receptors (although at very low levels), whereas neighboring Purkinje neurons contain Type I receptors (Simpson *et al.*, 1996). Cerebellar granule cells grown in culture possess a ryanodine-sensitive SER Ca^{2+} pool; however, studies in this system are limited in that after 4 days in

culture the ryanodine receptor agonist caffeine no longer produces Ca^{2+} release from the SER (Irving *et al.*, 1992).

The SER IP_3-releasable Ca^{2+} pool also plays a role in elevations of $Ca^{2+}{}_i$ in a variety of cells, including neurons. The homotetrameric IP_3 receptors, while related structurally to ryanodine receptors, differ in that their subunits are smaller (305-313 kDa *per* monomer) and have a 4-10 times smaller conductance than ryanodine receptors (Marks, 1997). Of the 3 known receptor subtypes, both cerebellar granule and Purkinje neurons possess Type I IP_3 receptors, with particularly high levels in cerebellar Purkinje cells (Sharp *et al.*, 1999). While IP_3 is the primary agonist at these receptors, their function is also antagonized by heparin and decavanadate. Activation of phosphoinositide-specific phospholipase C (PLC) causes the hydrolyis of phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol. The IP_3 then acts at IP_3 receptors on the surface of the SER membrane to cause release of Ca^{2+} from the SER. These receptors are also sensitive to local cytosolic Ca^{2+} levels in a bell-shaped fashion, with activation at low $[Ca^{2+}]$, inactivation at higher $[Ca^{2+}]$, and peak activity at approximately 300 nM (Bezprozvanny *et al.*, 1991). Their activity is complicated further by their interaction with nearby ryanodine receptors such that release of Ca^{2+} from the IP_3 pool can elicit CICR from ryanodine receptors, thus magnifying the Ca^{2+} signal (Irving *et al.*, 1992).

Both the IP_3 and ryanodine-sensitive Ca^{2+} pools are filled by SER Ca^{2+}-ATPases (SERCAs) which allow for rapid uptake of cytoplasmic Ca^{2+}. Although there are 5 subtypes known to date, they all possess varying degrees of sensitivity to the inhibitors thapsigargin and 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone (BHQ). Thapsigargin causes

an elevation of $[Ca^{2+}]_i$ by inhibiting the SERCA, which leads to emptying the SER Ca^{2+} stores (Thastrup *et al.*, 1990). In cerebellar granule cells, there is some distinction between the SERCAs filling the IP_3 and ryanodine pools in that BHQ will affect the IP_3 but not the ryanodine pool (Simpson *et al.*, 1996), suggesting that a separate filling mechanism exists for the IP_3 pool. Under physiological conditions, release of SER Ca^{2+} does not harm the cell; however, several lines of evidence suggest that prolonged loss of SER Ca^{2+} levels can have deleterious effects on normal cell function. For example, depletion of SER Ca^{2+} can disrupt normal protein synthesis pathways, as evidenced by the inhibition of general protein synthesis following rapid release of SER Ca^{2+} (Brostrom and Brostrom, 1990). Depletion of SER Ca^{2+} can also cause the release of a specific deoxyribonuclease involved in DNA degradation, which could contribute to apoptotic cell death (Peitsch *et al.*, 1993). Further, depletion of SER Ca^{2+} content with thapsigargin has been shown to cause cell death in a variety of cell types, including neurons (Levick *et al.*, 1995; Wei and Perry, 1996; Wei *et al.*, 1998). It is thus not surprising that many cells possess a mechanism for refilling the SER Ca^{2+} stores *via* store-operated (or capacitative) Ca^{2+} entry (SOCE) (Putney, 1986). When the SER becomes depleted of Ca^{2+}, there is a sensing mechanism in the SER that detects the Ca^{2+} decrease and, through an as yet unidentified signal, sends a signal to plasma membrane Ca^{2+} channels to activate Ca^{2+} influx to replenish the stores (Putney, 1986; Ma *et al.*, 2000). The affected Ca^{2+} channels are neither the classic receptor-operated nor voltage-gated channels and are thus termed store-operated Ca^{2+} channels (SOCCs) (Kiselyov *et al.*, 1998). Although this system was originally identified in non-excitable cells, it is also found in neuronal preparations, including cerebellar granule neurons (Chavis *et al.*, 1995; Simpson *et al.*, 1995).

Interestingly, recent evidence suggests that opening of SOCCs may be due to physical interactions between IP_3 receptors (Kiselyov *et al.*, 1998; Ma *et al.*, 2000) and/or ryanodine receptors (Usachev and Thayer, 1999; Kiselyov *et al.*, 2000) with the SOCCs themselves.

In addition to interactions between the SER and the plasma membrane, there is also increasing evidence for interaction between SER Ca^{2+} pools and mitochondria. Release of Ca^{2+} from the IP_3 pool is followed closely by increased mitochondrial matrix Ca^{2+} in response to the microdomain of high $[Ca^{2+}]$ generated near the mouth of the IP_3 receptor channels (Miyata *et al.*, 1991). Use of Ca^{2+}-sensitive photoproteins simultaneously targeted to the mitochondrial matrix and SER indicated that in living HeLa cells there is a close relationship between release of Ca^{2+} from the SER and increased matrix Ca^{2+} of mitochondria which were in close apposition to IP_3 receptors (Rizzuto *et al.*, 1998). In fact, Ca^{2+} released from the IP_3 pool caused a greater increase in mitochondrial matrix $[Ca^{2+}]$ than in the bulk cytosol, whereas intracellular perfusion of a buffered Ca^{2+} solution increased $[Ca^{2+}]_i$ of the SER and mitochondria to the same extent, suggesting preferential Ca^{2+} uptake of IP_3 Ca^{2+} by mitochondria. It has been hypothesized that mitochondria located near IP_3 receptors may function to buffer released Ca^{2+} to prevent receptor inactivation by high $[Ca^{2+}]$, thereby potentiating IP_3 receptor activity (Rizzuto *et al.*, 1998; Simpson and Russell, 1998a).

v) MeHg and the Smooth Endoplasmic Reticulum

In disrupting $[Ca^{2+}]_i$ homeostasis, MeHg causes Ca^{2+} release from intracellular stores into the cytosol in cerebellar granule cells (Sarafian, 1993; Marty and Atchison,

1997), synaptosomes (Kauppinen *et al.*, 1989; Denny *et al.*, 1993) and NG108-15 cells (Hare *et al.*, 1993; Hare and Atchison, 1995). In T lymphocytes (Tan *et al.*, 1993) and NG108-15 cells (Hare and Atchison, 1995), most of this Ca^{2+} originates in the SER. Both organic and inorganic mercurials cause rapid release of Ca^{2+} from muscle SER-derived vesicles (Abramson *et al.*, 1983). The release of SER Ca^{2+} may result from the observed inhibition of the SER Ca^{2+}-ATPase by MeHg, presumably resulting from its binding to critical thiols in the Ca^{2+} pump (Abramson *et al.*, 1983; Chiu *et al.*, 1983), or from the ability of MeHg to cause non-specific damage to lipid membranes through lipid peroxidation (Sarafian and Verity, 1991; Verity *et al.*, 1994; InSug *et al.*, 1997). However, the Ca^{2+} release may also occur by more specific mechanisms mediated by intracellular Ca^{2+} channels located on the SER membrane, such as the IP_3 and ryanodine receptors.

There are three subtypes of IP_3 receptor (Types I, II and III) which are differentially expressed throughout the CNS. In rat brain, Type I is the primary neuronal type, expressed in large quantities in the cerebellar Purkinje neurons as well as in the neighboring granule cells (which also express Type III receptors) (Sharp *et al.*, 1999). Type II receptors are found only in glia, while Type III receptors are enriched in limbic and basal forebrain regions, particularly in the nerve terminals (Sharp *et al.*, 1999). Additionally, there is a developmental time-source for the expression of receptor subtypes within the CNS, such that during embryonic development, only the Type III receptor is expressed in large quantities, while the Type I receptor is expressed highly only postnatally (Sharp *et al.*, 1999). Expression of the Type III receptor is hypothesized to play a role in the normal onset of programmed cell death in the brain during

development (Sharp *et al.*, 1999; Blackshaw *et al.*, 2000). T cells that are deficient in IP_3 Type I receptors are resistant to multiple inducers of apoptosis, suggesting that these receptors may play a role in Ca^{2+}-mediated cell death (Jayaraman and Marks, 1997; Marks, 1997). Type III IP_3 receptor expression is augmented in lymphocytes undergoing cell death, and Type III IP_3 receptors are expressed at high levels during developmental apoptosis in postnatal cerebellar granule cells, chick dorsal root ganglia and embryonic hair follicles (Blackshaw *et al.*, 2000). Introduction of a Type III IP_3 receptor antisense oligonucleotide prevents cell death caused by removal of nerve growth factor in chick dorsal root ganglia, implicating the Type III receptor in neuronal death processes (Blackshaw *et al.*, 2000). Thus, IP_3 receptors are involved not only in $Ca^{2+}{}_i$ signaling but also in neuronal development and death.

The differential expression of these receptors becomes important when addressing MeHg neurotoxicity, as the second messenger IP_3 and its receptor have been implicated as playing a role in MeHg-induced alterations of $[Ca^{2+}]_i$. In rat cerebellar granule cells in culture, exposure to 5 μM MeHg for 30 min resulted in a twofold increase in cellular inositol levels which potentially contributes to the observed elevation of $[Ca^{2+}]_i$ observed in this system (Sarafian, 1993). Presumably, this increased $[IP_3]$ contributes to the release of intracellular Ca^{2+} that was observed within the same time frame (Sarafian, 1993). In rat cerebellar membrane preparations, MeHg stimulates binding of [^{3}H]1,4,5-triphosphate and [^{3}H]inositol 1,3,4,5-tetrakisphosphate binding to IP_3 receptors (Chetty *et al.*, 1996). Additionally, rats treated with 2.5 mg/kg body wt/day MeHg for 7 days exhibited an increase in binding of [^{3}H]IP_3 to cerebellar membranes; this increased binding was not observed following treatment with inorganic mercury or lead (Chetty *et*

al., 1996). MeHg-induced alterations in IP_3 production are not limited to the nervous system but are also found on the immune system. In rat T lymphocytes, MeHg causes sustained elevations in $[Ca^{2+}]_i$ which, like those observed in cerebellar granule cells, consist of an intracellular and extracellular component (Tan *et al.*, 1993). The release of $Ca^{2+}{}_i$ was largely blocked by the IP_3 receptor antagonist heparin (Tan *et al.*, 1993). In NG108-15 cells, depletion of the IP_3-sensitive Ca^{2+} pool reduced, but did not abolish, the MeHg-induced increase in $[Ca^{2+}]_i$ (Hare and Atchison, 1995). Interestingly, in these cells, MeHg did not cause an increase in $[IP_3]$, suggesting that MeHg may interact directly with the receptor to cause Ca^{2+} release. Finally, in mouse lymphocytes, exposure to 1 μM MeHg for 1 hr resulted in a 50% reduction in the rate of inositol metabolism, suggesting that once $[IP_3]$ becomes elevated, MeHg maintains $[IP_3]$ at higher-than-normal levels (Nakatsuru *et al.*, 1985). Thus, MeHg causes elevated $[IP_3]$ in a variety of neurons and other cells.

Ca^{2+} release from the SER can also occur by opening of ryanodine receptor-linked channels localized to the SER membrane. Ryanodine receptors, like IP_3 receptors, are expressed as three different isoforms (I, II and III) which are expressed differentially in the CNS. Ryanodine receptors are involved in the process of CICR, in which cytosolic Ca^{2+} activates ryanodine receptors to cause Ca^{2+} release from the SER (Irving *et al.*, 1992), and have also been implicated as mediators of neuronal death (Marks, 1997). There is also limited evidence that MeHg targets ryanodine receptors to cause Ca^{2+} release from the SER. Inhibition of Ca^{2+} release through ryanodine receptors causes minor reductions in the amplitude of the MeHg-induced $[Ca^{2+}]_i$ increase in NG108-15

cells (Hare and Atchison, 1995). However, the role of ryanodine receptors in MeHg-induced cell death has not yet been investigated.

vi) MeHg and Mitochondria

A number of studies indicate that MeHg targets several parameters of mitochondrial function, including respiration and oxidative phosphorylation. Additionally, MeHg has serious effects on the regulation of mitochondrial Ca^{2+}. In mitochondria derived from rat forebrain, MeHg causes release of $^{45}Ca^{2+}$ from preloaded mitochondria while preventing further Ca^{2+} uptake (Levesque and Atchison, 1991). MeHg-induced alterations in neurotransmitter release appear to result primarily from perturbation of mitochondrial Ca^{2+} regulation, with minimal or no contribution of Ca^{2+} from the SER in hippocampal slices (Gasso *et al.*, 2000) and the rat neuromuscular junction (Levesque and Atchison, 1988).

More recent evidence suggests that MeHg causes opening of the MTP, which has been implicated in apoptotic cell death caused by a wide variety of toxic insults (Ichas and Mazat, 1998; Lemasters *et al.*, 1998). Excess accumulation of Ca^{2+} within the mitochondrial lumen, production of reactive oxygen species, and depletion of ATP are all activators of the MTP both in cells and in isolated mitochondria (see Bernardi *et al.*, 1998, for review). In MeHg toxicity, there is substantial evidence for the induction of all three of these conditions; thus it is not surprising that in recent studies with human monocytes (InSug *et al.*, 1997) and lymphoid cells (Shenker *et al.*, 2000), there is evidence of MeHg-induced opening of the MTP. Additionally, transfection of the murine GT1-7 hypothalamic neuronal cell line with the MTP inhibitor *bcl-2* reduces cell death

caused by MeHg, suggesting an important role for the MTP in MeHg-induced neuronal death (Sarafian *et al.*, 1994).

MeHg is also widely recognized as causing oxidative stress. MeHg-induced elevations of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals and hydrogen peroxide, are observed in human monocytes (InSug *et al.*, 1997), human T cells (Shenker *et al.*, 1998), and murine and rat brain (Ali *et al.*, 1992; Yee and Choi, 1994). MeHg also causes lipid peroxidation in rat cerebellar granule cells in suspension (Sarafian and Verity, 1991). The antioxidant vitamin E provides some protection against neuronal damage *in vivo* (Chang *et al.*, 1978; Usuki *et al.*, 2001), suggesting that ROS contribute to cellular damage caused by MeHg. The ability of a cell to handle ROS may be correlated with its sensitivity to MeHg. MeHg-induced increases in free radicals occur in brain regions specifically sensitive to the effects of MeHg, including the cerebellum (LeBel *et al.*, 1990; LeBel *et al.*, 1992). Additionally, cells expressing increased glutathione (GSH) exhibit decreased sensitivity to MeHg-induced damage (Miura *et al.*, 1994). However, there is considerable evidence that MeHg-induced oxidative stress may not be the determinant factor in MeHg-induced neuronal death in specific brain regions. Vitamin E prevents MeHg-induced increases in ROS and lipid peroxidation but does not protect against cell death in rat cerebellar granule cells (Sarafian and Verity, 1991). Additionally, modulation of intracellular GSH levels using N-acetylcysteine (to increase GSH) or L-buthionine-(S,R)-sulfoximine (BSO) (to decrease GSH) did not alter the neurotoxic effects of MeHg in primary cultures of rat embryonic CNS cells (Ou *et al.*, 1999). Taken together, these studies suggest that while

MeHg causes increased levels of ROS in both adult and developing brains, the resultant damage is not a determinant factor in the selective neurotoxicity of this compound.

vii) MeHg-Induced Interaction Between SER and Mitochondria Ca^{2+} Stores

There is growing evidence that interactions between SER and mitochondria are important in determining cell survival. In permeabilized RBL-2H3 cells (Csordas *et al.*, 1999), permeabilized hepatocytes (Hajnoczky *et al.*, 1999) and rat pulmonary smooth muscle cells (Drummond and Tuft, 1999), Ca^{2+} release from the IP_3 receptor results in a strong Ca^{2+} signal at the mitochondria. In HeLa cells transfected with specifically targeted, Ca^{2+} sensitive green fluorescent proteins, Ca^{2+} release from IP_3 receptors results in a higher localized concentration of Ca^{2+} at the outer surface of nearby mitochondria than is observed in the cytosol (Rizzuto *et al.*, 1998), revealing the preferential signal received by mitochondria from the SER. This Ca^{2+} signal between the SER and mitochondria can be translated into a pro-apoptotic signal; in a human tumor cell line, activation of IP_3 receptors releases Ca^{2+} which causes opening of the MTP in mitochondria, with subsequent release of cytochome c and promotion of apoptotic cell death (Szalai *et al.*, 1999). Mitochondria also communicate with the SER in a negative feedback system which alters SER Ca^{2+} uptake and release (Landolfi *et al.*, 1998), indicating the complex nature of this signaling pathway. Thus, any MeHg-induced changes in the $[Ca^{2+}]$ of one intracellular store must be considered in the context of its effects on the other store, and *vice versa*, in order to account for the complicated pattern of Ca^{2+} signaling which occurs within neurons.

viii) Non-Ca^{2+} Divalent Cations

Elevation of intracellular concentrations of non-Ca^{2+} divalent cations is also observed during MeHg exposure in NG108-15 cells (Hare *et al.*, 1993; Denny and Atchison, 1994), rat cerebellar granule cells (Marty and Atchison, 1997), and synaptosomes (Denny and Atchison, 1994). In synaptosomes, the non-Ca^{2+} cation was identified using ^{19}F-NMR as Zn^{2+} (Denny and Atchison, 1994). The elevated $[Zn^{2+}]_i$ was possibly due to the displacement of endogenous Zn^{2+} by MeHg from the numerous proteins which bind Zn^{2+} with cysteine sulfhydryl groups (Hunt *et al.*, 1984; Vallee and Auld, 1990). Zn^{2+} itself can be neurotoxic (Manev *et al.*, 1997; Cookson and Shaw, 1999), and has recently been shown to induce opening of the MTP in cortical neurons (Sensi *et al.*, 2000). In rats exposed to MeHg there was an elevated $[Zn^{2+}]$ in the brain which was not found in control or inorganic Hg-exposed animals (Muto *et al.*, 1991). MeHg also caused elevated $[Zn^{2+}]$ in the kidney, another sensitive target organ during MeHg toxicity (Muto *et al.*, 1991). In spite of the intriguing possibility that disruption of $[Zn^{2+}]_i$ is a decisive factor in the selective toxicity of MeHg, several lines of evidence argue against this hypothesis. First, the non-Ca^{2+} component of the MeHg-induced increase in cerebellar granule cells is much smaller in amplitude than the Ca^{2+} component (Marty and Atchison, 1997). Second, chelation of non-Ca^{2+} divalent cations with the cell-permeant heavy metal chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) does not protect against cell death caused by low micromolar concentrations of MeHg at 3.5 hr post-exposure (Marty and Atchison, 1998). Finally, in cerebellar granule neurons in culture, Zn^{2+}-mediated cell death was found to depend on protein synthesis (Manev *et al.*, 1997). MeHg inhibits protein synthesis in cerebellar neurons (Sarafian *et*

al., 1984; Sarafian and Verity, 1985), cerebral cortex synaptosomes (Verity *et al.*, 1977), and brain cortex slices (Yoshino *et al.*, 1966), suggesting that the mechanism by which Zn^{2+} exerts its toxic effects is non-functional during MeHg exposure. Thus, the contribution of elevated $[Zn^{2+}]_i$ to MeHg-induced neurotoxicity is minimal at best.

C) Rat Cerebellar Granule Neurons in Primary Culture as a Model for the Selective Neurotoxicity of MeHg

As described above, MeHg causes preferential degeneration of the granule cell layer of the cerebellar cortex in humans and experimental animals alike (Hunter and Russell, 1954; Syversen *et al.*, 1981; Leyshon and Morgan, 1991). While studies performed *in vivo* provide the closest parallel to human exposures, they do not provide enough information regarding the mechanisms underlying the selective neurotoxicity of MeHg. Additionally, experiments performed *in vivo* are complicated by the large number of interacting variables between different types of neurons within the cerebellar cortex. Thus, the experiments in this dissertation were performed in rat cerebellar granule cells in primary culture to allow for in-depth exploration of the actions of MeHg in these cells. Because cerebellar granule cells are the most populous neuronal type in the cerebellum, and are affected by several diseases and toxicants, they have been well characterized *in vivo*. When isolated from neonatal rats (approximately 6-7 days old), cerebellar granule cells grown in culture continue to develop and mature; they extend processes which bifurcate into fibers resembling the parallel fibers found in the cerebellar cortex, and make functional synapses with neighboring neurons. The presence and activity of many plasma membrane and intracellular ion channels, receptors and proteins have been well

characterized for the first 14 days post-isolation, with developmental profiles compiled for many cellular proteins (Gonzalez *et al.*, 1992; Aronica *et al.*, 1993; Aronica *et al.*, 1993; Aronica *et al.*, 1993; Resink *et al.*, 1994; Xia *et al.*, 1995; de Luca *et al.*, 1996). In the experiments performed for this dissertation, all cells were used at days 6-8 post-isolation to allow for the maturation of cells. At this time in culture, granule cells express L, N, P/Q and R types of voltage-gated Ca^{2+} channels; M2 and M3 muscarinic receptors; metabotropic glutamate receptors; excitatory amino acid (glutamate) receptors (N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-5-isoxazole propionate (AMPA), and kainate); $GABA_A$ receptors; Type I IP_3 receptors; and Type II ryanodine receptors, among other receptors and channels (Meier *et al.*, 1984; Alonso *et al.*, 1990; Pizzi *et al.*, 1991; Aronica *et al.*, 1993; Aronica *et al.*, 1993; Randall and Tsien, 1995; Simpson *et al.*, 1996; Randall and Tsien, 1997; Mhyre *et al.*, 2000; Mogensen and Jorgensen, 2000). Thus, the cells express many of the channels and receptors which characterize functionally mature cerebellar granule cells, making granule cells is primary culture a useful model for the study of MeHg neurotoxicity.

The use of granule cells in culture is limited, however, by the lack of normal interactions with other neurons that are present in the intact animal. In particular, there is a relative lack of astrocytes in the culture, which comprise less than 10% of the cells in the culture (Marty and Atchison, 1997). Astrocytes have been intensively studied as a potential mediator of MeHg neurotoxicity (Aschner, 1996). Astrocytes concentrate MeHg and, when exposed to MeHg, release the neurotransmitter glutamate (Oyake *et al.*, 1966; Garman *et al.*, 1975; Aschner *et al.*, 1994; Aschner *et al.*, 2000). Excess glutamate causes excitotoxic death in cerebellar granule cells by causing influx of Ca^{2+}_e through

NMDA receptors (Ankarcrona *et al.*, 1995; Budd and Nicholls, 1996a; Nicholls *et al.*, 1999). Thus, MeHg-induced release of glutamate from astrocytes may contribute to the toxicity of MeHg *in vivo*. In granule cells in primary culture, inhibition of ion flux through NMDA, kainite and AMPA receptors does not alter MeHg-induced elevations of $[Ca^{2+}]_i$ (Marty and Atchison, 1997); however, these cells are grown in the relative absence of astrocytes, which would be present *in vivo*. The lack of astrocytes in the culture is a disadvantage when comparing the results obtained in the culture system to those obtained in the whole animal. However, in examining the relative sensitivity of granule cells to MeHg-induced damage, the lack of astrocytes is advantageous, as it removes a potentially contributing factor to MeHg-induced neurotoxicity which is probably not unique to the cerebellar cortex. Additionally, granule cells in culture undergo cell death at much lower concentrations of MeHg than is observed in other less-sensitive cell types, suggesting that granule cells are inherently more sensitive to MeHg-induced cell death in the absence of any external contributing factors (Park *et al.*, 1996; Marty and Atchison, 1998). Thus, the use of granule cells in primary culture provides an opportunity to identify potential molecular targets which confer the unique sensitivity of this cell type to the effects of MeHg.

CHAPTER TWO

INTERACTION OF METHYLMERCURY WITH MUSCARINIC RECEPTORS CAUSES CALCIUM RELEASE FROM THE SMOOTH ENDPLASMIC RETICULUM INOSITOL-1,4,5-TRIPHOSPHATE RECEPTORS IN RAT CEREBELLAR GRANULE NEURONS

ABSTRACT

The contribution of Ca^{2+} release from the SER to MeHg-induced cation dysregulation was studied using single-cell microfluorimetry of granule cells *in vitro* loaded with fura-2. Depletion of SER Ca^{2+} with thapsigargin (10 μM) prior to MeHg (0.2-1.0 μM) exposure decreased the amplitude of MeHg-induced release of $[Ca^{2+}]_i$ stores by approximately 30%, indicating that SER Ca^{2+} is released by MeHg. However, the time-to-onset of $[Ca^{2+}]_i$ elevations by MeHg was unaffected by thapsigargin treatment. When the muscarinic receptors and inositol-1,4,5-triphosphate (IP_3) receptors were down-regulated by 24 hr pretreatment with bethanechol (BCh, 1 mM), MeHg-induced release of $Ca^{2+}{}_i$ was again reduced by 30-40%. BCh pretreatment significantly hastened the time-to-onset of the MeHg-induced release of $Ca^{2+}{}_i$ without altering the influx of $Ca^{2+}{}_e$. Atropine (10 μM) significantly delayed the time-to-onset and reduced the amount of $[Ca^{2+}]_i$ released into the cytosol by MeHg, suggesting that MeHg interacts with muscarinic receptors and produces IP_3 , which subsequently releases Ca^{2+} from the SER. Ryanodine (10 μM) did not affect the time-to-onset or amplitude of MeHg-induced increases in $[Ca^{2+}]_i$. Thus, MeHg interacts with muscarinic receptors with the end result of Ca^{2+} release from the SER, which contributes to the MeHg-induced loss of $[Ca^{2+}]_i$ regulation.

INTRODUCTION

As discussed in Chapter One, MeHg causes an early release of Ca^{2+} from at least one intracellular source in cerebellar granule cells in culture (Marty and Atchison, 1997). One possible source is the SER, which is a high-affinity, low-capacity Ca^{2+} pool filled by the SERCA (Irving *et al.*, 1992; Gunter and Gunter, 1994). Under normal conditions, Ca^{2+} release from the SER into the cytosol occurs *via* the IP_3 receptor and/or the ryanodine receptor. In NG108-15 cells, most of the MeHg-induced first-phase is due to release of Ca^{2+} from the IP_3-sensitive pool in the SER, with some contribution from the ryanodine-sensitive pool; however, emptying this pool with the SERCA inhibitor thapsigargin does not eliminate the first phase, suggesting that at least one additional source contributes to the first phase Ca^{2+} increase (Hare and Atchison, 1995). In granule cells, the contribution of the SER to MeHg-induced elevations of $[Ca^{2+}]_i$ is examined for the first time in the experiments presented in this chapter.

At the SER, both organic and inorganic mercurials cause rapid release of Ca^{2+} from muscle SER-derived vesicles (Abramson *et al.*, 1983). In granule cells in culture, MeHg causes an increase in $[IP_3]$ at low micromolar concentrations (Sarafian, 1993), although this increase in $[IP_3]$ is not observed in all neuronal models (Hare and Atchison, 1995) and has only been observed in granule cells after exposure to 5 μM MeHg for 30 min, which does not shed light on whether there is an early increase in $[IP_3]$ (Sarafian, 1993). The elevation of $[IP_3]$ could be due to binding of MeHg to muscarinic receptors, which has been observed in several neuronal systems (Eldefrawi *et al.*, 1977; Von Burg *et al.*, 1980; Abd-Elfattah and Shamoo, 1981; Castoldi *et al.*, 1996; Candura *et al.*, 1997). Granule cells express M2 and M3 muscarinic receptors, with the M3 receptors linked to

PLC (Fukamauchi *et al.*, 1991; Fohrman *et al.*, 1993). MeHg is also reported to stimulate IP_3 binding to its receptor in rat cerebellar membrane preparations, suggesting that MeHg interacts with the IP_3 receptor itself (Chetty *et al.*, 1996).

The experimental approach taken to study the contribution of SER Ca^{2+} was similar to that used by Hare and Atchison (1994) and Marty and Atchison (1997), in which different receptor agonists and antagonists were used to inhibit different portions of Ca^{2+} signaling pathways during MeHg exposure. First, the SERCA inhibitor thapsigargin was applied prior to MeHg application to deplete the SER of its Ca^{2+} content (Thastrup *et al.*, 1990; Irving *et al.*, 1992). Second, the muscarinic and IP_3 receptors were down-regulated prior to MeHg exposure using the muscarinic agonist bethanechol (BCh); this approach was taken to observe the contribution of the entire M3-PLC-IP_3 receptors pathway to MeHg-induced increases in $[Ca^{2+}]_i$ (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Wojcikiewicz *et al.*, 1994; Simpson *et al.*, 1996; Fukamauchi *et al.*, 2000). Third, the nonspecific muscarinic antagonist atropine was used to determine if MeHg interacts with these receptors to causes elevations of $[Ca^{2+}]_i$ (Yan *et al.*, 1995). Finally, the ryanodine receptor antagonist ryanodine was used to inhibit Ca^{2+} mobilization through these channels (Irving *et al.*, 1992). The results presented here indicate that MeHg interacts with muscarinic receptors to cause Ca^{2+} release from the SER IP_3-sensitive Ca^{2+} pool, and that this Ca^{2+} contributes to the whole-cell elevation of $[Ca^{2+}]_i$ observed during MeHg neurotoxicity.

MATERIALS AND METHODS

Materials and Experimental Solutions. Deoxyribonuclease I (DNase I) and type II trypsin were purchased from Worthington Biochemicals (Freehold, NJ). Cell culture supplies, including Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY). SK&F 96365 was purchased from Calbiochem (La Jolla, CA). Fura-2 acetoxymethylester (fura-2 AM) was purchased from Molecular Probes (Eugene, OR). Thapsigargin, carbamyl-β-methylcholine chloride (bethanechol), carbamylcholine (carbachol), atropine, ryanodine, poly-D-lysine and cytosine-β-arabinofuranoside were purchased from Sigma (St. Louis, MO). Methyl mercuric chloride (MeHg) was purchased from ICN Biochemicals Inc. (Aurora, OH) and dissolved in deionized water.

Granule cell isolation. Cerebellar granule cells are isolated as described previously (Marty and Atchison, 1997). Approximately 5-10 7-day-old Sprague Dawley rats were used *per* isolation. Each rat was decapitated, then the skin was cut down the dorsal midline from the neck to the nose. Using the flat end of the scissors, the skull was carefully peeled back to reveal the brain. The cerebellum was cut along each side using scissors, then gently lifted away from the rest of the brain. Each extracted cerebellum was placed in an ice-cold beaker containing Ca^{2+}-Mg^{2+} free Hank's Buffered Saline Solution (CMF-HBSS). CMF-HBSS contains (mM): 5.36 KCl, 0.44 KH_2PO_4, 136.89 NaCl, 20.02 HEPES, 0.59 EDTA, 5.5 *d*-glucose, and 4.17 $NaHCO_3$ (pH 7.3, 37°C). Once all cerebella had been dissected, the meninges were removed and the tissue finely minced using sterile scissors. The tissue was then added to a beaker containing 0.25%

trypsin (w/v) and digested at 37°C in a shaking water bath for 15 min. Cold (4°C) DMEM with 5% fetal bovine serum and 0.04% DNase I (w/v) was added to inactivate the trypsin. The cell suspension was centrifuged at 134*g* for 5 min (4°C), then the pellet was resuspended in DMEM with 5% FBS and 0.04% DNase I and triturated approximately 15 times with a wide-bore fire-polished Pasteur pipette. The cell suspension was allowed to sit for 4 min on ice, then the supernatant (which contained the dissociated cells) was transferred to a centrifuge tube on ice. The addition of FBS-DNase followed by trituration, settling of debris and transfer of supernatant was performed two more times. The collected supernatant was gently underlaid with 4% BSA and 3.82% $MgSO_4$ (dissolved in CMF-HBSS) and the tube was centrifuged at 134*g* at 4°C for 5 min. The resulting pellet was resuspended in the plating medium that consists of DMEM with 10% FBS, 25 mM KCl, 50μM GABA, 50μM kainate, 5 μg/ml insulin, 100 U/ml penicillin and 50 μg/ml streptomycin. Cells were plated at a density of 2.0-2.2x10^6 cells/35 mm dish, each containing a 25 mm glass coverslip coated with 0.1 mg/ml poly-D-lysine. After 21 hours, 10 μM cytosine-β-arabinofuranoside was added to inhibit glial cell proliferation. Penicillin-streptomycin was not added at this or subsequent medium changes because streptomycin alters Ca^{2+} channel function (Atchison *et al.*, 1988; Redman and Silinsky, 1994). Cells for all experiments were used at 6-8 days *in vitro* to allow for cell attachment and maturation.

Measuring changes in $[Ca^{2+}]_i$. Unless otherwise noted, the standard buffer used for experimental solutions is HEPES Buffered Saline (HBS) which contains (mM): 150 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 20 *d*-glucose, and 20 HEPES (free acid) (pH 7.3 at room temperature of 23-25°C). The 40 mM K^+ solution contains the same components

as HBS except with 40 mM K^+ and 115.4 mM NaCl. The low-$[Ca^{2+}]$, EGTA-containing buffer (EGTA-HBS) has the same components as HBS minus $CaCl_2$ and plus 20 μM EGTA. For all experiments, pharmacological agents are dissolved in either HBS or EGTA-HBS, with appropriate controls for any additional solvents used (DMSO or ethanol, maximum final solvent of 0.01% (v/v), for all experiments). In a cell-free system, fura-2 did not interact with MeHg or any of the agents used (BCh, atropine, thapsigargin and ryanodine) at concentrations used in the present study (results not shown).

To measure changes in $[Ca^{2+}]_i$, cells were loaded with 3-4 μM fura-2 AM in HBS for 1 hr at 37°C, followed by perfusion with HBS for 30 min. Digital fluorescent images were obtained using a Nikon Diaphot microscope (Nikon, Tokyo, Japan) or an Olympus IX70 microscope (Olympus Optical Co., Tokyo, Japan), coupled to an IonOptix system (Milton, MA) with an Open Perfusion Micro-Incubator (Medical Systems Corp.), maintained at 37°C. For each experiment, changes in emitted fluorescence at excitation wavelengths of 340 and 380 nm were monitored simultaneously in multiple soma (3-10 cells *per* experiment) within the same microscopic field. The fluorescence ratio ($F_{340/380}$) indicated the approximate amount of $[Ca^{2+}]_i$, however the data were not converted to $[Ca^{2+}]_i$ due to fura-2 interactions with other divalent cations, such as Zn^{2+}, a cation known to play a role in the MeHg response (Hare *et al.*, 1993; Denny and Atchison, 1994).

All experiments began with a 1 min wash with HBS (to establish baseline fluorescence), followed by 1 min exposure to 40 mM K^+ solution; only cells which exhibited a reversible increase in $[Ca^{2+}]_i$ following the K^+ exposure were considered to be viable for the experiment. The K^+ solution was washed out for 3 min with HBS, then one

of the experimental protocols was followed. Pharmacological agents were used either to empty the SER of its Ca^{2+} content or block Ca^{2+} release from receptors on the SER membrane. To empty the SER prior to addition of MeHg, the SERCA inhibitor thapsigargin (10 μM) was applied for 5 min immediately prior to MeHg (0.2-1.0 μM) in HBS or EGTA-HBS (Irving *et al.*, 1992; Simpson *et al.*, 1996). To block the muscarinic receptors, the nonspecific muscarinic receptor antagonist atropine (10 μM) was applied for 10 min prior to, as well as during, exposure to 0.2-1.0 μM MeHg in HBS or EGTA-HBS (Xu and Chuang, 1987; Yan *et al.*, 1995). To inhibit Ca^{2+} release from the ryanodine receptors, 10 μM ryanodine was applied for 10 min prior to, as well as during, exposure to 0.2-1.0 μM MeHg in HBS or EGTA-HBS (Irving *et al.*, 1992). To down-regulate the M3 muscarinic receptors and thus subsequently the IP_3 receptors, 1 mM bethanechol (BCh) was added directly to the growth media 24-30 hr prior to loading cells with fura-2 (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Wojcikiewicz *et al.*, 1994; Simpson *et al.*, 1996; Fukamauchi *et al.*, 2000). For BCh experiments, the fura-2 AM was added directly to the growth media, thus cells were in BCh-free solution for the 30 min HBS rinse and the 5 min viability test with 40 mM K^+. In these experiments, MeHg (0.2-1.0 μM) in HBS or EGTA-HBS was perfused immediately following the 3 min K^+ solution wash period.

Following exposure to MeHg, the time-to-onset of the first and second $Ca^{2+}{}_i$ phases was determined: the first phase (from release of $Ca^{2+}{}_i$ pools) was measured from the point at which the ratioed fluorescence irreversibly leaves baseline, and the second phase began at the point at which the 380 nm fluorescence sharply drops. For experiments performed in the absence of $Ca^{2+}{}_e$, the amplitude of the fura-2 ratio increase was

determined as the fluorescence increase above baseline, and was normalized to the peak fura-2 ratio caused by the 1 min exposure to 40 mM K^+ ("normalized ratio"). Ratio data was normalized to remove slight variations in fluorescence intensity resulting from differences in cell size and fura-2 loading efficiency. For both time-to-onset and normalized ratio experiments, data from each cell observed in an experiment were averaged to provide mean time-to-onset for that dish of cells (n=1). In order to minimize differences between cell isolates, experiments using MeHg alone and MeHg with the pharmacological agent were run on the same day, and experiments using the same agents were performed in at least two separate cell isolates.

Statistics. Comparisons of mean time-to-onset for "MeHg" *vs* the corresponding "MeHg plus inhibitor" cells are made using Student's paired t-test, with values of $p<0.05$ considered to be statistically significant.

RESULTS

The SER contributes Ca^{2+} to the MeHg-induced release of Ca^{2+}_i. To determine whether the SER contributed to the first-phase $[Ca^{2+}]_i$ elevation, the SER was depleted of its Ca^{2+} content with the SERCA inhibitor thapsigargin. To verify that thapsigargin abolished agonist-induced release of Ca^{2+} from this organelle, experiments were performed using the nonspecific muscarinic agonist bethanechol (BCh, 1 mM), which activates M3 muscarinic receptors to cause production of IP_3 and subsequent release of Ca^{2+} from the IP_3 receptor (Figure 2.1). A 5 min application of 10 μM thapsigargin abolished the BCh-activated increase in $[Ca^{2+}]_i$ in 13 of 15 cells examined (from 3 dishes *per* treatment and two separate cell isolations), indicating the effectiveness of thapsigargin in reducing the SER Ca^{2+} pool (Table 2.1). Thapsigargin (10 μM) was then applied for 5 min immediately prior to MeHg (0.2-1.0 μM). In the presence of Ca^{2+}_e, thapsigargin did not alter the time-to-onset of the first-phase release of $[Ca^{2+}]_i$ or the second-phase influx of $[Ca^{2+}]_e$ at any MeHg concentration tested (Figure 2.2). The time-to-onset for the first-phase increase in fura-2 ratio between neurons treated with MeHg only and MeHg plus thapsigargin was not different for 0.2, 0.5 or 1.0 μM MeHg. Additionally, thapsigargin did not change the time-to-onset of the second-phase Ca^{2+} influx for 0.2, 0.5 or 1.0 μM MeHg. In nominally Ca^{2+}_e-free solution ("EGTA-HBS"), thapsigargin again did not alter the time-to-onset of the first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5 or 1.0 μM MeHg (Figure 2.3).

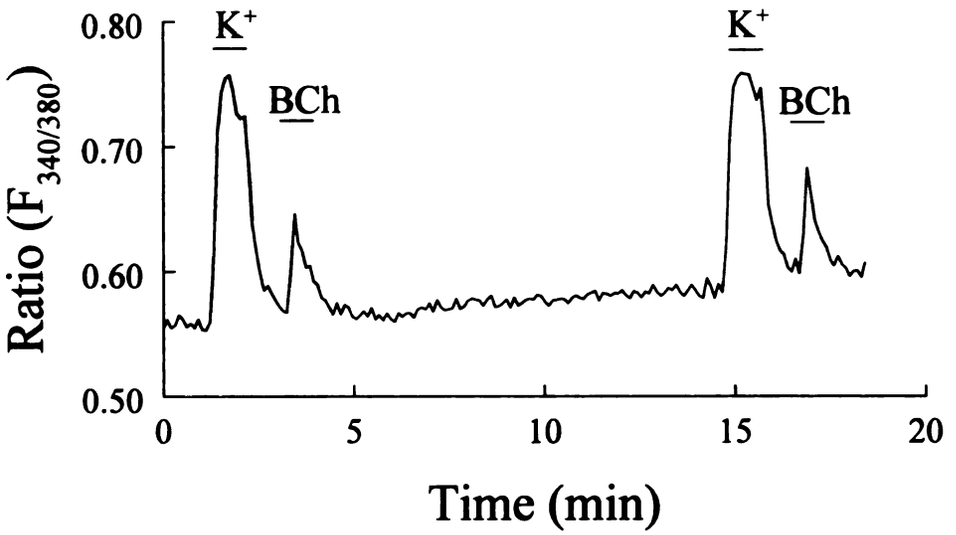

Figure 2.1. Alternating exposures to 40 mM K^+ and 1 mM BCh cause increases in $[Ca^{2+}]_i$ in cerebellar granule cells. Representative changes in fura-2 fluorescence ("Ratio") caused by alternating exposure to 40 mM K^+ (1 min) and 1 mM BCh (1.5 min), in a single granule cell. The amplitude of the fluorescence change in response to the BCh is normalized to the response to the preceding K^+ exposure as a relative measure of the amount of Ca^{2+} released from the IP_3-sensitive Ca^{2+} pool. This protocol was used to test the effect of pharmacological agents on release of Ca^{2+} from the IP_3-sensitive Ca^{2+} pool (see Table 2.1).

INHIBITOR	NORMALIZED RATIO (NO INHIBITOR)	NORMALIZED RATIO (WITH INHIBITOR)	PERCENT DECREASE
No inhibitor (control)	0.71±0.11	0.56±0.11	21.1%
Thapsigargin (10 μM)	0.59±0.08	0.04±0.03*	93.2%
BCh (1 mM)	0.13±0.01	0.03±0.01*	76.9%
Atropine (10 μM)	0.42±0.03	0.004±0.004*	99.3%
Ryanodine (10 μM)	0.51±0.09	0.24±0.04*	53.0%

Table 2.1. Effect of pharmacological inhibitors on peak fura-2 ratio induced by 1.5 min 1 mM BCh in cerebellar granule cells. Cells were exposed to alternating challenges of 40 mM K^+ (1 min) and 1 mM BCh (1.5 min), as shown in Figure 2.1. The pharmacological inhibitor was then applied, and the K^+-BCh protocol repeated. The peak fura-2 ratio response to the BCh treatment was normalized to the preceding response to the K^+ depolarization ("normalized ratio"). The peak BCh response was compared in the same cell in the absence or presence of inhibitor. Because not all cells responded to BCh, comparisons were made only in cells which responded to the initial BCh exposure. For the 24 hr 1 mM BCh experiments, comparisons were made between cells receiving 24 hr BCh pretreatment and cells receiving 24 hr vehicle pretreatment. Because the experimental protocol for the 24 hr BCh experiments prevented comparisons within the same cell, all cells were included in this data, including control cells that did not respond to the BCh treatment, to prevent any experimental bias. Thus, the normalized ratios under control conditions are lower than those observed for the other pharmacological agents due to the inclusion of non-responding cells in the 24 hr BCh data. For the ryanodine experiments, 1 mM carbachol was used instead of 1 mM BCh. Results are presented as mean±SEM (n = 3). The asterisk (*) indicates a value significantly different from the response prior to addition of the pharmacological agent ($p<0.05$).

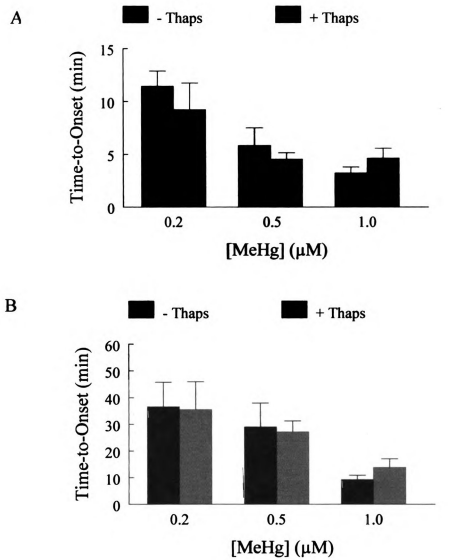

Figure 2.2. Thapsigargin does not affect the time-to-onset of MeHg-induced increases in $[Ca^{2+}]_i$. A) Pretreatment with 10 μM thapsigargin ("thaps") for 5 min did not alter the time-to-onset of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, or 1.0 μM MeHg. (B) In the same cells, pretreatment with 10 μM thapsigargin for 5 min also did not alter the time-to-onset of the MeHg-induced second-phase influx of Ca^{2+}_e caused by 0.2, 0.5, or 1.0 μM MeHg. Results are presented as mean ± SEM (n=3-5).

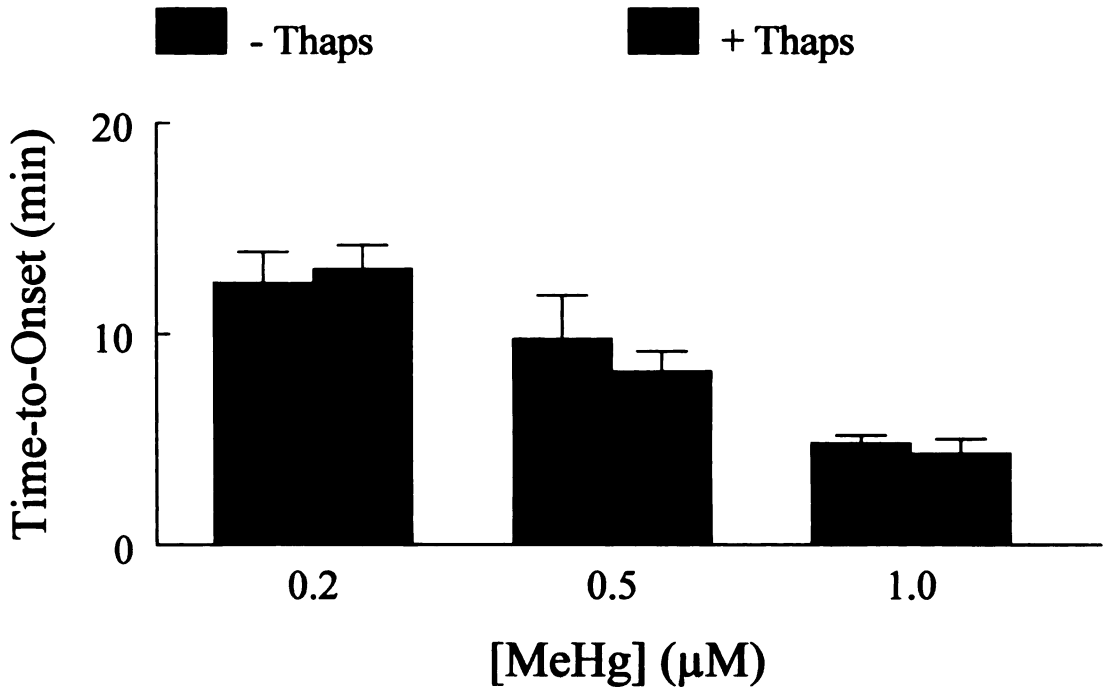

Figure 2.3. Thapsigargin does not affect the time-to-onset of MeHg-induced $[Ca^{2+}]_i$ increases in the absence of Ca^{2+}_e. Pretreatment with 10 μM thapsigargin ("thaps") for 5 min did not alter the time-to-onset of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, or 1.0 μM MeHg, measured in the absence of Ca^{2+}_e. Results are presented as mean ± SEM (n=3-5).

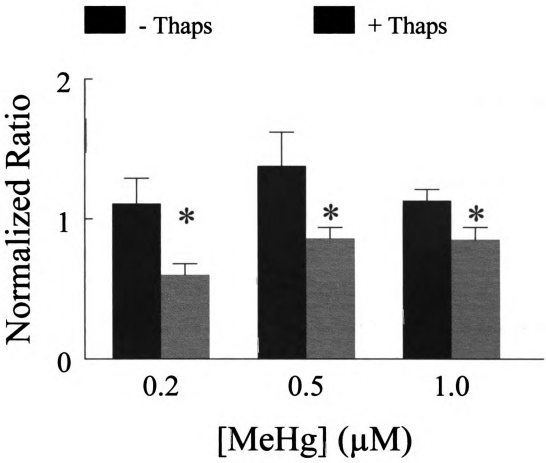

Figure 2.4. Thapsigargin decreases the amplitude of MeHg-induced fura-2 ratio increases, as measured in the absence of Ca^{2+}_e. Pretreatment with 10 μM thapsigargin ("thaps") for 5 min significantly decreased the amplitude of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, and 1.0 μM MeHg. The fura-2 ratio increase was normalized to the amplitude of the 40 mM K^+ response ("Normalized Ratio"). Results are presented as mean ± SEM (n=3-5). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

However, there was a significant decrease in the amplitude of the first phase caused by 0.2 μM MeHg (from a normalized ratio of 1.11±0.2 to 0.60±0.08), 0.5 μM MeHg (from 1.38±0.24 to 0.86±0.08), and 1.0 μM MeHg (from 1.13±0.08 to 0.85±0.09) (Figure 2.4). This represents a decrease in the first-phase ratio amplitude of approximately 46%, 38% and 25% caused by 0.2, 0.5 and 1.0 μM MeHg, respectively.

Down-regulation and desensitization of the M3-IP_3 receptor pathway decreases the MeHg-induced increase in $[Ca^{2+}]_i$. A 24 hr application of 1 mM carbachol causes down-regulation and desensitization of M2 and M3 muscarinic receptors, and IP_3 receptors, in cerebellar granule cells in culture (Fohrman *et al.*, 1993; Simpson *et al.*, 1994). BCh, like carbachol, is a non-specific muscarinic agonist and thus would be expected to have similar effects; BCh, however, has the advantage of being more selective for muscarinic receptors with minimal activity at nicotinic ACh receptors. Pretreatment with 1 mM BCh for 24 hr significantly inhibited the Ca^{2+} increase in response to a 1.5 min exposure to 1 mM BCh from 0.13±0.01 to 0.03±0.01, a 77% loss of the IP_3 response (Table 2.1). The 24 hr BCh treatment was then tested for its effect on MeHg-induced increases of $[Ca^{2+}]$. BCh pretreatment hastened the time-to-onset of the first-phase increase in fura-2 ratio for 0.2 and 0.5μM MeHg, but did not change the time-to-onset of $[Ca^{2+}]_i$ increases caused by 1.0 μM MeHg (Figure 2.5A). However, the time-to-onset of the second-phase influx of Ca^{2+}_e was not changed for MeHg as compared to MeHg plus 24 hr BCh pretreatment for 0.2 μM, 0.5 0r 1.0 μM MeHg (Figure 2.5B).

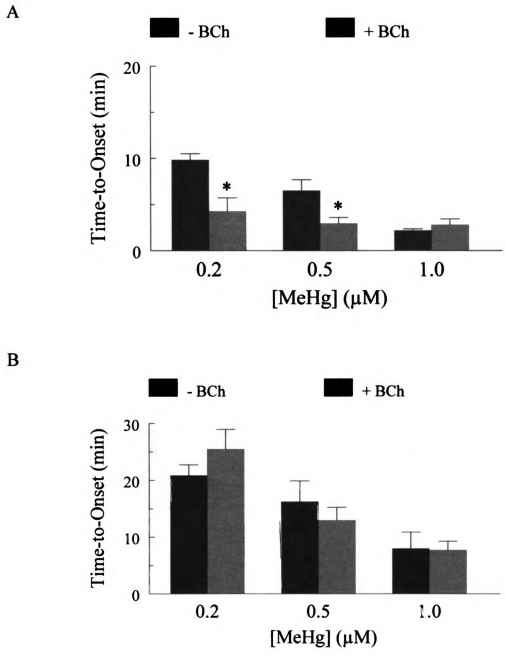

Figure 2.5. Pretreatment with BCh hastens the MeHg-induced release of $[Ca^{2+}]_i$ without affecting the onset of MeHg-induced influx of Ca^{2+}_e. A) Pretreatment with 1 mM BCh for 24 hr significantly hastened the time-to-onset of the first-phase MeHg-induced release of Ca^{2+}_i caused by 0.2 and 0.5, but not 1.0 μM, MeHg. B) In the same cells, 1 mM BCh pretreatment did not alter the time-to-onset of the second-phase influx of Ca^{2+}_e at any MeHg concentration examined. Results are presented as mean ± SEM (n=4-6). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

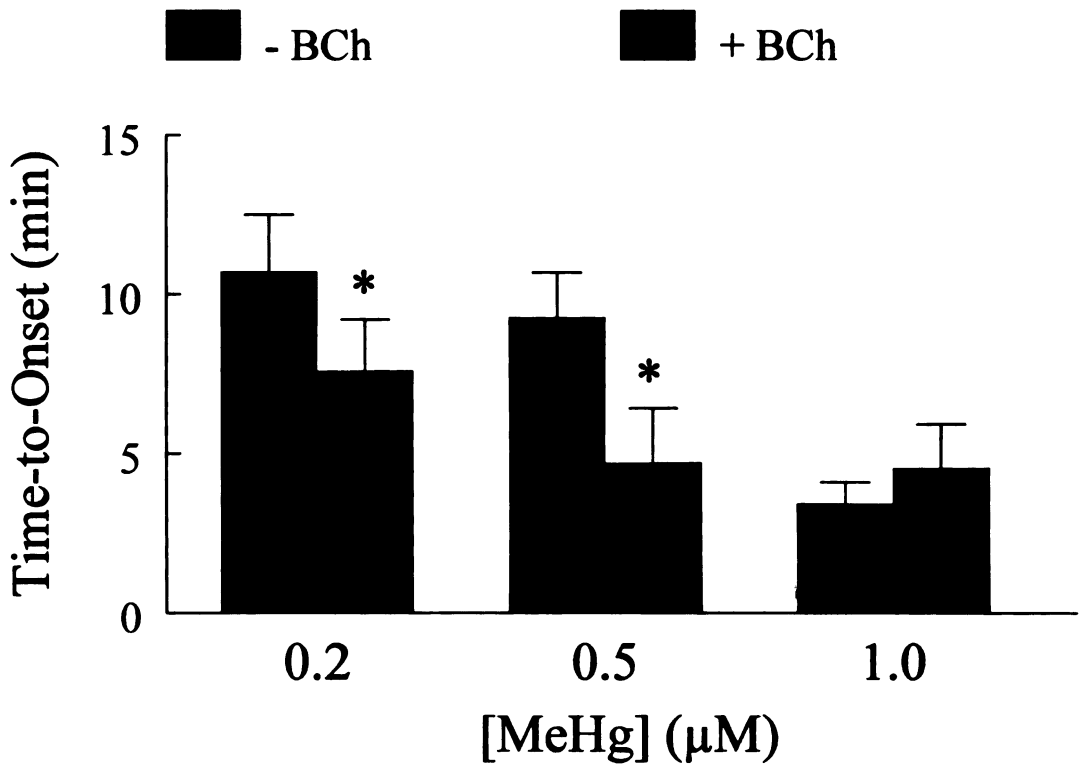

Figure 2.6. Pretreatment with BCh hastens the MeHg-induced release of $[Ca^{2+}]_i$, as measured in the absence of Ca^{2+}_e. Pretreatment with 1 mM BCh for 24 hr significantly hastened the time-to-onset of the first-phase increase in $[Ca^{2+}]_i$ caused by 0.2 and 0.5, but not 1.0, μM, MeHg. Results are presented as mean ± SEM (n=4-5). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

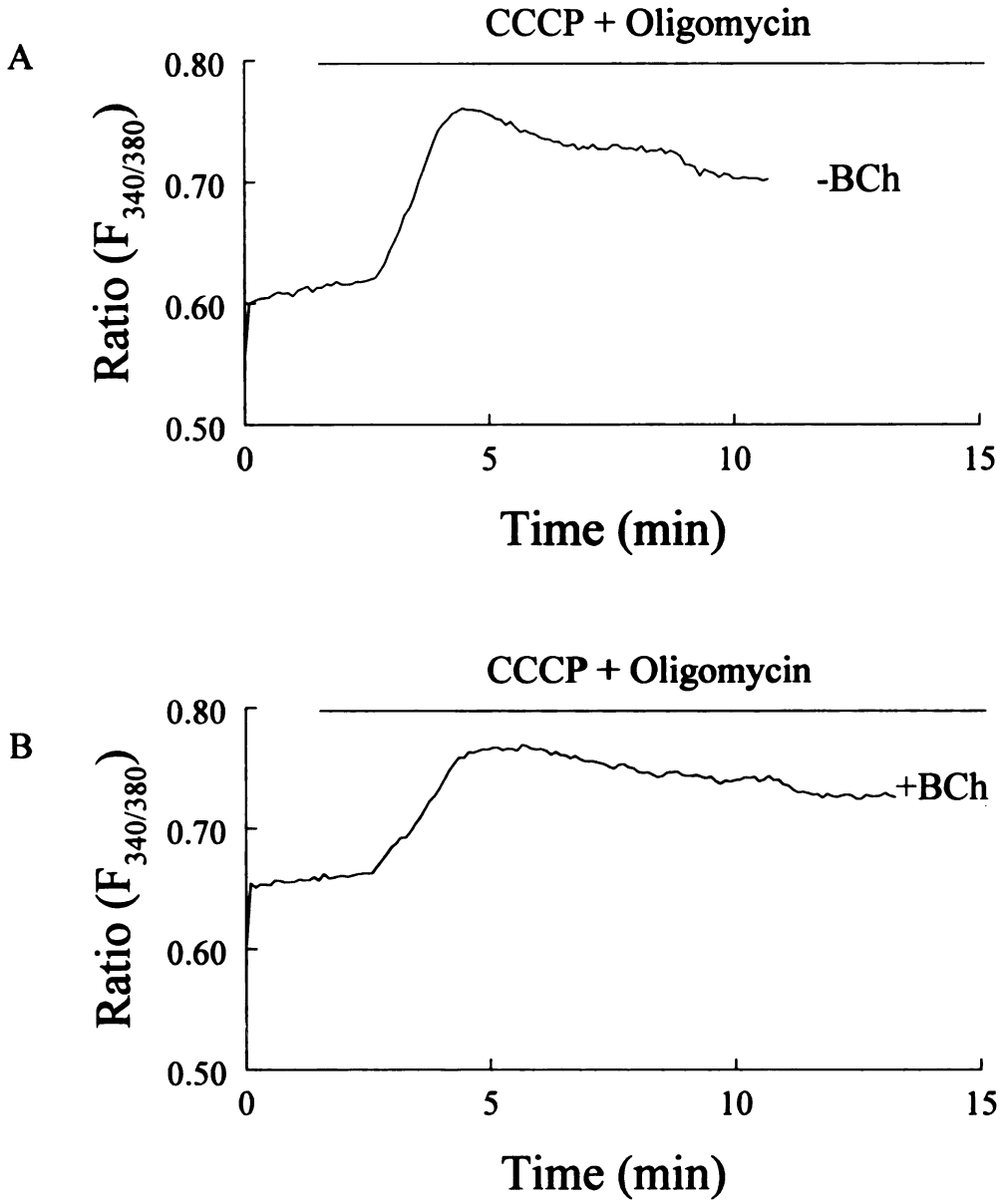

Figure 2.7. Pretreatment with BCh (1 mM, 24 hr) does not increase the [Ca^{2+}] within mitochondria. Representative changes in fura-2 fluorescence in response to mitochondrial depolarization with carbonyl cyanide m-chlorophenylhydrazone (CCCP) (5 μM) and oligomycin (10 μM). Granule cells were treated with vehicle ("-BCh") or 1 mM BCh ("+BCh") for 24 hr. CCCP and oligomycin were then applied to depolarize the empty the mitochondria of their Ca^{2+} content (see Chapter Four) to determine the amount of Ca^{2+} in these organelles following BCh treatment.

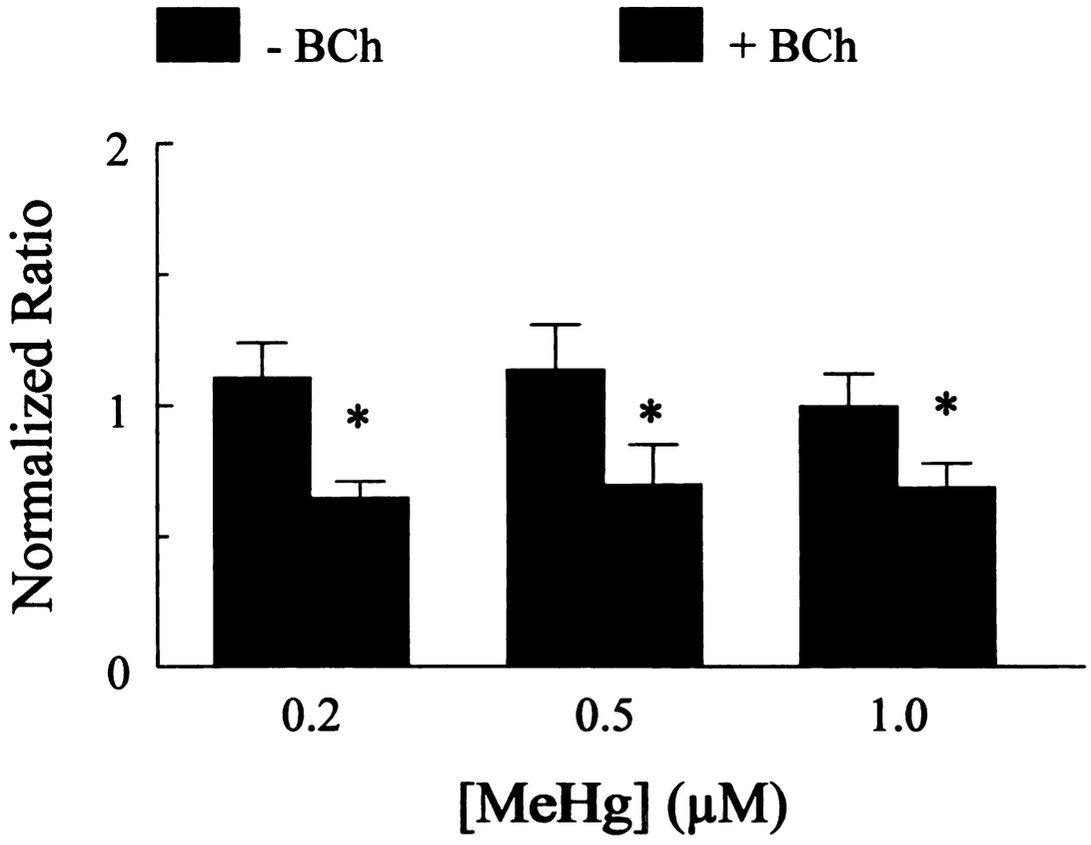

Figure 2.8. Pretreatment with BCh decreases the amplitude of the fura-2 ratio increase caused by MeHg, as measured in the absence of Ca^{2+}_e. Granule cells were treated with 1 mM BCh for 24 hr prior to 0.2-1.0 μM MeHg in EGTA-HBS. Results are presented as mean ± SEM (n=4-5). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

In the absence of Ca^{2+}_e, 24 hr pretreatment with BCh again hastened the time-to-onset of the first-phase ratio increase for 0.2 and 0.5 μM MeHg but not 1.0 μM MeHg (Figure 2.6). The hastened onset of the first phase was not due to an increased amount of Ca^{2+} in the mitochondria, as mitochondrial membrane depolarization with 5 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 10 μM oligomycin indicated no difference in the amount of mitochondrial Ca^{2+} content in cells treated with BCh as compared to untreated cells (Figure 2.7). Like thapsigargin, BCh pretreatment also decreased the amplitude of the first-phase fura-2 ratio increase caused by 0.2 μM MeHg (from 1.11±0.13 to 0.65±0.06), 0.5 μM MeHg (from 1.14±0.17 to 0.70±0.15), and 1.0 μM MeHg (from 1.00±0.12 to 0.69±0.09) (Figure 2.8). This represents a decrease in the first-phase ratio amplitude of approximately 41%, 39% and 31% caused by 0.2, 0.5 and 1.0 μM MeHg, respectively.

Blocking muscarinic receptors with atropine also decreases the MeHg-induced increase in $[Ca^{2+}]_i$. To determine whether MeHg causes Ca^{2+} release from the SER *via* binding to the M3 muscarinic receptor, experiments were performed using the nonspecific muscarinic receptor antagonist atropine. Atropine blocked the response to a brief (1.5 min) 1 mM challenge with BCh, indicating the effectiveness of this compound in blocking muscarinic receptors (Table 2.1). Application of 10 μM atropine for 10 min prior to, as well as during, exposure to MeHg in the presence of Ca^{2+}_e indicated that atropine significantly delayed the time-to-onset of the first-phase ratio increase caused by 0.2 and 0.5 μM MeHg, but not did not change the time-to-onset of elevations of $[Ca^{2+}]_i$ caused by 1.0 μM MeHg (Figure 2.9A). Atropine did not alter the time-to-onset of the second-phase influx of Ca^{2+}_e caused by 0.2, 0.5 or 1.0 μM MeHg (Figure 2.9B).

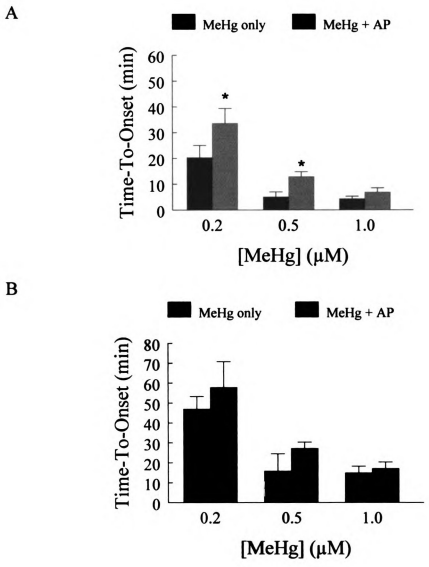

Figure 2.9. Atropine ("AP") delays the first-phase release of Ca^{2+}_i without affecting the second-phase influx of Ca^{2+}_e caused by MeHg. A) Granule cells were treated with atropine (10 μM) for 10 min prior to, as well as during, exposure to MeHg. Atropine significantly delayed the time-to-onset of the first-phase MeHg-induced release of Ca^{2+}_i caused by 0.2 and 0.5, but not 1.0 μM, MeHg. B) In the same cells, atropine did not alter the time-to-onset of the second-phase influx of Ca^{2+}_e at any MeHg concentration examined. Results are presented as mean ± SEM (n=4). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

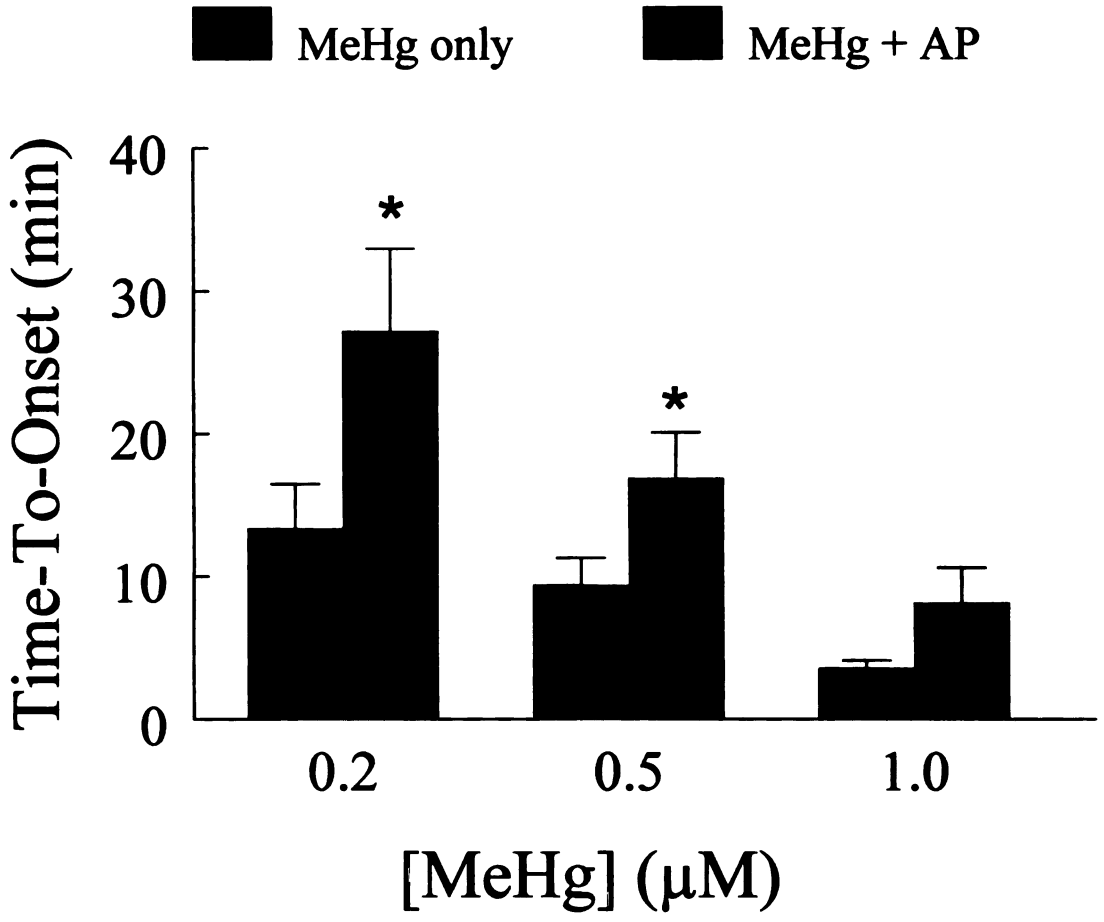

Figure 2.10. Atropine ("AP") delays the time-to-onset of the MeHg-induced first-phase release of Ca^{2+}_i, as measured in the absence of Ca^{2+}_e. Treatment with atropine (10 μM) for 10 min prior to, as well as during, exposure to MeHg significantly delayed the time-to-onset of the $[Ca^{2+}]_i$ increase caused by 0.2 and 0.5, but not 1.0, μM MeHg. Results are presented as mean ± SEM (n=3-6). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

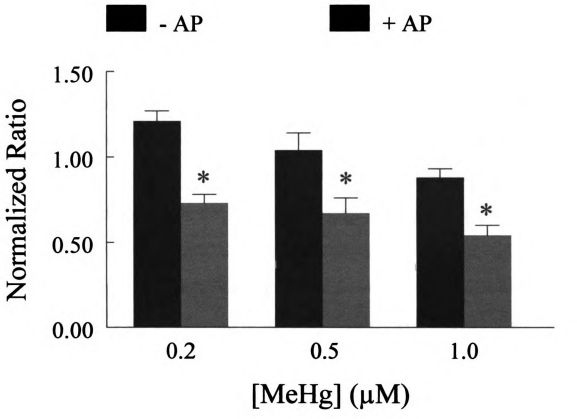

Figure 2.11. Atropine ("AP") decreases the amplitude of the fura-2 ratio increase caused by MeHg, as measured in the absence of Ca^{2+}_e. Granule cells were treated with atropine (10 μM) for 10 min prior to, as well as during, exposure to MeHg. Results are presented as mean ± SEM (n=3-6). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

In cells treated with MeHg in EGTA-HBS, atropine again delayed the time-to-onset of the first-phase ratio increase caused by 0.2 and 0.5 μM MeHg but did not change the time-to-onset of the first phase caused by 1.0 μM MeHg (Figure 2.10). Additionally, atropine caused a significant decrease in the first-phase amplitude caused by 0.2 μM MeHg (from a normalized ratio of 1.21±0.06 to 0.73±0.05), 0.5 μM MeHg (from 1.04±0.10 to 0.67±0.09), and 1.0 μM MeHg (from 0.88±0.05 to 0.54±0.06) (Figure 2.11). This represents a decrease in the first-phase ratio amplitude of approximately 40%, 36% and 39% caused by 0.2, 0.5 and 1.0 μM MeHg, respectively.

Effect of ryanodine on MeHg-induced $[Ca^{2+}]_i$ elevations. Granule cells in culture exhibit a developmental profile in the ability of their ryanodine receptors to respond to agonists, such that after 4 days in culture, they lose the ability to respond to ryanodine receptor agonists such as caffeine (Irving *et al.*, 1992); unpublished observation). However, these receptors are still functional and participate in CICR, as evidenced by the ability of 10 μM ryanodine to inhibit CICR initiated from IP_3 receptors in granule cells in culture (Irving *et al.*, 1992). Because all experiments in this dissertation were used at days 6-8 *in vitro*, it was impossible to test directly the ability of ryanodine, and thus experiments were performed to test the ability of 10 μM ryanodine to inhibit CICR initiated by release of Ca^{2+} from the IP_3 receptors when activated with 1 mM carbachol for 1 min. In agreement with Irving *et al.*, (1992) ryanodine inhibited elevations of $[Ca^{2+}]_i$ initiated by release of Ca^{2+} through the IP_3 receptor, as evoked by the muscarinic agonist BCh (Table 2.1). Treatment with 10 μM ryanodine for 10 min prior to, as well as during, treatment with 0.2-1.0 μM MeHg, did not change the time-to-onset of the first-phase ratio increase for 0.2, 0.5 or 1.0 μM MeHg (Figure 2.12A).

Ryanodine also did not delay the time-to-onset of the second-phase Ca^{2+} influx caused by 0.2, 0.5 or 1.0 μM MeHg (Figure 2.12B). In the nominally Ca^{2+}-free buffer, ryanodine again did not change the time-to-onset of the first-phase ratio increase caused by 0.2, 0.5 or 1.0 μM MeHg (Figure 2.13). Ryanodine also failed to decrease amplitude of the first-phase ratio increase caused by MeHg at any concentration examined (Figure 2.14).

Contribution of store-operated Ca^{2+} entry (SOCE). Experiments were performed to determine whether MeHg-induced emptying of the SER triggers opening of store-operated Ca^{2+} channels (SOCC), thereby contributing to the second-phase influx of Ca^{2+}_e. As shown in Figure 2.15, treatment with 10 μM thapsigargin for 10 min, followed by 1 mM EGTA dissolved in HBS, causes a noticeable decline in the baseline fura-2 ratio. Immediately following replacement of the EGTA solution with HBS (which contains 1.8 mM Ca^{2+}), the baseline fura-2 ratio returns to near-pretreatment levels, which is consistent with opening of SOCCs. Experiments were then performed to determine whether the return to normal $[Ca^{2+}]_i$ was due to opening of SOCCs. The SOCC inhibitor SK&F-96365 was tested for its ability to inhibit Ca^{2+} entry through SOCCs. SK&F-96365 is a known inhibitor of SOCCs (Lo and Thayer, 1995); however, SK&F-96365 is reported to also interact with voltage-operated Ca^{2+} channels (VOCCs), inhibiting Ca^{2+} movement through these channels (Koch *et al.*, 1994). For this reason, the effect of 10 μM SK&F-96365 on VOCCs was examined by adding the compound during plasma membrane depolarization-induced influx of Ca^{2+}_e caused by 1 min exposure to 40 mM K^+. As seen in Figure 2.16, SK&F-96365 significantly decreased Ca^{2+} entry through VOCCs by approximately 50%; thus, this compound could not be used to study the contribution of SOCE to MeHg-induced influx of Ca^{2+}_e.

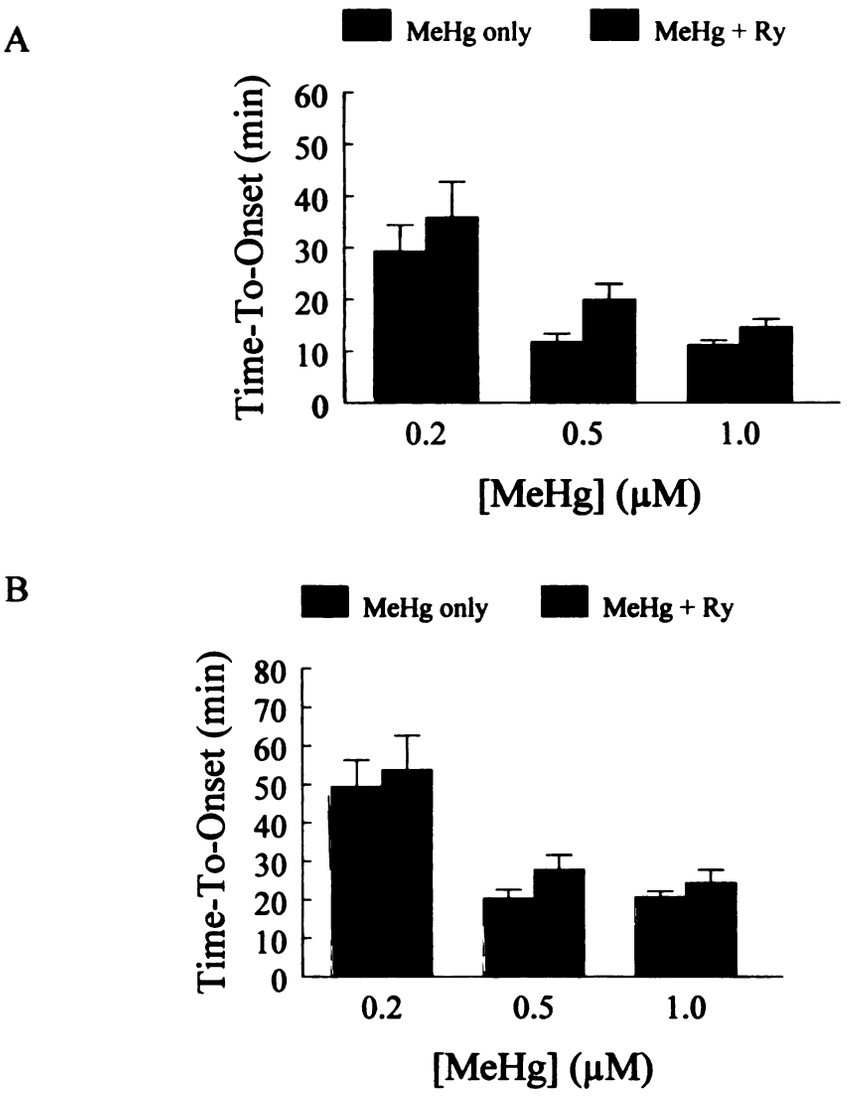

Figure 2.12. Ryanodine ("Ry") does not affect the time-to-onset of MeHg-induced increases in $[Ca^{2+}]_i$. A) Treatment with 10 μM ryanodine for 10 min prior to, as well as during MeHg exposure, did not alter the time-to-onset of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, or 1.0 μM MeHg. (B) In the same cells, treatment with 10 μM ryanodine also did not alter the time-to-onset of the MeHg-induced second-phase influx of Ca^{2+}_e caused by 0.2, 0.5, or 1.0 μM MeHg. Results are presented as mean ± SEM (n=6-7).

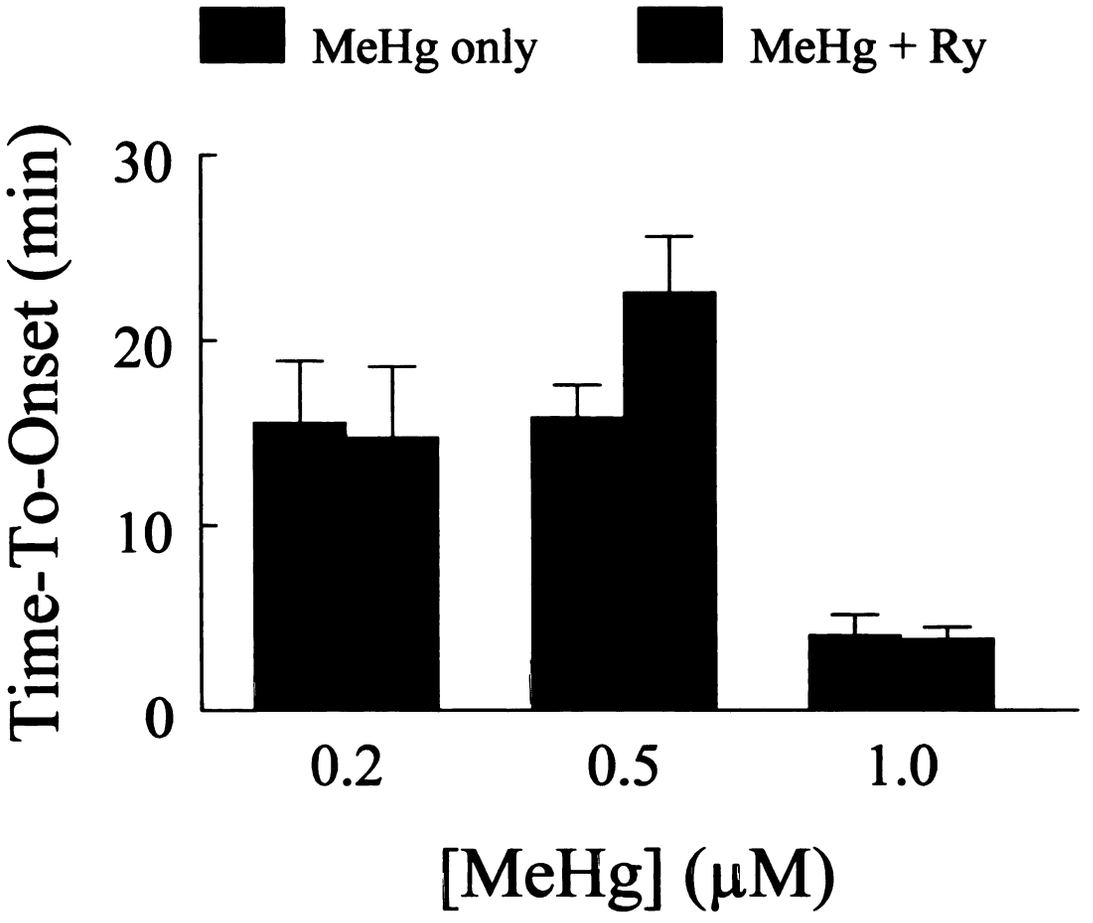

Figure 2.13. Ryanodine ("Ry") does not affect the time-to-onset of the MeHg-induced first-phase release of Ca^{2+}_i, as measured in the absence of Ca^{2+}_e. Treatment with ryanodine (10 μM) for 10 min prior to, as well as during, exposure to MeHg significantly does not alter the time-to-onset of the $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, or 1.0 μM MeHg. Results are presented as mean ± SEM (n=4-7).

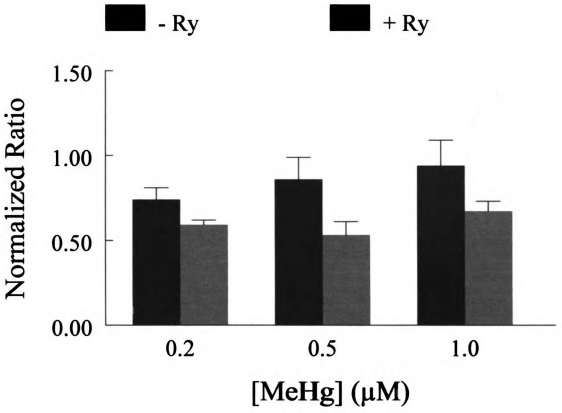

Figure 2.14. Ryanodine does not affect the amplitude of MeHg-induced increases in $[Ca^{2+}]_i$ as measured in the absence of Ca^{2+}_e. Ryanodine caused slight decreases in the amplitude of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2, 0.5, and 1.0 μM MeHg; however, the differences were not statistically significant. Results are presented as mean ± SEM (n=4-7).

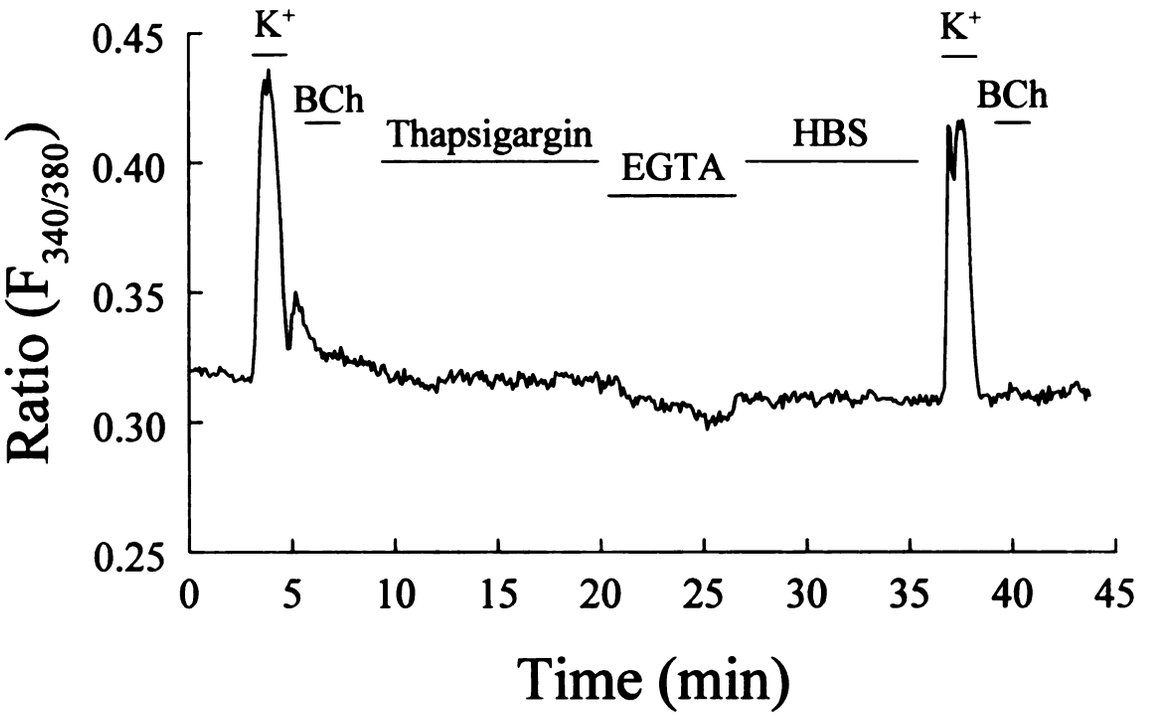

Figure 2.15. Depletion of SER Ca^{2+} triggers SOCE in rat cerebellar granule cells. Granule cells were exposed to 40 mM K^+ (1 min) followed by 1 mM BCH (1.5 min) to release Ca^{2+} from the IP_3-sensitive Ca^{2+} pool. Thapsigargin (10 μM, 10 min) followed by a high concentration of EGTA (1 mM, 5 min) caused a noticeable decline in the baseline $[Ca^{2+}]_i$. Returning the cells to a Ca^{2+}-containing medium (HBS) caused an immediate return to baseline $[Ca^{2+}]_i$, which is characteristic of SOCE. The loss of SER Ca^{2+} content with this treatment is confirmed by the loss of response to a second exposure to 1 mM BCh (1.5 min).

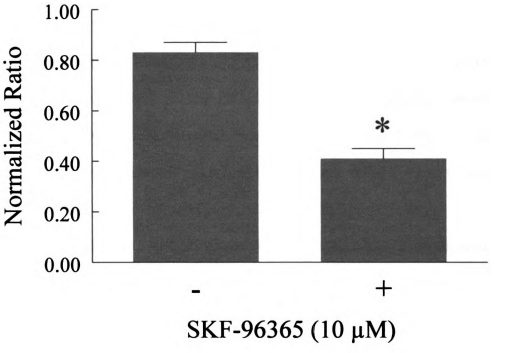

Figure 2.16. SK&F 96365 inhibits depolarization-stimulated Ca^{2+} influx through VOCCs. Granule cells were exposed to 40 mM K^+ for 1 min, in the presence or absence of 10 μM SK&F-96365, and the amplitude of the peak $[Ca^{2+}]_i$ response to the K^+ depolarization was measured. Results are presented as mean ± SEM (n=4). The asterisk (*) indicates a value significantly different from the corresponding vehicle control data ($p < 0.05$).

DISCUSSION

MeHg increases $[Ca^{2+}]_i$ in rat cerebellar granule neurons, which contributes to MeHg-induced neuronal death (Marty and Atchison, 1997; Marty and Atchison, 1998). Because loss of Ca^{2+} regulation is a pivotal step in both apoptosis and necrosis, these experiments sought to identify the Ca^{2+} source in granule cells to understand the mechanism by which MeHg causes elevations of $[Ca^{2+}]_i$ in cerebellar granule cells. MeHg interacts with muscarinic ACh receptors (Castoldi *et al.*, 1996; Coccini *et al.*, 2000), causes generation of IP_3 in rat cerebellar granule cells in culture (Sarafian, 1993), and causes Ca^{2+} release through the IP_3 receptor in NG108-15 cells and rat T lymphocytes (Tan *et al.*, 1993; Hare and Atchison, 1995); thus, these experiments examined whether MeHg interacts with these receptors to cause Ca^{2+} release from the SER.

Data presented in this chapter reveals that Ca^{2+} release from the SER by MeHg contributes approximately 30-40% to the observed increase in fura-2 ratio during the initial phase of MeHg exposure. This differs from results obtained in NG108-15 cells, in which emptying the SER with thapsigargin and bradykinin prior to MeHg treatment reduced the MeHg-induced first-phase ratio increase by 68% (Hare and Atchison, 1995). As was found in NG108-15 cells, the ryanodine receptor Ca^{2+} pool in granule cells contributes minimal Ca^{2+} during MeHg exposure (Hare and Atchison, 1995). All four drug treatments (thapsigargin, BCh, atropine and ryanodine) decreased the first-phase ratio amplitude to a similar extent for all MeHg concentrations examined, suggesting that MeHg activates a common signaling pathway which involves muscarinic receptors, IP_3 receptors and ryanodine receptors.

While pharmacological manipulation of the SER with different compounds caused similar decreases in the amount of Ca^{2+} released from within the cell by MeHg, there was a complicated pattern of effects on the time-to-onset of the initial increase in $[Ca^{2+}]_i$. Atropine delayed the time-to-onset of the initial $[Ca^{2+}]_i$ elevation, suggesting that the MeHg-induced elevation of $[Ca^{2+}]_i$ is initiated by biding of MeHg to muscarinic receptors. Because granule cell M3 receptors are coupled to G_q, which leads to generation of IP_3 through PLC, while M2 receptors are linked to G_i, which inhibits adenylate cyclase and thus is not involved in Ca^{2+} regulation, the effect of atropine is likely due to inhibition of M3 receptors (Whitham *et al.*, 1991; Doble *et al.*, 1992; Contrera *et al.*, 1993; Fohrman *et al.*, 1993). These results support the observations that MeHg binds multiple subtypes of muscarinic receptors in both the central and peripheral nervous systems (Abd-Elfattah and Shamoo, 1981; Castoldi *et al.*, 1996; Coccini *et al.*, 2000) and causes increased $[IP_3]$ in cerebellar granule neurons *in vitro* (Sarafian, 1993). The binding of MeHg to muscarinic receptors may partially explain the selective neurotoxicity of specific cell types within the central nervous system (CNS). In rats, granule cells express the highest density of muscarinic receptors in the cerebellar cortex, as assessed by [^{3}H]quinuclidinyl benzilate binding (Neustadt *et al.*, 1988). *In vivo* studies indicate that MeHg causes an up-regulation of muscarinic receptors in both the cerebellum and hippocampus, which tend to accumulate MeHg and exhibit neuronal death following MeHg exposure (Coccini *et al.*, 2000). In granule cells, inhibition of muscarinic receptors protects against apoptosis induced by growth in non-depolarizing conditions (Yan *et al.*, 1995), suggesting that muscarinic receptor activation can activate cell death in granule cells. Because the primary downstream target of M3 receptors is the

SER-localized IP_3 receptor, the functional consequence of MeHg activation of M3 receptors would be Ca^{2+} release from the SER, which would then contribute to the whole-cell $[Ca^{2+}]_i$ elevation observed during MeHg exposure.

Depletion of the SER Ca^{2+} store with the smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin, while decreasing the amount of $Ca^{2+}{}_i$ released into the cytosol, did not alter the time-to-onset of the initial Ca^{+} increase. Complete emptying of the SER by thapsigargin would be expected to delay or prevent the MeHg-induced first-phase increase of $[Ca^{2+}]_i$ if it originated from the SER, as is suggested by the atropine data. However, this would only be the case if thapsigargin completely empties the SER of its Ca^{2+} content. In these experiments, it was found that a 5 min treatment with 10 μM thapsigargin abolished the response to treatment with 1 min 1 mM BCh, indicating loss of responsiveness of the M3 muscarinic receptor-IP_3 receptor pathway. This was presumably due to store depletion. However, in experiments performed with rat cerebellar granule cells in primary culture, 20 min treatment with 10 μM thapsigargin only reduced the SER Ca^{2+} store by approximately 50% in spite of the complete abolishment of response to 1 mM carbachol (Masgrau *et al.*, 2000). The authors concluded that granule cells may contain a thapsigargin-insensitive SER Ca^{2+} pool; this Ca^{2+} pool could be the initial target of MeHg, thereby contributing to the observed $[Ca^{2+}]_i$ increases irrespective of thapsigargin treatment. The total SER Ca^{2+} pool in granule cells can be subdivided into pharmacologically, and possibly physically, distinct subsets, making the existence of a thapsigargin-insensitive pool plausible (Simpson *et al.*, 1996). Thus, the ability of thapsigargin treatment to decrease the amplitude of the first-phase $[Ca^{2+}]_i$ increase without affecting the time-to-onset of the

initial $[Ca^{2+}]_i$ elevation suggests that the thapsigargin-sensitive store does contribute to the overall $[Ca^{2+}]_i$ increase without necessarily being the initial target.

In order to examine the contribution of Ca^{2+} released from the IP_3 receptor, cells were treated with BCh (1 mM, 24 hr) to down-regulate the SER IP_3 receptor. In granule cells *in vitro*, 24 hr treatment with 0.1-1 mM carbachol is well documented as causing down-regulation and desensitization of M2, M3 and Type I IP_3 receptors (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Simpson *et al.*, 1994; Simpson *et al.*, 1996), thus a 24 hr pretreatment with 1 mM BCh should cause a similar down-regulation of these receptors. The 24 hr BCh treatment caused a 77% decrease in the peak Ca^{2+} response to a brief 1 mM BCh treatment (Table 2.1), which agreed with the 77±14% loss of carbachol-induced response caused by 24 hr 1 mM carbachol exposure (Simpson *et al.*, 1996). These same authors found that while 24 hr carbachol treatment caused a 65% reduction in agonist-induced accumulation of IP_3, it did not abolish the response (Simpson *et al.*, 1996), suggesting that 24 hr BCh treatment might inhibit but not abolish the MeHg-induced release of $Ca^{2+}{}_i$ if it occurs through the IP_3 receptor. Accordingly, exposure to 1 mM BCh decreased the amplitude of the first-phase $Ca^{2+}{}_i$ elevation, indicating that loss of IP_3 receptors corresponded with a decreased response to MeHg. Surprisingly, BCh treatment hastened the release of $Ca^{2+}{}_i$ during MeHg exposure. The BCh treatment does not alter the amount of Ca^{2+} release by preloaded mitochondria, thus the BCh treatment does not shift Ca^{2+} from the SER to the mitochondria. Because 24 hr carbachol treatment reduces the number of IP_3 receptors by approximately 80% (Simpson *et al.*, 1994) and reduces but does not abolish the IP_3 accumulation following muscarinic receptor activation

(Simpson *et al.*, 1996), the remaining IP_3 receptors could exhibit increased activity to compensate for the lost receptors.

Depletion of Ca^{2+}_i stores can trigger entry of Ca^{2+}_e *via* store-operated Ca^{2+} entry (SOCE) in a variety of cells, including neurons. Previous experiments indicated that MeHg causes influx of Ca^{2+}_e which cannot be completely blocked by voltage-gated Ca^{2+} channel blockers (Marty and Atchison, 1997), leading to the hypothesis that at least part of this Ca^{2+} influx was through activation of SOCE. Rat cerebellar granule cells do undergo SOCE when the SER is depleted of Ca^{2+} by a combination of thapsigargin and high-EGTA medium; however, direct attempts to block SOCE with SK&F 96365 were unsuccessful, as this compound did not inhibit SOCE while causing significant inhibition of Ca^{2+}_e entry through voltage-gated Ca^{2+} channels. MeHg-induced activation of SOCE in granule cells is doubtful, however, because of the lack of effect any SER targeting compound on the MeHg-induced influx of Ca^{2+}_e. Because none of the pharmacological agents used in this study altered the MeHg-induced Ca^{2+} influx, this suggests (but remains untested) that SOCE does not play a role in the observed loss of Ca^{2+}_i regulation.

CHAPTER THREE

ACUTE METHYLMERCURY EXPOSURE CAUSES NEURONAL DEATH IN RAT CEREBELLAR GRANULE CELLS THROUGH INTERACTION WITH INOSITOL-1,4,5-TRIPHOSPHATE RECEPTORS

ABSTRACT

Low concentrations of methylmercury (MeHg) cause Ca^{2+}-dependent cell death in rat cerebellar granule cells *in vitro*. As demonstrated in Chapter Two, MeHg interacts with muscarinic ACh receptors to cause the release of Ca^{2+} from the SER, primarily through the inositol-1,4,5-triphosphate (IP_3) receptor, in cerebellar granule cells. Experiments presented in this chapter examined whether this Ca^{2+} release plays a role in MeHg-induced cell death in granule cells. Acute exposure to increasing concentrations of MeHg (0.2-1.0 μM) caused a corresponding increase in cell death at 24.5 hr post-exposure. Prior down-regulation of the M3 muscarinic receptor-IP_3 receptor pathway with the muscarinic receptor agonist bethanechol (BCh, 1 mM) provided significant protection against MeHg-induced cell death. This protection was reversed by the non-specific muscarinic receptor antagonist atropine (10 μM) and the M3 receptor antagonist 4-diphenylacetoxyl-N-methylpiperidine methiodide (10 μM), but not the M2 receptor antagonist methoctramine (10 μM) or neuronal nicotinic receptor antagonist dihydro-β-erythroidine hydrobromide (10 μM). Atropine alone (10 μM) was not protective against MeHg-induced neuronal death. Acute or 24 hr pre-treatment with the IP_3-receptor antagonist xestospongin C (XeC) (1 μM) was also not neuroprotective, with 24 hr XeC itself causing significant cell death. Inhibition of the SER ryanodine receptors with 10 μM ryanodine was also not protective, indicating that MeHg-induced Ca^{2+} release from this store does not play a significant role in cell death. These results indicate that inhibition of MeHg-induced Ca^{2+} release from the SER protects against MeHg-induced cell death.

INTRODUCTION

Results of experiments described in Chapter Two indicate that a significant portion of the MeHg-induced increase of $[Ca^{2+}]_i$ in granule cells originates from the SER, as released through the IP_3 receptor. This release of Ca^2 from the IP_3-sensitive Ca^{2+} pool appears to be due to interaction of MeHg with muscarinic receptors, although the possibility of a direct interaction between MeHg and intracellular Ca^{2+} channels is not ruled out. The next set of experiments was designed to test the hypothesis that MeHg-induced Ca^{2+} release from the SER plays a role in MeHg-induced cell death in cerebellar granule cells. To test this hypothesis, pharmacological inhibitors of specific receptors and ion channels involved in $Ca^{2+}{}_i$ signaling pathways were tested for their ability to prevent MeHg-induced cell death. The receptors and channels examined in these experiments include muscarinic ACh receptors, IP_3 receptor-linked channels and ryanodine receptor-linked channels.

Granule cells express a high density of M2 and M3 muscarinic receptors; activation of the M3 receptor leads to generation of IP_3 by activation of PLC (Alonso *et al.*, 1990; Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Simpson *et al.*, 1994). Muscarinic receptors of all subtypes contain two critical thiol groups within the receptor's active site, suggesting that MeHg could potentially bind to, and activate, these receptors (Castoldi *et al.*, 1996; Coccini *et al.*, 2000). This interaction between MeHg and the muscarinic receptors may affect MeHg-induced cell death, as granule cell death caused by a number of toxicants is mediated by the M3 receptors (Galli *et al.*, 1995; Yan *et al.*, 1995; de Luca *et al.*, 1996; Lin *et al.*, 1997).

The functional consequence of activating M3 muscarinic receptors is the production of IP_3 and subsequent release of Ca^{2+} release from the IP_3–sensitive store in the SER. There is growing evidence that the release of Ca^{2+} through these channels plays a critical role in neuronal death caused by a variety of toxicants (Marks, 1997; Szalai *et al.*, 1999; Blackshaw *et al.*, 2000). T cells lacking Type I IP_3 receptors are resistant to apoptosis induced by dexamethasone, T-cell receptor stimulation, ionizing radiation, and Fas (Jayaraman and Marks, 1997). Resistance to apoptosis caused by these agents is reversed by elevating $[Ca^{2+}]_i$, suggesting that the IP_3 receptor's importance may be in the release of Ca^{2+} into the cytosol rather than activation of a protein-mediated mechanism (Jayaraman and Marks, 1997). In permeabilized HepG2 cells, Ca^{2+} signals from the IP_3 receptor are directly translated into elevations of mitochondrial $[Ca^{2+}]$, opening of the MTP and subsequent release of the pro-apoptotic cytochrome c into the cytosol (Szalai *et al.*, 1999). Thus, MeHg-induced Ca^{2+} release through the IP_3 receptors could be a critical step in MeHg-induced cell death.

In addition to SER-localized IP_3 receptors, ryanodine receptors also influence neuronal viability (Marks, 1997) and contribute a small amount of Ca^{2+} to the whole-cell $[Ca^{2+}]_i$ increase caused by MeHg (previous chapter). Inhibition of Ca^{2+} release from the ryanodine receptor-activated store is protective in several *in vitro* and *in vivo* models of neuronal damage (Wei and Perry, 1996; Marks, 1997; Wei *et al.*, 2000). Thus these receptors could potentially be important in MeHg-induced neuronal death.

The purpose of these experiments was to determine whether inhibition of specific components of the SER-linked Ca^{2+} signaling pathways altered MeHg-induced cell death in rat cerebellar granule neurons in primary culture. The first set of experiments

examined the effect of SER store depletion on MeHg-induced cell death using the SERCA inhibitor thapsigargin. The second set of experiments examined the effect of down-regulation of both the IP_3 receptor and the muscarinic receptors using the nonspecific muscarinic agonist bethanechol (BCh). Specific portions of this signaling pathway were also examined using specific muscarinic receptor subtype antagonists and the IP_3 receptor inhibitor XeC. The final set of experiments examined the role of the ryanodine receptors in MeHg-induced cytotoxicity using the ryanodine receptor inhibitor ryanodine.

MATERIALS AND METHODS

The materials and solutions for isolation of cerebellar granule cells are described in detail in Chapter Two. Unless specifically stated, all solutions were identical to those described in Chapter Two. The methods and solutions described below are specific to this chapter.

Materials and experimental solutions. The Live/Dead Eukolight viability/cytotoxicity kit was purchased from Molecular Probes (Eugene, OR). 4-diphenylacetoxyl-N-methylpiperidine methiodide (4-DAMP), dihydro-β-erythroidine hydrobromide (DHE), and methoctramine hydrochloride (methoctramine) were purchased from Sigma (St. Louis, MO). Xestospongin C (XeC) was purchased from Calbiochem (La Jolla, CA). CellLocate coverslips were purchased from Eppendorf Scientific (Madison, WI).

Experimental solutions were prepared in sterile-filtered HBS. MeHg was prepared as a 5 mM stock solution in deionized water and diluted to working concentrations. Thapsigargin was prepared as a 1 mM stock in ethanol. All other pharmacological inhibitors were prepared as stock solution in deionized water. Control solutions contained an equal volume of vehicle in sterile HBS.

Rat cerebellar granule cell isolation procedure. Cerebellar granule cells were isolated as described in Chapter Two and were plated at a density of 1.8×10^8 cells/35 mm^2 dish, each containing a 13 mm CellLocate coverslip coated with 0.1 mg/ml poly-D-lysine.

Cells were maintained at 37°C in 5% CO_2 for 6-8 days *in vitro* to allow for cell maturation (Aronica *et al.*, 1993).

Measurement of granule cell viability. The cell viability protocol was designed to parallel experimental conditions in previous experiments examining MeHg-induced elevations of $[Ca^{2+}]_i$ in granule cells in primary culture (Marty and Atchison, 1998). To replicate these conditions, all cells were incubated for 90 min at 37°C in HBS to mimic the fura-2 AM incubation (1 hr) and HBS perfusion rinse (30 min) of prior $[Ca^{2+}]_i$ measurement experiments. Cells were then exposed to HBS (76 min), 0.2 μM MeHg (76 min), 0.5 μM MeHg (45 min) or 1.0 μM MeHg (38 min). MeHg exposure duration and concentrations were based on previous experiments by Marty and Atchison (1997), which determined these times sufficient for approximately 97% of cells to undergo MeHg-induced release of intracellular Ca^{2+} stores and influx of Ca^{2+}_e. For all experiments, appropriate solvent and drug controls were performed at the same time for 76 min each, as this was the maximum MeHg exposure interval. Following MeHg exposure, cells were returned to conditioned medium (to bind excess MeHg) for 24 hr. Previous experiments indicated no significant loss of cell viability immediately following exposure and significant cell death at 24 hr post-exposure, thus cells were examined at 24 hr in these experiments. At 24 hr after treatment, cells were removed from conditioned medium, rinsed twice with HBS and overlaid with Live/Dead Eukolight Viability/Cytotoxicity reagents (Molecular Probes, Eugene, OR) for 30 min at 37°C. As per the kit instructions, the reagent buffer contains 2 μM calcein acetoxymethylester (calcein-AM) and 4 μM ethidium homodimer which labels healthy cells green (calcein-

AM) and dead cells red (ethidium homodimer), both dissolved in HBS. Following the 30 min exposure, cells were examined using a Nikon Diaphot epifluorescence microscope and the number of live and dead cells in 8 randomly selected CellLocate grids were counted in order to determine percent viability for that coverslip. Because of the 30 min period in Live/Dead reagent, cells were counted at 24.5 hr after the cessation of MeHg treatment.

To determine whether emptying of the SER Ca^{2+} pool inhibited MeHg-induced neuronal death, cells were treated with 10 μM thapsigargin for 5 min immediately prior to 0-1.0 μM MeHg. Previous work demonstrated that this protocol empties the thapsigargin-sensitive SER Ca^{2+} store (Simpson *et al.*, 1996); previous chapter). Following MeHg treatment, cells were returned to conditioned medium for 24 hr, and counted at 24.5 h post-exposure.

To determine if BCh treatment protected cells from MeHg-induced neuronal death, BCh was added directly to the growth medium to give a final concentration of 1 mM BCh. Similar exposure to 1 mM carbachol down-regulates the M3 receptor in granule cells as a result of continued receptor stimulation, thus 24 hr of 1 mM BCh would be expected to cause a similar down-regulation (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Wojcikiewicz *et al.*, 1994; Simpson *et al.*, 1996; Fukamauchi *et al.*, 2000). At 24 hr after application of BCh, cells were treated with MeHg alone or MeHg plus 1 mM BCh. For each set of cells exposed to MeHg there were corresponding HBS, 1 mM BCh, and vehicle control cells which were treated and counted at the same time as the MeHg-treated cells. For cells treated with BCh, all HBS washes contained 1 mM BCh; additionally, the conditioned medium to which they were returned after MeHg treatment

contained 1 mM BCh for the entire 24 hr post-treatment period. In experiments in which BCh was added with a specific cholinergic receptor antagonist, the antagonist was added for 10 min prior to the initial application of 1 mM BCh, as well as during BCh treatment and HBS wash. The antagonists tested were atropine (nonspecific muscarinic receptor antagonist), methoctramine (M2 antagonist), 4-DAMP (M3 antagonist) and DHE (neuronal nicotinic ACh receptor antagonist), all at a final concentration of 10 μM. The concentrations of each antagonist were based on the results of Yan *et al.* (1995) and Lin *et al.* (1997), who used these compounds to inhibit cell death induced by non-depolarizing growth medium and mastoparan, respectively, in rat cerebellar granule neurons in primary culture. In a similar set of experiments, atropine was added directly to the conditioned medium to a final concentration of 10 μM 10 min prior to, as well as during, exposure to 0-1.0 μM MeHg.

In the next experiment, the reversible IP_3–receptor inhibitor XeC (1 μM) was added for 10 min prior to, as well as during, treatment with 0-1.0 μM MeHg. This concentration of XeC was chosen based on the results of Netzeband *et al.*, 1999, who found 1 μM XeC sufficient to prevent Ca^{2+} release from the IP_3 receptor in rat cerebellar granule neurons in primary culture. Finally, cells were treated with the ryanodine receptor inhibitor ryanodine (10 μM) for 10 min prior to, as well as during, exposure to 0-1.0 μM MeHg. This concentration of ryanodine has previously been shown to be effective at inhibiting Ca^{2+}-induced Ca^{2+} release from ryanodine receptors in cerebellar granule cells (Irving *et al.*, 1992).

Statistics. To account for variability between cell isolates, each set of experiments was performed in at least two separate isolates. Further, all experiments within a set (HBS only, drug only, and MeHg with or without drug) were performed on the same day (n=1), allowing the data to be paired for statistical comparison. For each exposure group, the percent viability (number of live cells/total number of cells x 100) was determined, and these percentages were normalized using an angular transformation. Values are presented as the mean $\pm$ SEM. Comparisons of cell viability were made using a repeated measures analysis of variance (ANOVA) followed by Tukey's procedure for post-hoc comparisons to compare MeHg to MeHg + inhibitor, with $p < 0.05$ considered to be statistically significant.

RESULTS

These experiments were designed to examine the role of SER Ca^{2+} in MeHg-induced death of cerebellar granule neurons. In the first set of experiments, the effect of MeHg on granule cell viability was determined both immediately and 24 hr after exposure to 0-2 μM MeHg. Cell viability is similar to control immediately following MeHg treatment; however, at 24 hr post-exposure MeHg causes increasing cell death in a concentration-dependent manner (Figure 3.1). These results confirm earlier findings by Marty and Atchison (1998). Because MeHg does not immediately cause neuronal death, all cell viability assays were performed at 24 hr post-exposure to MeHg.

The next experiment sought to determine whether emptying of the SER Ca^{2+} pool with the SERCA inhibitor thapsigargin was protective against cell death at 24.5 h post-exposure to 0-1 μM MeHg. Cells were treated with 10 μM thapsigargin for 5 min immediately prior to MeHg; this exposure was based on earlier experiments in which this treatment depleted the SER of its Ca^{2+} content (previous chapter). However, this brief exposure to thapsigargin was itself toxic, causing cell death at 24.5 h post-exposure (results not shown).

In the next set of experiments, the nonspecific muscarinic agonist BCh (1 mM) was applied for 24 hr prior to MeHg treatment to down-regulate the muscarinic-IP_3 receptor pathway. Application of 1 mM carbachol is well documented as causing down-regulation and desensitization of M2, M3 and subsequently, IP_3 receptors in granule neurons (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Wojcikiewicz *et al.*, 1994; Simpson *et al.*, 1996; Fukamauchi *et al.*, 2000). Treatment with 1 mM BCh significantly

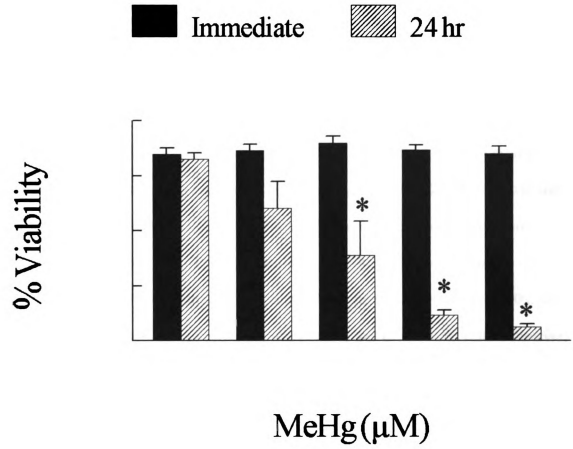

Figure 3.1. MeHg-induced cell death at 30 min and 24 hr post-exposure. Immediately following exposure to 0.2-2.0 μM MeHg, there is no apparent loss of granule cell viability. However, at 24 hr post-exposure, there is a concentration-dependent increase in cell death. Results are presented as mean ± SEM (n = 4). (*) significantly different from the corresponding 0 μM MeHg data ($p < 0.05$).

inhibits agonist-induced release of Ca^{2+} through granule cell IP_3 receptors (previous chapter), suggesting that BCh, like carbachol, also causes down-regulation of the receptors in this pathway.

In the first experiment, cells were treated with 1 mM BCh for 24 hr, followed by 0–1 μM MeHg for the durations described earlier. As seen in Figure 3.2, increasing concentrations of MeHg killed an increasing percentage of cells at 24.5 h post-exposure. The loss of cell viability was consistent with that observed in previous experiments (Marty and Atchison, 1998). Treatment with 1 mM BCh significantly protected cells from MeHg-induced cell death at all concentrations examined. Furthermore, at 0.2 and 0.5 μM MeHg the incidence of cell death was equal to that of untreated cells. However, at 1 μM MeHg, the protection provided by 1 mM BCh was not as great as at the lower MeHg concentrations. To determine whether the protection provided by BCh was due to action at muscarinic receptors, the nonspecific muscarinic antagonist atropine (10 μM) was applied for 10 min prior to BCh, as well as during the 24 hr BCh treatment for 24 hr prior to exposure to MeHg. Cell viability was again measured at 24 hr post-MeHg exposure. As seen in Figure 3.3, addition of atropine prevented the protection afforded by 1 mM BCh (Figure 3.2), resulting in no significant difference in the number of cells surviving MeHg alone or MeHg plus BCh and atropine.

Granule cells express M2 and M3 receptors (Alonso *et al.*, 1990), thus the protection provided by BCh could be through either or both of these receptors. In a similar protocol to the BCh plus atropine protocol, the M2 antagonist methoctramine (10 μM) was applied for 10 min prior to, as well as during, treatment with 1 mM BCh.

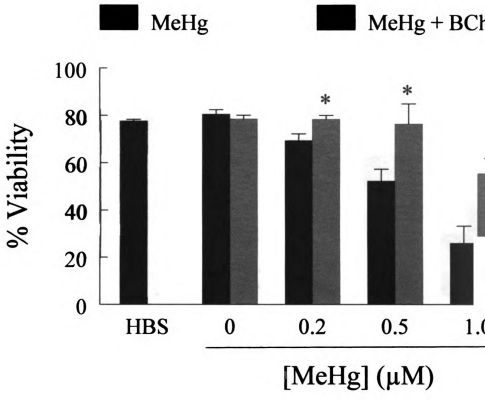

Figure 3.2. 24 hr pretreatment with 1 mM BCh protects against MeHg-in death. Granule cells exhibit a concentration-dependent decrease in survival a post-exposure to increasing concentrations of MeHg. Cell death is attenuated pre-exposure to 1 mM BCh. Results are presented as mean±SEM (n=4). (*)S different from MeHg-only pair ($p < 0.05$).

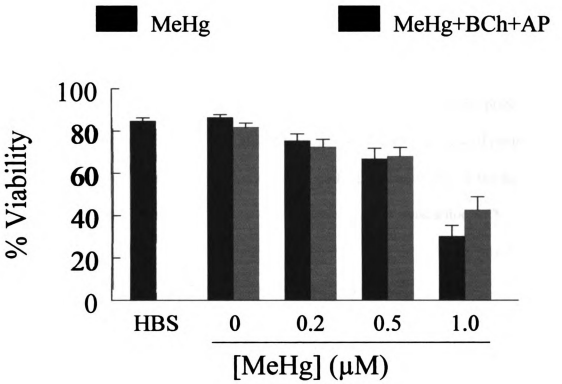

Figure 3.3. Atropine plus BCh does not protect against MeHg-induced cell death. In contrast to the protection afforded by BCh alone (Figure 3.2), application of 10 μM atropine for 10 min prior to, as well as during, the 1 mM BCh pre-exposure period is not protective against cell death caused by 0.2-1.0 μM MeHg. Results are presented as mean±SEM (n = 6).

However, application of BCh and methoctramine alone for 24 hr resulted in a significant increase in the incidence of cell death, thus preventing examination of whether the protective effect of BCh was due to action at the M2 receptors (data not shown). Application of the M3 receptor antagonist 4-DAMP (10 μM) did prevent any protection by 1 mM BCh in a manner similar to atropine (Figure 3.4). Again, the lack of protection was complete at lower MeHg concentrations (0.2 and 0.5 μM) and partial at the higher MeHg concentration (1 μM). The actions of BCh were through muscarinic ACh receptors and not nicotinic ACh receptors, as the nicotinic receptor antagonist DHE (10 μM) did not affect the neuroprotective effects of BCh (Figure 3.5).

The 24 hr BCh treatment down-regulated both the M3 receptor and the IP_3-receptor, thus the next experiment sought to determine whether preventing MeHg from interacting with the M3 receptor would protect the cells from dying. To this end, cells were exposed to atropine alone (10 μM) for 10 min prior to, as well as during, exposure to MeHg. Cell viability was again measured at 24.5 h post-exposure. Unlike the 24 hr BCh pretreatment, atropine alone did not protect against MeHg-induced cell death (Figure 3.6). This is in contrast to its ability to decrease significantly MeHg-induced elevations in $[Ca^{2+}]_i$ cerebellar granule neurons (previous chapter).

Because atropine alone was not neuroprotective, the next step was to block the IP_3-receptor during MeHg exposure to determine whether MeHg interacts directly with this receptor in granule cells. The reversible IP_3-receptor inhibitor XeC (1 μM) was applied for 10 min prior to, as well as during, exposure to MeHg. As seen in Figure 3.7, acute application of XeC also did not prevent MeHg-induced cell death. Pretreatment for 24 hr with 1 μM XeC, which would presumably down-regulate

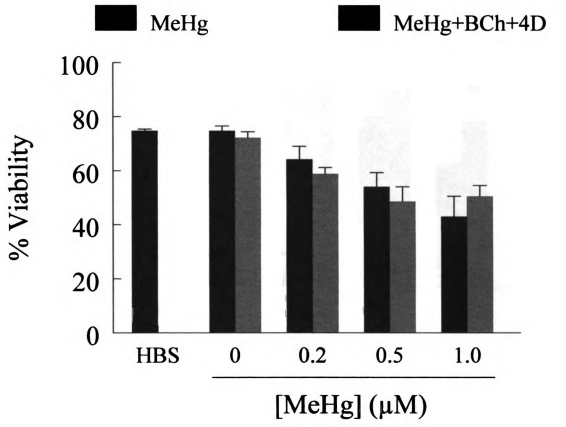

Figure 3.4. 4-DAMP plus BCh does not protect against MeHg-induced cell death. In contrast to the protection afforded by BCh alone (Figure 3.2), application of the M3-specific antagonist 4-DAMP for 10 min prior to, as well as during, the 1 mM BCh pre-exposure period does not protect against MeHg-induced cell death at 24 hr post-exposure. Results are presented as mean±SEM (n=4).

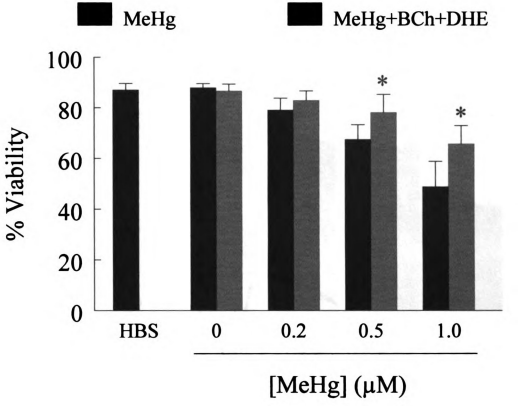

Figure 3.5. The nicotinic receptor antagonist DHE does not affect BCh-mediated protection against MeHg-induced cell death. The protective effect of 1 mM BCh was not due to action at nicotinic ACh receptors, because inclusion of the nicotinic receptor antagonist DHE (10 μM) for 10 min prior to, as well as during, MeHg exposure did not alter the effect of BCh. Results are presented as mean±SEM (n=8). (*)Significantly different from MeHg-only control ($p < 0.05$).

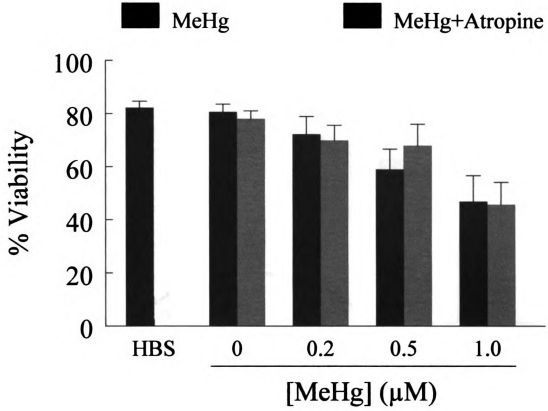

Figure 3.6. Atropine alone does not protect against MeHg-induced cell death. A brief pretreatment with atropine (10 min, 10 μM), as well as treatment with atropine during MeHg exposure, did not affect MeHg-induced cell death at 24.5 h post-exposure. Results are presented as mean±SEM (n=6).

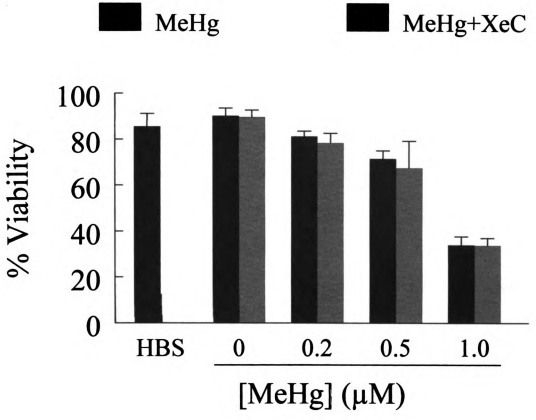

Figure 3.7. Xestospongin C does not protect against MeHg-induced cell death. Pretreatment with xestospongine C (XeC) (10 min, 1 μM), as well as inclusion of XeC in the MeHg solution, did not affect MeHg-induced cell death at 24.5 h post-exposure. Results are presented as mean±SEM (n=4).

the IP_3 receptors, was also not neuroprotective and was itself neurotoxic (data not shown).

Finally, the participation of ryanodine receptors in MeHg-induced neuronal death was examined. Previous results indicated that inhibition of Ca^{2+} release through the ryanodine receptors did not affect MeHg-induced increases of $[Ca^{2+}]_i$ in granule cells (previous chapter). To test whether ryanodine receptors are involved in MeHg-induced cell death, granule cells were exposed to 10 μM ryanodine for 10 min prior to, as well as during, exposure to MeHg, and again cell viability was determined at 24.5 h post-exposure. In results similar to those of the Ca^{2+} imaging experiments, ryanodine did not protect against MeHg-induced neuronal death (Figure 3.8).

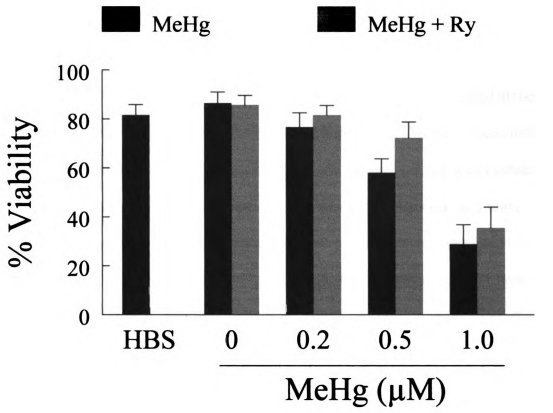

Figure 3.8. Ryanodine does not protect against MeHg-induced cell death. Application of 10 μM ryanodine for 10 min prior to, as well as during, exposure to MeHg did not protect against cell death at 24.5 hr post-exposure to 0.2, 0.5 or 1.0 μM MeHg. Results are presented as mean±SEM (n=4).

DISCUSSION

The purpose of these experiments was to elucidate the mechanism by which MeHg causes neuronal death in cerebellar granule cells which are highly sensitive to MeHg. The experiments focused on Ca^{2+} signaling pathways which converged at the SER Ca^{2+} pool, as this Ca^{2+}_i store has recently been implicated in MeHg-induced loss of $[Ca^{2+}]_i$ homeostasis in granule cells (previous chapter). While there has been evidence for MeHg interacting with various components of this signaling network, including muscarinic receptors, IP_3 receptors and ryanodine receptors (Hare and Atchison, 1995; Castoldi *et al.*, 1996; Candura *et al.*, 1997; Coccini *et al.*, 2000), this is the first report in which cytotoxicity in cerebellar granule cells resulting from specific interactions of MeHg with these receptors is examined.

In the first set of experiments, the SERCA inhibitor thapsigargin was used to examine the effect of depleting the SER of its Ca^{2+} content prior to MeHg exposure. Not surprisingly, thapsigargin treatment was itself neurotoxic, suggesting that disruption of SER Ca^{2+} regulation can induce neuronal death in granule cells. Thapsigargin-induced cell death could be due to a thapsigargin-induced increase in cytosolic $[Ca^{2+}]$ or induction of a SER "stress" response (Mattson *et al.*, 2000); however, the mechanism of thapsigargin-induced cell death was not explored further.

In the next set of experiments, 24 hr BCh was used to down-regulate the muscarinic and IP_3 receptors for 24 hr prior to MeHg exposure (Fohrman *et al.*, 1993; Fukamauchi *et al.*, 1993; Simpson *et al.*, 1994). Similar treatment with 1 mM carbachol is well documented as causing down-regulation and desensitization of both the M3 and IP_3 receptors in rat cerebellar granule neurons (Fohrman *et al.*, 1993; Fukamauchi *et al.*,

1993; Wojcikiewicz *et al.*, 1994; Simpson *et al.*, 1996; Fukamauchi *et al.*, 2000). As described in the previous chapter, 24 hr treatment with 1 mM BCh caused loss of granule cell responsiveness to subsequent muscarinic receptor stimulation by 1 mM BCh, suggesting that BCh has the same effect as carbachol. BCh was chosen based on its higher affinity for muscarinic receptors and lesser interaction with nicotinic ACh receptors than carbachol. In this study, 24 hr pretreatment with 1 mM BCh provided significant protection against MeHg-induced neuronal death at 24.5 hr post-exposure, indicating the importance of this pathway in the neurotoxicity of MeHg. Experiments using muscarinic receptor-specific antagonists suggest that the protective effect of BCh is mediated through the M3 receptor, as the protection provided by BCh is reversed by the M3-prefering antagonist 4-DAMP. The protection is also not mediated through an effect on nicotinic ACh receptors, as it is not reversed by DHE. However, the contribution of M2 receptors was not determined due to the toxicity of the M2 antagonist methoctramine itself and cannot be ruled out by these experiments.

Experiments were then performed in an attempt to differentiate between MeHg interactions with muscarinic receptors and with the IP_3 receptor. Atropine alone did not protect against MeHg-induced neurotoxicity, which was surprising given the known interactions of MeHg with muscarinic receptors. As described in Chapter Two, atropine delayed the onset of MeHg-induced elevations of $[Ca^{2+}]_i$. Additionally, both *in vivo* and *in vitro* studies suggest that MeHg binds to multiple subtypes of cholinergic receptors, inhibiting radioligand binding to both nicotinic and muscarinic receptors in a competitive fashion (Zhang and Wu, 1994; Castoldi *et al.*, 1996; Coccini *et al.*, 2000). At two weeks after a 16 day exposure to low concentrations of MeHg (0.5-2.0 mg/kg/day), rats exhibit

an increase in the number of muscarinic receptors without a change in receptor affinity in both the cerebellum and hippocampus, suggesting receptor up-regulation to compensate for loss of functional receptors *via* irreversible binding by MeHg (Coccini *et al.*, 2000). Autoradiography of MeHg accumulation in mice indicates that MeHg accumulates preferentially in these two brain regions (Berlin and Ullberg, 1963), suggesting a possible correlation between the selective neurotoxicity of MeHg within specific areas of the CNS, and location of specific muscarinic receptor subtypes. However, the results presented here suggest that the binding of MeHg to muscarinic receptors does not explain the selective targeting of neurons during toxicity, as block of these receptors with atropine did not protect against cell death. Alternatively, MeHg may have higher affinity for muscarinic receptors than does atropine, resulting in displacement of atropine by MeHg and subsequent promotion of neurotoxicity. MeHg competes with M1- and M2-prefering antagonists in rat cortical membranes, and demonstrates higher affinity for the M1 receptor (IC_{50} = 3.4 μM) *vs.* the M2 receptor (IC_{50} = 149 μM) (Castoldi *et al.*, 1996). MeHg also binds to cholinergic (presumably nicotinic) receptors isolated from *Torpedo* with high affinity and blocks binding of [^{3}H]acetylcholine to the receptor (Eldefrawi *et al.*, 1977). In guinea pig intestinal smooth muscle, MeHg inhibits contractions induced by stimulation of cholinergic nerves or by external application of ACh, suggesting a high affinity for muscarinic receptors (Fukushi and Wakui, 1985). Because MeHg cannot be removed from cholinergic receptors by mere wash with a MeHg-free solution, it is very likely that MeHg has higher affinity for muscarinic receptors than does atropine; however, the ability of MeHg to replace atropine on the muscarinic receptor has not yet been examined directly.

Attempts to block the IP_3 receptor with XeC also did not prevent MeHg-induced cell death. Given the protective effect of BCh and its reversibility by atropine, this result was also surprising. The IP_3 receptor has been implicated in MeHg-induced loss of $[Ca^{2+}]_i$ homeostasis in NG108-15 neuroblastoma cells (Hare and Atchison, 1995), T cells (Tan *et al.*, 1993), and cerebellar granule neurons (Sarafian, 1993); previous chapter. Indeed, T cells that are deficient in IP_3 receptors are resistant to apoptosis induced by a number of agents (Jayaraman and Marks, 1997; Marks, 1997). In cerebellar granule cells in culture, neurons which have a relative deficiency in IP_3 receptors are resistant to low-$[K^+]$- induced apoptosis (Oberdorf *et al.*, 1997), suggesting that this receptor plays a critical role in granule cell death. Given that MeHg does increase $[IP_3]$, and that down-regulation of the M3 and IP_3 receptors protects against MeHg-induced neuronal death, the lack of protection afforded by xestospongin C may reflect a higher affinity for the IP_3 receptor by MeHg. Alternatively, the results suggest that MeHg-induced Ca^{2+} release through the IP_3 receptor does not contribute to MeHg-induced neurotoxicity, although this conclusion is not supported by the 24 hr BCh data.

Inhibition of Ca^{2+} release through the ryanodine receptor did not protect against MeHg-induced cell death. This was not surprising, given that ryanodine receptors contribute only a fraction of the $[Ca^{2+}]_i$ increase observed in granule cells following MeHg exposure (previous chapter). Ryanodine receptors also do not contribute significantly to MeHg-induced $[Ca^{2+}]_i$ increases in NG108-15 cells (Hare and Atchison, 1995), suggesting that these receptors do not play a significant role in MeHg-induced neurotoxicity. Most likely, their primary contribution is through participation in Ca^{2+}-induced Ca^{2+}-release originating in IP_3 receptors and/or mitochondria.

In summary, the experiments described in Chapters Two and Three indicate that MeHg causes Ca^{2+} release from the SER which contributes to MeHg-induced cell death of rat cerebellar granule cells. While the SER does not contribute the majority of the Ca^{2+}_i released during exposure, inhibition of this Ca^{2+} release is neuroprotective. As will be discussed in greater detail in Chapter Four, the real significance of the release of SER Ca^{2+} may be in its effects on mitochondria by contributing to mitochondrial Ca^{2+} dysregulation and thereby promoting of cell death through mitochondrial dysfunction.

CHAPTER FOUR

DISRUPTION OF MITOCHONDRIAL CALCIUM SIGNIFICANTLY CONTRIBUTES TO METHYLMERCURY-INDUCED ELEVATIONS OF $[Ca^{2+}]_i$

ABSTRACT

The contribution of Ca^{2+}_m to whole-cell $[Ca^{2+}]_i$ elevations caused by acute exposure to low concentrations of methylmercury (MeHg) was examined in rat cerebellar granule cells. In granule cells loaded with fura-2 to observe changes in whole-cell $[Ca^{2+}]_i$, prior depletion of Ca^{2+}_m using CCCP and oligomycin significantly decreased the amplitude of $[Ca^{2+}]_i$ release from intracellular stores, as well as delayed the time-to-onset of whole-cell $[Ca^{2+}]_i$ elevations, caused by 0.5 μM MeHg. Oligomycin delayed the MeHg-induced influx of Ca^{2+}_e, suggesting that ATP depletion triggers Ca^{2+} influx. In granule cells loaded with rhod-2, MeHg exposure caused a biphasic increase in fluorescence. The initial increase in fluorescence was not dependent on Ca^{2+}_e and was abolished by mitochondrial depolarization, indicating uptake of Ca^{2+} from a non-mitochondrial Ca^{2+}_i source. The secondary increase was associated with spreading of the dye from a punctate staining to whole-cell distribution, and was significantly delayed by thapsigargin and CsA, suggesting that Ca^{2+} released from the SER contributes to MeHg-induced opening of the MTP. It is concluded that MeHg causes an initial release of Ca from the SER which is buffered by mitochondria, leading to elevated $[Ca^{2+}]_m$ and opening of the MTP in rat cerebellar granule neurons.

INTRODUCTION

As discussed in the previous chapters, acute exposure to low concentrations of MeHg causes release of Ca^{2+} from at least one intracellular store into the cytosol. Depletion of SER Ca^{2+} content reduced the amplitude of the MeHg-induced $Ca^{2+}{}_i$ release by less than half; thus, at least one other intracellular store contributes to the MeHg-induced $[Ca^{2+}]_i$ elevations. The next two chapters discuss experiments designed to examine the contribution of $Ca^{2+}{}_m$ to MeHg-induced increases in $[Ca^{2+}]_i$ and cell death.

Mitochondria are "excitable organelles" that can accumulate a large amount of Ca^{2+} within the lumen and, upon excess accumulation, release $Ca^{2+}{}_m$ into the cytosol *via* reversal of the uniporter and/or opening of the MTP (Nicholls, 1978; Thayer and Miller, 1990; Budd and Nicholls, 1996b). Not only does disruption of $Ca^{2+}{}_m$ contribute to opening of the MTP and release of pro-apoptotic factors (such as cytochrome c) into the cytosol, but release of sequestered Ca^{2+} contributes to cytosolic elevations of $[Ca^{2+}]_i$ which promotes cell death through activation of Ca^{2+}-dependent proteases (calpains and caspases), phospholipases and endonucleases which degrade cellular protein and DNA (Cohen and Duke, 1984; Orrenius and Nicotera, 1994; Waring and Sjaarda, 1995; Moran *et al.*, 1999). Recent studies indicate that elevation of $Ca^{2+}{}_m$ is a critical step during a variety of neurotoxic insults, including glutamate excitotoxicity, generation of reactive oxygen species, and exposure to heavy metals, including manganese and mercury (Chavez and Holguin, 1988; Chavez *et al.*, 1989; Richter and Kass, 1991; Nicholls and Budd, 1998; Gavin *et al.*, 1999; Kruman and Mattson, 1999; Verity, 1999)

Several parameters of mitochondrial function are affected by MeHg. MeHg binds to mitochondria with high affinity, both *in vivo* and *in vitro* (Yoshino *et al.*, 1966; Chang

and Hartmann, 1972; O'Kusky, 1983). Further, MeHg causes irreversible loss of mitochondrial membrane potential in human monocytes (InSug *et al.*, 1997), isolated nerve terminals (Bondy and McKee, 1991), NG108-15 neuroblastoma cells (Hare *et al.*, 1993), and guinea pig cerebral cortex synaptosomes (Kauppinen *et al.*, 1989). Inhibition of Ca^{2+}_m uptake and release inhibits MeHg-induced increases in the frequency of spontaneous neurotransmitter release in hemidiaphragm nerve terminals (Levesque and Atchison, 1988), and attenuates the uptake of ^{45}Ca and release of $^{45}Ca^{2+}$ from preloaded, isolated mitochondria Ca^{2+} uptake (Levesque and Atchison, 1991). MeHg is also well documented as inhibiting mitochondrial respiration, decreasing cellular ATP content and causing production of reactive oxygen species, all of which can contribute to mitochondria-triggered cell death (Kauppinen *et al.*, 1989; Sarafian *et al.*, 1989; Levesque and Atchison, 1991; Sarafian and Verity, 1991; Yee and Choi, 1996). Because the Ca^{2+}_i chelator BAPTA protects against MeHg-induced cell death in rat granule cells *in vitro*, suggesting a Ca^{2+}-dependent process (Marty and Atchison, 1998), there is a strong possibility that MeHg-induced changes in Ca^{2+}_m may be critical in MeHg-induced neuronal death.

The experiments described in this chapter examined whether mitochondria contribute Ca^{2+}_m to the first-phase $[Ca^{2+}]_i$ increase during acute exposure to low concentrations of MeHg. Because alterations in cellular and mitochondrial Ca^{2+} regulation are critical events in both apoptosis and necrosis, the experiments were designed to characterize the effects of MeHg on Ca^{2+}_m in the intact neuron. The first approach was to determine the relative contribution of Ca^{2+}_m to whole-cell elevations of $[Ca^{2+}]_i$ to determine whether mitochondria are a significant target in these cells. The next

experiments examined the effect of MeHg on regulation of Ca^{2+}_m in the intact cell, including interactions between intracellular Ca^{2+} stores in the mitochondria and SER during MeHg exposure. The results presented here suggest that MeHg targets both the mitochondria and SER during MeHg exposure, with Ca^{2+} release from the SER contributing to Ca^{2+} release from the mitochondria *via* opening of the MTP. Further, these experiments indicate that Ca^{2+}_m contributes the bulk of the cytosolic $[Ca^{2+}]_i$ observed during MeHg exposure, highlighting the importance of mitochondria during MeHg neurotoxicity.

MATERIALS AND METHODS

The materials and solutions for isolation of cerebellar granule cells and measurement of changes in $[Ca^{2+}]_i$ are described in detail in Chapter Two. Unless specifically stated, all solutions were identical to those described in Chapter Two. The methods and solutions described below are specific to this chapter.

Materials and solutions. Rhod-2 acetoxymethylester (rhod-2 AM) and tetramethylrhodamine ethyl ester (TMRE) were purchased from Molecular Probes (Eugene, OR). Cyclosporin A (CsA), CCCP and oligomycin were purchased from Sigma (St. Louis, MO). For all experiments, pharmacological agents are dissolved in either HBS or EGTA-HBS, with appropriate controls for any additional solvents used (DMSO or ethanol, with maximum solvent of 0.01% (w/v) for all experiments). In a cell-free system, none of the pharmacological agents altered fura-2 fluorescence.

Measurement of $[Ca^{2+}]_i$. Cells were loaded with fura-2 for 1 hr, and rinsed in HBS for 30 min, as described in Chapter Two. Changes in cellular fluorescence were measured using the IonOptix system described in Chapter Two. As before, following exposure to MeHg, the times-to-onset of the first and second phases of increased $Ca^{2+}{}_i$ were determined. Additionally, in experiments performed in the nominally Ca^{2+}-free buffer (EGTA-HBS), the amplitude of $[Ca^{2+}]_i$ release caused by MeHg was normalized to the peak fluorescence ratio amplitude of the 1 min exposure to 40 mM K^+ to provide a "normalized ratio" relative to K^+-induced depolarization response. For both time-to-onset and normalized fluorescence ratio, the data from each trial were averaged to

provide a mean time-to-onset or mean normalized ratio for that dish of cells (n=1). In order to minimize differences between cell isolates, experiments using MeHg alone and MeHg with a given pharmacological agent were run on the same day, and experiments using the same agents were replicated using at least two separate cell isolates.

Measurement of $[Ca^{2+}]_m$. To measure changes in $[Ca^{2+}]_m$, cells were loaded with 1 μM rhod-2 AM for 1 hr at room temperature (23-25°), followed by overnight incubation in normal culture medium (18-24 hr) (Trost and Lemasters, 1997; Peng *et al.*, 1998; Simpson and Russell, 1998a). Because rhod-2 will fluoresce in both the cytosol and mitochondria, this protocol was used to ensure that mitochondrial loading occurred and provide time for the cytosolic dye to leak out of the cell. Cells were imaged as in the fura-2 experiments, except that the excitation wavelength was 540 nm and the emission wavelength was 590 nm. Efficiency of mitochondrial loading was confirmed using an 8 min exposure to 5 μM CCCP to uncouple oxidative phosphorylation, and 10 μM oligomycin to dissipate ψ_m; this combination prevents mitochondrial Ca^{2+} uptake during Ca^{2+} influx caused by K^+ depolarization (40 mM), thereby abolishing changes in rhod-2 fluorescence. Like fura-2, rhod-2 data are analyzed for time-to-onset of fluorescence changes, with the time-to-onset of the initial elevation determined as the point at which fluorescence rose above baseline, and the secondary elevation (from spreading of rhod-2 fluorescence from a punctate stain to whole-cell fluorescence) as the point at which there is a sharp increase in rhod-2 fluorescence. The times-to-onset of each phase of fluorescence were calculated for all cells monitored within an experiment and were averaged to provide mean time-to-onset for that dish of cells (n=1).

Measurement of Ψ_m. The fluorescent dye tetramethylrhodamine ethyl ester (TMRE) was used to observe changes in Ψ_m. TMRE preferentially loads into mitochondria with normal membrane potentials (around –160 mV) and loses fluorescence when Ψ_m becomes depolarized (Farkas *et al.*, 1989). Granule cells were loaded with 100 nM TMRE in HBS for 30 min at 37°C, then rinsed in 10 nM TMRE in HBS for 30 min. Cellular fluorescence was then measured continuously using the IonOptix system. In these experiments, perfusion solutions contained 10 nM TMRE to maintain dye availability for the mitochondria. Fluorescence was measured relative to an initial fluorescence of 100%. Control experiments indicated that TMRE fluorescence remained stable for at least 1 hr of observation (data not shown).

Statistics

Comparisons of mean time-to-onset for "MeHg" *vs.* the corresponding "MeHg plus inhibitor" cells were made using Student's paired t-test, with values of $p<0.05$ considered to be statistically significant.

RESULTS

Neonatal granule cells in culture exposed to a low concentration of MeHg undergo a characteristic biphasic increase in $[Ca^{2+}]_i$, with the first phase due to release of Ca^{2+} from at least one intracellular store, and the second phase due to influx of Ca^{2+}_e (Marty and Atchison, 1997). To examine the contribution of the mitochondrial Ca^{2+} store, the mitochondria were depolarized with 5 μM CCCP and 10 μM oligomycin for 8 min immediately prior to exposure to 0.5 μM MeHg. Because mitochondria accumulate Ca^{2+} only when their inner membranes are sufficiently hyperpolarized, this depolarization causes both release of Ca^{2+}_m and prevention of further Ca^{2+} uptake (Budd and Nicholls, 1996b). Monitoring cells using TMRE fluorescence confirmed that this protocol was sufficient to depolarize completely the mitochondria (Figure 4.1A). The brief increase in TMRE fluorescence intensity is probably due to mitochondrial swelling, rather than mitochondrial membrane hyperpolarization, which accompanies the increased permeability of the inner mitochondrial membrane and subsequent increase in lumen volume (White and Reynolds 1996; Trost and Lemasters 1997). Further, experiments using cells loaded with fura-2 indicated that this protocol caused a large increase in $[Ca^{2+}]_i$ (Figure 4.1B). Following 8 min exposure to CCCP plus oligomycin, the cells did not respond to another application of CCCP plus oligomycin, indicating the irreversible loss of mitochondrial membrane potential, and successful depletion of Ca^{2+}_m, with this protocol (Figure 4.1C). Treatment with 10 μM oligomycin alone caused a more gradual loss of mitochondrial membrane potential, with a 75-85% loss of TMRE fluorescence at 20 min of exposure; TMRE fluorescence could be lowered further with 5 μM CCCP, indicating that oligomycin alone did not cause complete mitochondrial membrane

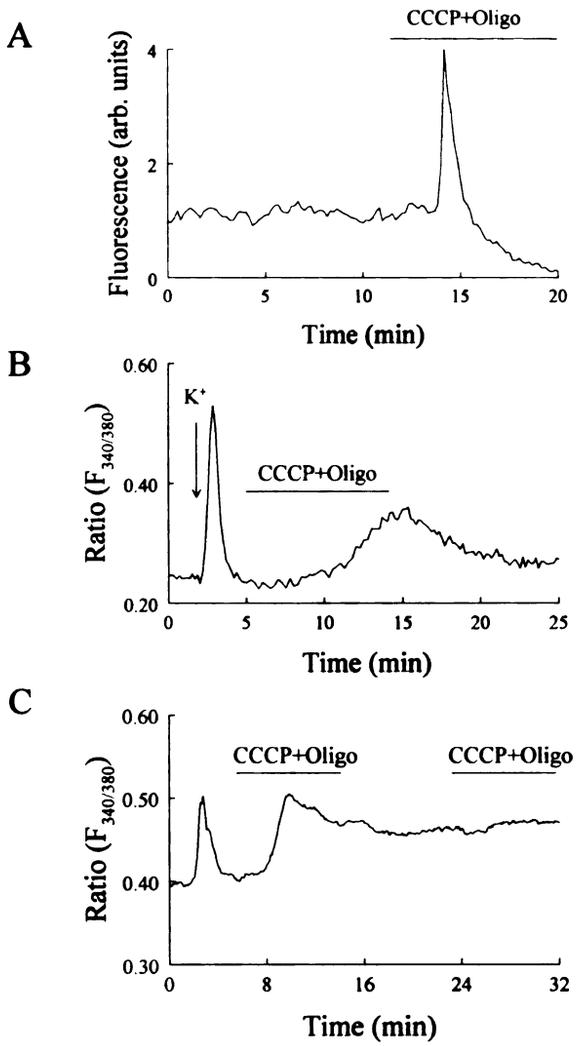

Figure 4.1. CCCP and oligomycin cause a rapid decrease in TMRE fluorescence with a concomitant increase in fura-2 fluorescence. A) Changes in TMRE fluorescence in a single cerebellar granule neuron in culture exposed to 5 μM CCCP and 10 μM oligomycin for 8 min. CCCP and oligomycin cause a rapid decrease in TMRE fluorescence (as the mitochondrial membrane becomes depolarized). In granule cells, the rapid loss of ψm caused by CCCP and oligomycin is irreversible for up to 60 min post-treatment. TMRE fluorescence is measured relative to the initial fluorescence intensity. B) Changes in fura-2 fluorescence ("Ratio ($F_{340/380}$)") in a single cerebellar granule neuron exposed to 5 μM CCCP and 10 μM oligomycin for 8 min. Mitochondrial membrane depolarization causes a large-amplitude increase in $[Ca^{2+}]_i$ which recovers to baseline $[Ca^{2+}]_i$ by the end of the 8 min exposure. C) Changes in fura-2 fluorescence ("Ratio ($F_{340/380}$)") in a single cerebellar granule neuron exposed to 5 μM CCCP and 10 μM oligomycin for 8 min, followed by 10 min HBS and another 8 min application of 5 μM CCCP and 10 μM oligomycin. The second exposure to these compounds does not result in additional increases in fura-2 ratio, indicating that a single application is sufficient to deplete Ca^{2+}_m.

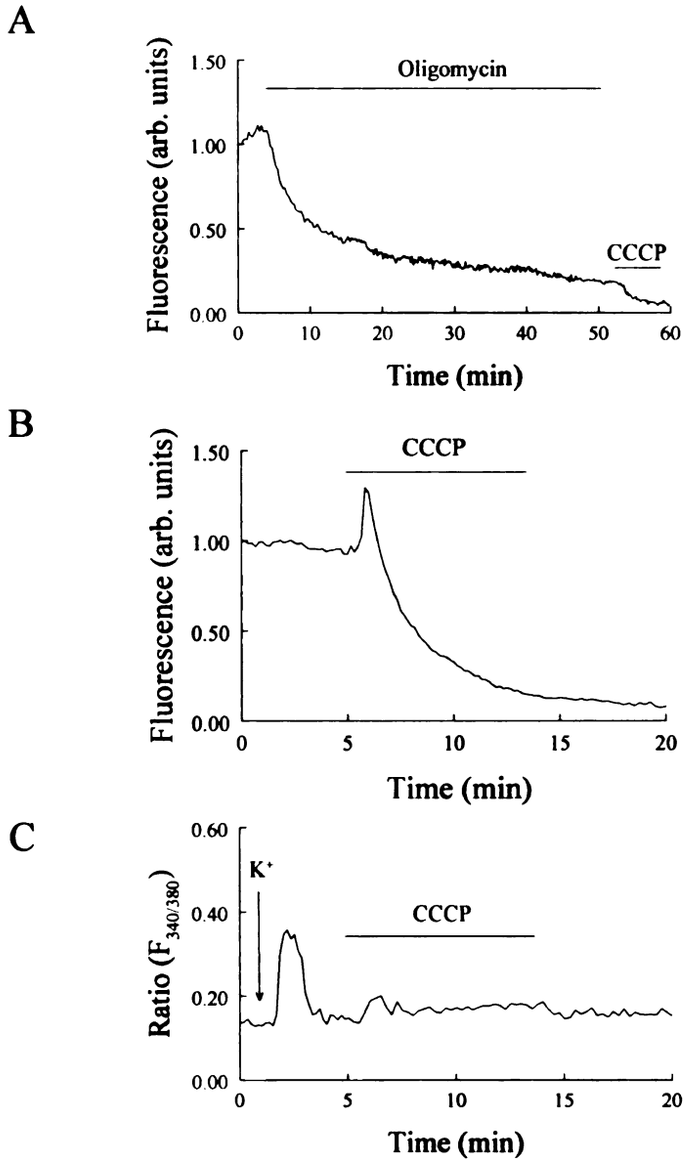

Figure 4.2 Effect of oligomycin on TMRE fluorescence, as well as effect of CCCP on TMRE and fura-2 fluorescence. A) Changes in TMRE fluorescence in a single cerebellar granule neuron in culture exposed to 10 μM oligomycin for 45 min. Oligomycin causes a gradual loss of TMRE fluorescence which is further lowered by the addition of 5 μM CCCP, indicating that oligomycin alone does not completely depolarize the mitochondrial membrane. TMRE fluorescence is measured relative to the initial fluorescence intensity. B) Changes in TMRE fluorescence in a single cerebellar granule neuron in culture exposed to 5 μM CCCP for 8 min. CCCP alone causes a rapid decrease in TMRE fluorescence which does not recover during wash with CCCP-free HBS. TMRE fluorescence is measured relative to the initial fluorescence intensity. C) Changes in fura-2 fluorescence ("Ratio ($F_{340/380}$)") in a single cerebellar granule neuron exposed to 5 μM CCCP for 8 min. CCCP alone causes a much smaller increase in $[Ca^{2+}]_i$ than CCCP and oligomycin together (see Figure 4.1B).

depolarization (Figure 4.2A). Application of 5 μM CCCP for 8 min did cause rapid, complete loss of TMRE fluorescence which did not recover after removal of CCCP and wash with HBS, indicating that CCCP alone causes rapid depolarization of the mitochondrial membrane (Figure 4.2B). However, unlike CCCP plus oligomycin, CCCP alone caused a much smaller increase in cytosolic $[Ca^{2+}]$ with an amplitude approximately half that caused by CCCP plus oligomycin (Figure 4.2C).

Next, the effect of depleting mitochondrial Ca^{2+} stores on MeHg-induced elevations of $[Ca^{2+}]_i$ was examined. Complete removal of $Ca^{2+}{}_m$ using 5 μM CCCP plus 10 μM oligomycin for 8 min delayed the time-to-onset of increases in $[Ca^{2+}]_i$ caused by 0.5 μM MeHg (from 12.6 ± 2.5 min to 37.7 ± 3.0 min) (Figure 4.3). The time-to-onset of the first-phase increase in $[Ca^{2+}]_i$ was not significantly altered by CCCP alone (11.5 ± 1.4 min *vs.* 10.7 ± 1.7 min) or oligomycin alone (9.8 ± 1.9 min *vs.* 17.4 ± 5.8 min) (Figure 4.3). Similarly, emptying of the mitochondria with CCCP and oligomycin prior to exposure to 0.5 μM MeHg in EGTA-HBS revealed a similar delay in the increased $[Ca^{2+}]_i$, delaying the onset of the first-phase $[Ca^{2+}]_i$ increase from 15.3±3.2 to 45.4±3.9 min (Figure 4.4A). Additionally, CCCP and oligomycin treatment significantly reduced the amplitude of the fura-2 ratio increase from a normalized ratio of 1.25±0.26 to 0.41±0.13, representing a 67% decrease in the ratio amplitude (Figure 4.4B). Thus complete removal of $Ca^{2+}{}_m$ is required to delay significantly the MeHg-induced release of Ca^{2+} from intracellular pools, but does not prevent an eventual increase in $[Ca^{2+}]_i$. Additionally, this data indicates that most of the $Ca^{2+}{}_i$ released by MeHg originated in the mitochondria.

The mitochondrial inhibitors had differing effects on the time-to-onset of the

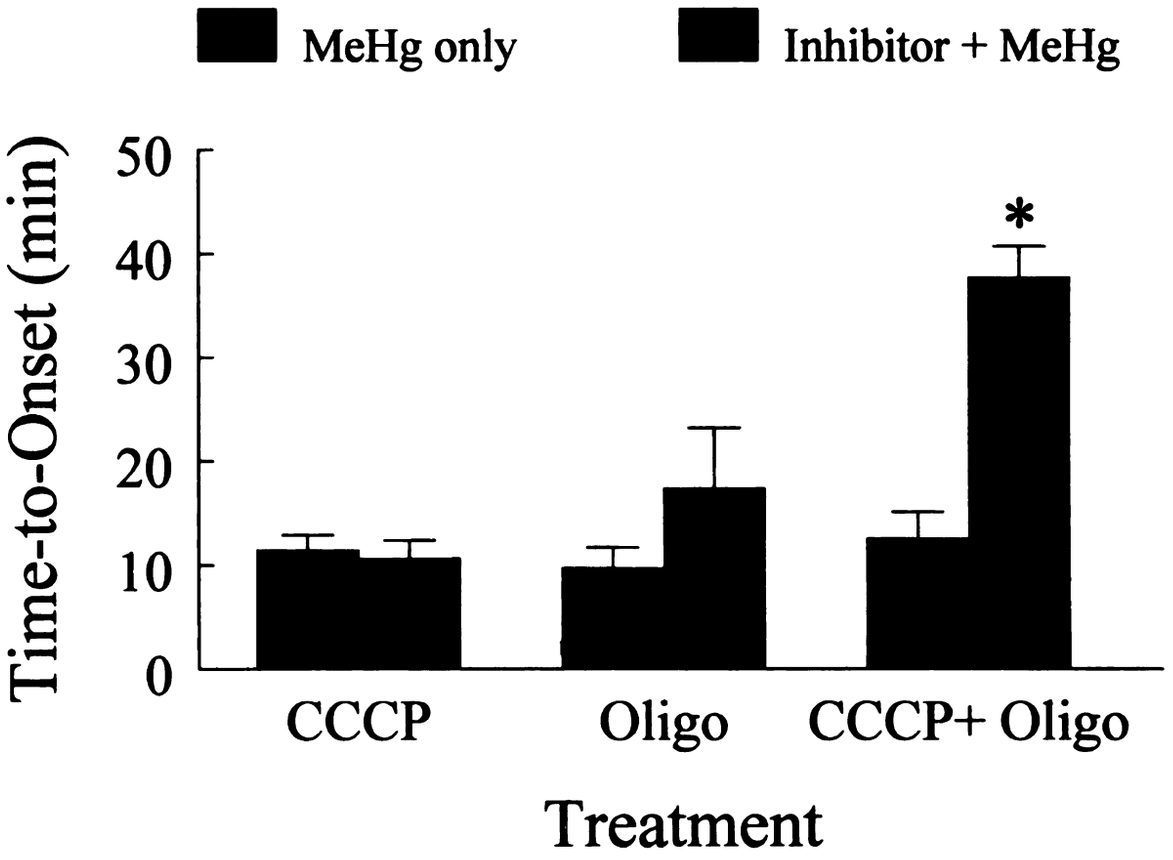

Figure 4.3. Effect of mitochondrial inhibitors on first-phase increase in $[Ca^{2+}]_i$ caused by 0.5 μM MeHg. Granule cells were pretreated with 5 μM CCCP (8 min), 10 μM oligomycin (8 min), or 5 μM CCCP plus 10 μM oligomycin (8 min) immediately prior to 0.5 μM MeHg. Time-to-onset of the $[Ca^{2+}]_i$ increase was compared between MeHg alone and MeHg plus inhibitor for each treatment. Results are presented as mean±SEM (n=5-8). The asterisk (*) indicates a value significantly different from MeHg-only control ($p<0.05$).

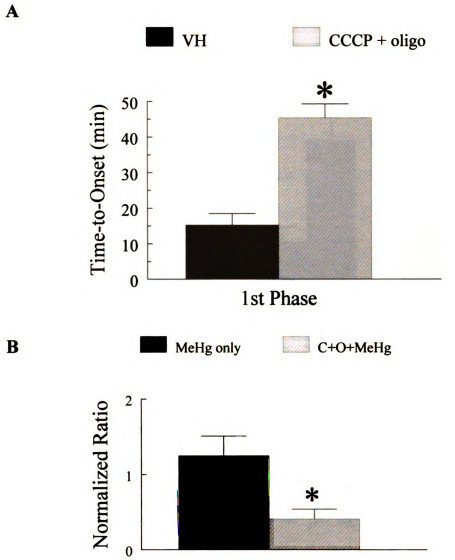

Figure 4.4. Effect of CCCP and oligomycin on first-phase time-to-onset and ratio amplitude induced by 0.5 μM MeHg in the absence of Ca^{2+}_e. A) Measurement of time-to-onset of $[Ca^{2+}]_i$ elevations caused by 0.5 μM MeHg in the absence of Ca^{2+}_e. Cells were pretreated with vehicle control ("VH") or 5 μM CCCP plus 10 μM oligomycin ("CCCP+oligo") for 8 min immediately prior to 0.5 μM MeHg. B) Measurement of the first-phase ratio amplitude in the absence of Ca^{2+}_e. Cells were exposed to 0.5 μM MeHg alone ("MeHg") or 5 μM CCCP plus 10 μM oligomycin (8 min) immediately prior to 0.5 μM MeHg ("C+O"). The amplitude of the first-phase ratio increase was normalized to the peak fura-2 response to the 1 min 40 mM K^+ test (at the beginning of each experiment). Results are presented as mean±SEM. (n=5). The asterisk (*) indicates a value significantly different from MeHg-only control ($p<0.05$).

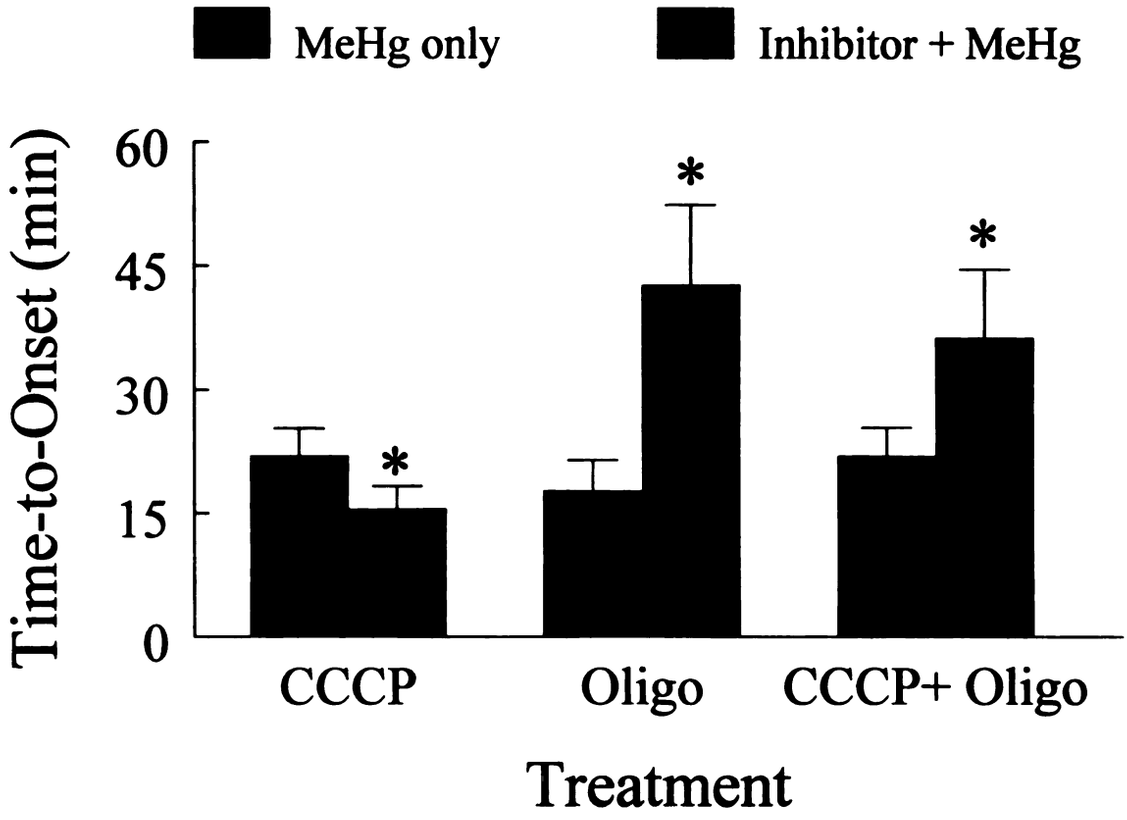

Figure 4.5. Comparison of time-to-onset of Ca^{2+}_e influx caused by 0.5 μM MeHg in the presence or absence of mitochondrial inhibitors. Granule cells were pretreated with 5 μM CCCP (8 min), 10 μM oligomycin (8 min), or 5 μM CCCP plus 10 μM oligomycin (8 min) immediately prior to 0.5 μM MeHg. Comparisons are made between MeHg alone and MeHg plus inhibitor for each treatment. Results are presented as mean±SEM (n=5-8). The asterisk (*) indicates a value significantly different from MeHg-only control ($p<0.05$).

influx of Ca^{2+}_e caused by 0.5 μM MeHg. Mitochondrial membrane depolarization without maintenance of cellular ATP levels using CCCP alone hastened the influx of Ca^{2+}_e caused by MeHg, from 22.0 ± 3.3 min to 15.6 ± 2.7 min (Figure 4.5). This Ca^{2+} influx may occur independently of any effects of MeHg, as mitochondrial membrane depolarization causes reversal of the mitochondrial ATPase, leading to conversion of ATP to ADP and depletion of ATP, thereby causing Ca^{2+} influx through loss of ATP for plasma membrane Ca^{2+} pumps (Nicholls and Scott, 1980). Oligomycin alone had the opposite effect, causing a significant delay in the time-to-onset of MeHg-induced Ca^{2+}_e influx, from 17.8 ± 3.7 min to 42.7 ± 9.7 min (Figure 4.5). Because oligomycin does not completely depolarize the mitochondria by 8 min but does maintain cellular ATP levels, this delayed influx of Ca^{2+}_e suggests that MeHg-induced depletion of cellular ATP triggers onset of the second-phase increase in $[Ca^{2+}]_i$. Application of CCCP plus oligomycin caused a similar delay in the time-to-onset of the MeHg-induced Ca^{2+} influx as that caused by oligomycin alone, from 22 ± 3.4 min to 36.3 ± 8.3 min (Figure 4.5). Thus, MeHg-induced influx of Ca^{2+}_e appears to be dependent on loss of cellular ATP content as a result of MeHg-induced alteration of mitochondrial function.

The next step was to examine the route by which MeHg causes uptake of Ca^{2+} into the mitochondria, and subsequent release of Ca^{2+}_m from the mitochondria into the cytosol. As described in Chapter Two, previous experiments suggested that MeHg causes release of Ca^{2+} from the SER *via* activation of muscarinic receptors linked to production of IP_3. Based on these results, it was hypothesized that MeHg caused Ca^{2+} to be released from the SER, which was then buffered by mitochondria. To test this hypothesis, the effect of MeHg on Ca^{2+}_m was examined using the fluorescent Ca^{2+}_m

indicator rhod-2. Cells were first loaded with fura-2 and exposed to increasing concentration of K^+ (10-100 mM) for 1 min each, to demonstrate that this protocol causes reversible increase in $[Ca^{2+}]_i$ which exhibit increasing fura-2 ratio amplitude as the $[K^+]$ increases (Figure 4.6A). Granule cells were then loaded with rhod-2 to monitor changes in $[Ca^{2+}]_m$. Control experiments using 1 min depolarizations with increasing concentrations of K^+ (10-100 mM) indicated that rhod-2 fluorescence increases during an increase in $[Ca^{2+}]_i$ and returns to baseline after removal of the K^+ solution (Figure 4.6B). Further, this increase in fluorescence was due to a change in $Ca^{2+}{}_m$ and not due to cytosolic loading of the dye as the increase in rhod-2 fluorescence caused by 1 min treatment with 40 mM K^+ was abolished by 8 min exposure to CCCP and oligomycin (Figure 4.6B).

As seen in Figure 4.7, granule cells loaded with rhod-2 exhibit a biphasic increase in rhod-2 fluorescence during exposure to 0.5 μM MeHg. Rhod-2 fluorescence increases to a similar intensity as that caused by 1 min depolarization with 40 mM K^+ (Figure 7A); this initial increase is abolished by 8 min exposure to CCCP and oligomycin, indicating that the initial increase in fluorescence is due to an increase in $Ca^{2+}{}_m$ (Figure 7B). The secondary increase in fluorescence is associated with a spreading of the dye from the mitochondria to the cytosol which was apparent both in the digital image recorded by the IonOptix software, and in the fluorescence emission recording. The dye movement was associated with an increase in rhod-2 fluorescence intensity above that caused by the 1 min 40 mM K^+ depolarization; thus we measured the secondary fluorescence increase as the point at which rhod-2 fluorescence became elevated above that of the 40 K^+-induced depolarization. Interestingly, treatment with CCCP and oligomycin abolished the initial

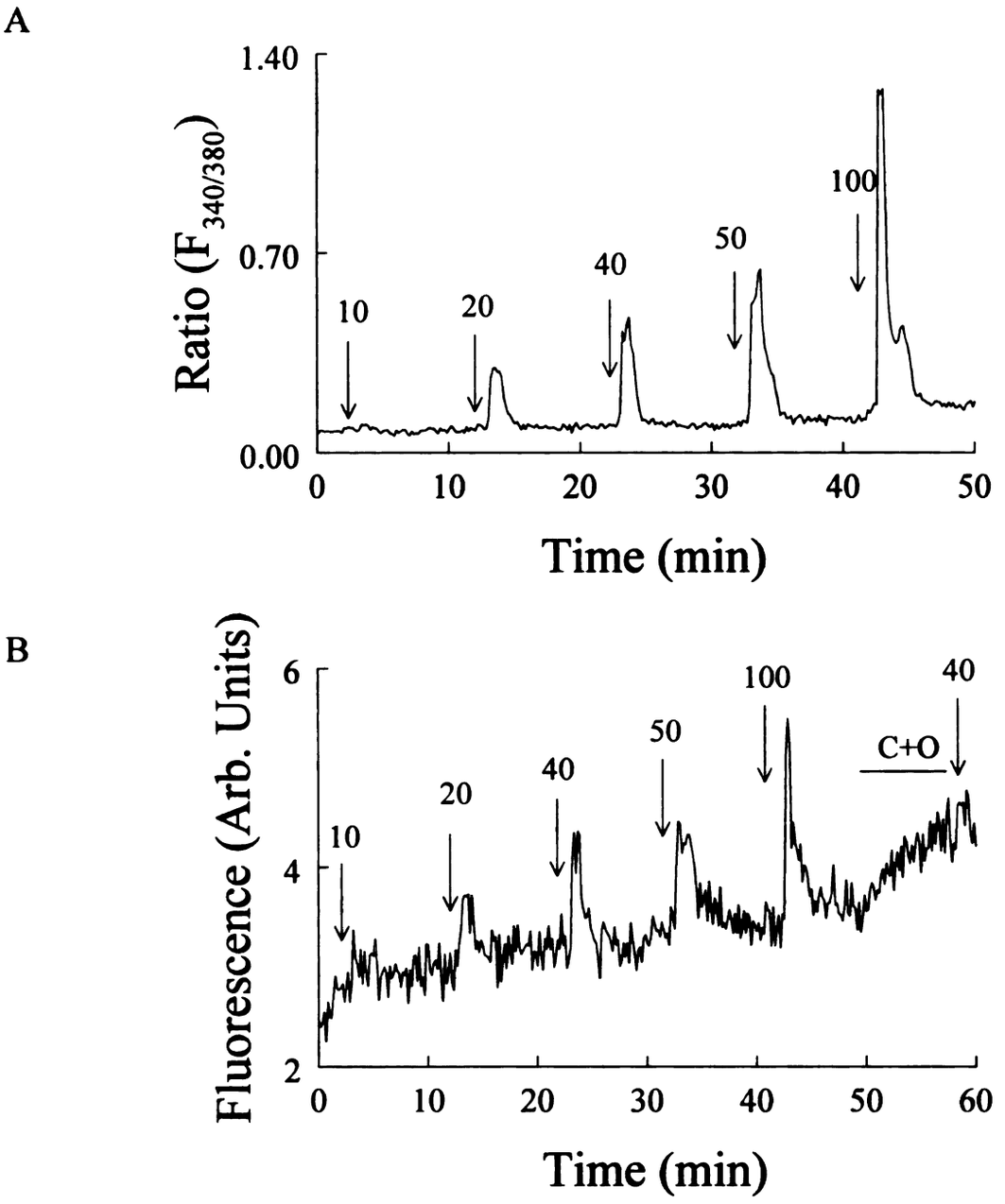

Figure 4.6. Changes in fura-2 and rhod-2 fluorescence in response to increasing concentrations of extracellular K^+. A) Representative example of changes in fura-2 fluorescence ("Ratio ($F_{340/380}$)") in a single cerebellar granule neuron exposed to increasing concentrations of K^+ (10-100 mM). Each K^+ solution was applied for 1 min starting at the arrow, with the [K^+] (mM) indicated by the number above the arrow. B) Representative example of changes in rhod-2 fluorescence ("Fluorescence (arb. units)") in a single cerebellar granule neuron exposed to increasing concentrations of K^+ (10-100 mM). Each K^+ solution was applied for 1 min starting at the arrow, with the [K^+] (mM) indicated by the number above the arrow. At the end of the experiment, 5 μM CCCP and 10 μM oligomycin were applied for 8 min, followed by another 1 min treatment with 40 mM K^+. CCCP and oligomycin abolished rhod-2 fluorescence changes in response to the K^+ depolarization, confirming that rhod-2 was localized within the mitochondria.

elevation of fluorescence but did not alter the time-to-onset of the secondary fluorescence increase associated with the dye spreading throughout the cell (Figure 4.9A). Because the initial increase of fluorescence did not occur until the spreading of dye throughout the cell occurs, the "first-phase" increase in rhod-2 fluorescence occurred simultaneously with the "second-phase" release of dye into the cytosol (Figure 4.8). When granule cells were exposed to 0.5 μM MeHg in the nominally Ca^{2+}-free EGTA-HBS, the pattern of rhod-2 fluorescence changes was similar, as MeHg again caused an initial fluorescence increase which was abolished by CCCP and oligomycin, and a secondary fluorescence increase from dye movement into the cytosol (Figure 4.9B).

Because MeHg caused an increase in Ca^{2+}_m in the absence of Ca^{2+}_e, it was hypothesized that the mitochondria were buffering Ca^{2+} that was released from another intracellular Ca^{2+} store, possibly the SER; thus, the next experiment examined the effect of SER Ca^{2+} depletion on MeHg-induced elevations of Ca^{2+}_m using the SERCA inhibitor thapsigargin (10 μM for 5 min). Thapsigargin treatment immediately prior to exposure to 0.5 μM MeHg did not affect the time-to-onset of the initial rhod-2 fluorescence increase, but did significantly delay the dye movement into the cytosol (Figure 4.10).

Finally, experiments were performed to test whether the release of rhod-2 from the mitochondria into the cytosol was due to opening of the MTP. Related experiments indicated that the MTP inhibitor CsA delays the MeHg-induced increase in $[Ca^{2+}]_i$ caused by 0.2 and 0.5, but not 1.0 μM MeHg (see Chapter Five). In granule cells loaded with rhod-2, application of 5 μM CsA for 10 min immediately before 0.5 μM MeHg did not alter the time-to-onset of the initial fluorescence increase but did significantly delay the

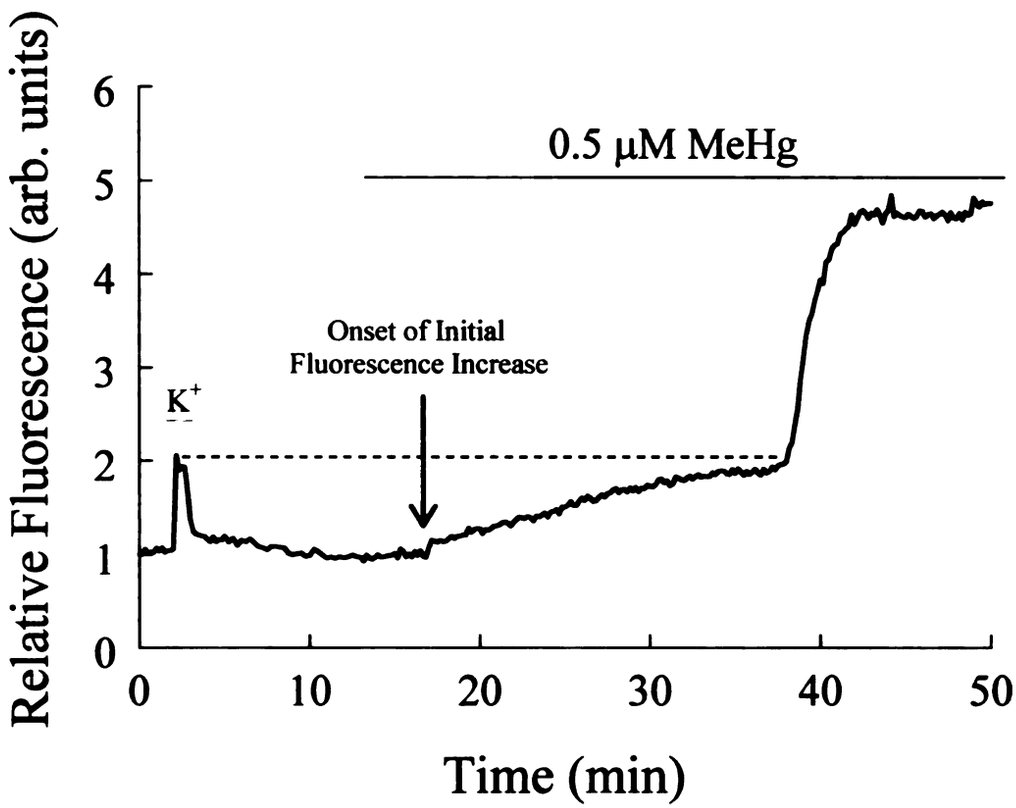

Figure 4.7. MeHg causes a biphasic increase in rhod-2 fluorescence in granule cells. Changes in rhod-2 fluorescence in a single neuron exposed to 0.5 μM MeHg. Cells were initially exposed to 1 mM K^+ to verify cell viability ("K^+"). During MeHg exposure, there is a biphasic increase in rhod-2 fluorescence: the initial rise plateaus at a similar fluorescence intensity as that caused by the 1 min 40 mM K^+; the secondary increase is associated with the dye spreading throughout the entire cytosol.

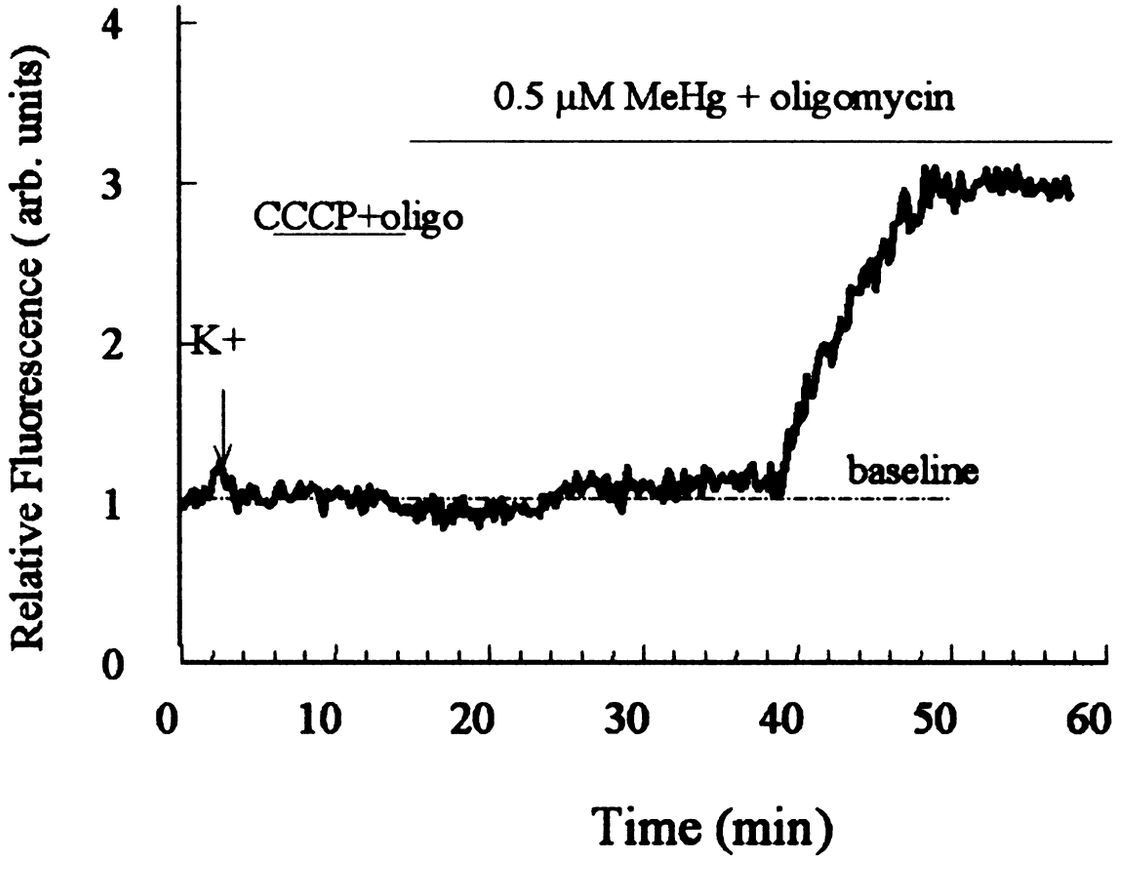

Figure 4.8. Effect of CCCP and oligomycin on MeHg-induced elevations of rhod-2 fluorescence. Changes in rhod-2 fluorescence in a single neuron exposed to 5 μM CCCP and 10 μM oligomycin ("C+O") for 8 min immediately before 0.5 μM MeHg. In these cells, the initial rise in rhod-2 fluorescence is absent; however, the secondary fluorescence increase from the dye spreading throughout the cytosol is still present.

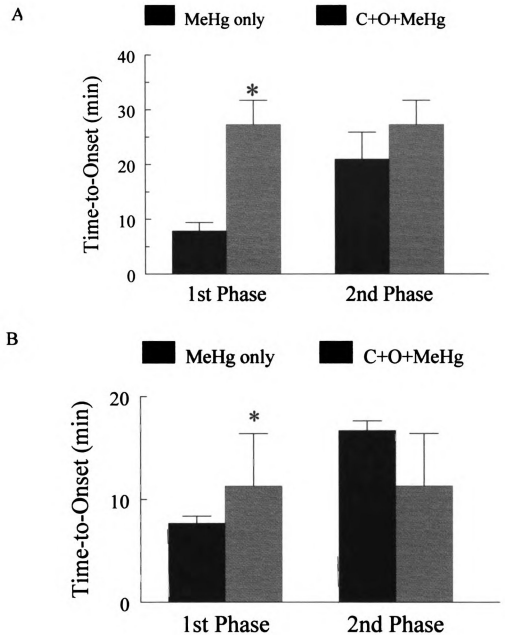

Figure 4.9. Mitochondrial membrane depolarization abolished the initial elevation of rhod-2 fluorescence without affecting the time-to-onset of the spreading of rhod-2 throughout the cytosol. A) Granule cells were pretreated with 5 μM CCCP plus 10 μM oligomycin (8 min) immediately prior to 0.5 μM MeHg. Time-to-onset of the $[Ca^{2+}]_i$ increase was compared between MeHg alone and MeHg plus CCCP and oligomycin. Results are presented as mean±SEM (n=4). B) Granule cells were pretreated with 5 μM CCCP plus 10 μM oligomycin (8 min) immediately prior to 0.5 μM MeHg in EGTA-HBS. Results are presented as mean ± SEM (n=3). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

spreading of rhod-2 throughout the cell (Figure 4.11). A comparison of the effect of CsA and thapsigargin on the interval between onset of first and second rhod-2 fluorescence increases is shown in Figure 4.12.

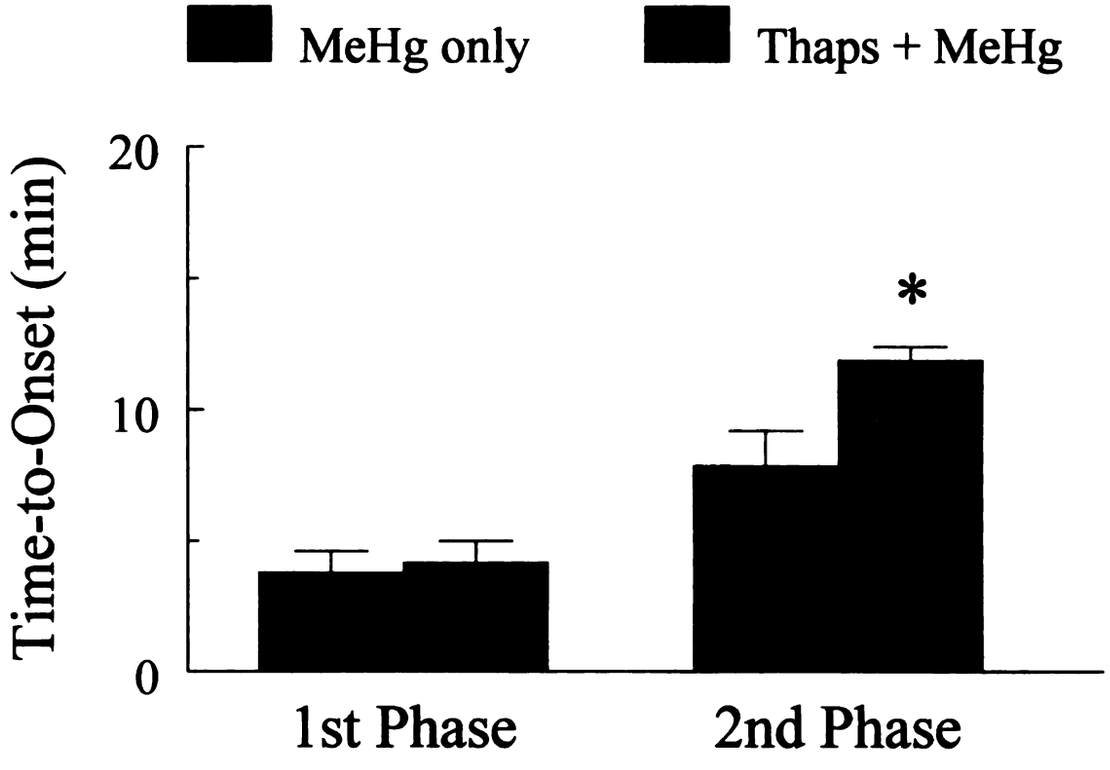

Figure 4.10. Thapsigargin delays the spreading of rhod-2 from punctate to whole-cell staining as caused by 0.5 μM MeHg. Granule cells were pretreated with 10 μM thapsigargin for 5 min immediately prior to 0.5 μM MeHg. The first-phase elevation is measured where rhod-2 fluorescence rises above baseline; the second-phase elevation is measured where the fluorescence rises above the fluorescence increase caused by the 1 min 40 mM K^+ test. Results are presented as mean±SEM (n=5). The asterisk (*) indicates a value significantly different from MeHg-only control ($p<0.05$).

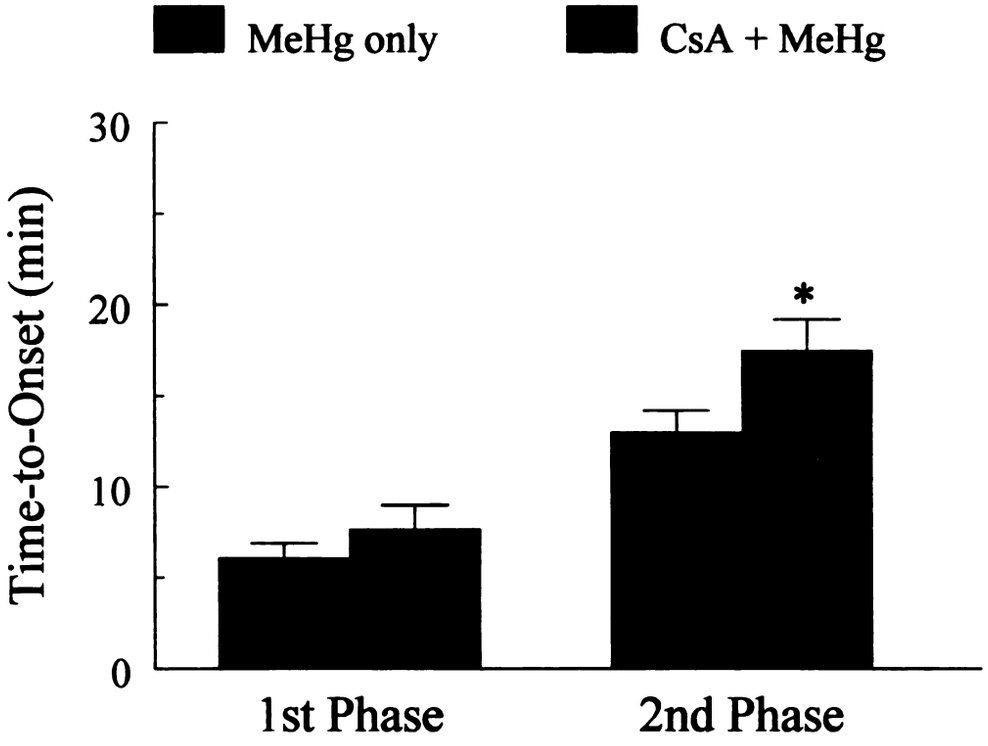

Figure 4.11. CsA delays the spreading of rhod-2 from punctate to whole-cell staining as caused by 0.5 μM MeHg. Granule cells were pretreated with 5 μM CsA immediately prior to 0.5 μM MeHg. The first-phase elevation is measured where rhod-2 fluorescence rises above baseline; the second-phase elevation is measured where the fluorescence rises above the fluorescence increase caused by the 1 min 40 mM K^+ test. Results are presented as mean±SEM (n=7). The asterisk (*) indicates a value significantly different from MeHg-only control ($p<0.05$).

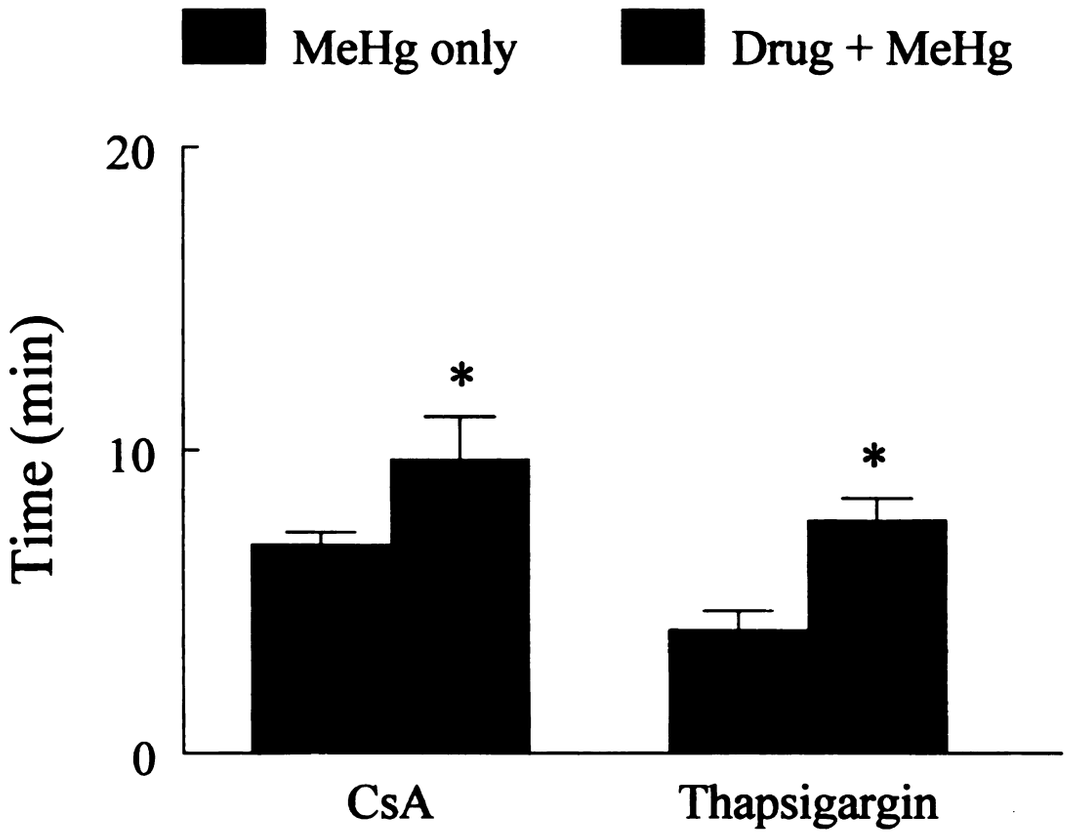

Figure 4.12. Effect of CsA and thapsigargin on interval between onset of first and second rhod-2 fluorescence increases. The time between the first- and second-phase increases in rhod-2 fluorescence indicates the amount of time between the initial uptake of Ca^{2+} into the mitochondrial lumen and the release of rhod-2 into the cytosol. Results are presented as mean ± SEM (n=5-7). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data ($p < 0.05$).

DISCUSSION

Disruption of $[Ca^{2+}]_i$ is an early step in MeHg neurotoxicity which apparently contributes to MeHg-induced cell death in rat cerebellar granule neurons (Marty and Atchison, 1998). These experiments indicate that the bulk of this $[Ca^{2+}]_i$ originates in the mitochondria. When the mitochondrial Ca^{2+} store is emptied following mitochondrial membrane depolarization with CCCP plus oligomycin, the amplitude of the whole-cell $[Ca^{2+}]_i$ elevation caused by MeHg is significantly reduced by approximately 65%. Removal of $Ca^{2+}{}_m$ does not abolish the MeHg-induced release of $[Ca^{2+}]_i$, indicating the participation of more than one intracellular Ca^{2+} source. The participation of more than one intracellular store agrees with results of experiments in T lymphocytes, in which MeHg causes release of Ca^{2+} from both mitochondria and SER (Tan *et al.*, 1993). However, the first-phase increase in fura-2 ratio is not comprised solely of Ca^{2+}, as there is at least one other divalent cation whose intracellular levels increase in response to MeHg (Denny *et al.*, 1993; Hare *et al.*, 1993; Marty and Atchison, 1997). In rat cortical synaptosomes, this cation was identified as Zn^{2+} by ^{19}F-NMR (Denny and Atchison, 1994). Zn^{2+} has recently been identified as a toxic cation capable of opening the MTP during neurotoxic insult (Wudarczyk *et al.*, 1999). Although non-Ca^{2+} divalent cations do not appear to contribute to MeHg-induced cell death in granule cells (Marty and Atchison, 1997), elevated Zn^{2+} could contribute to the opening of the MTP observed in this and other studies (Limke and Atchison, 2002). Thus, the non-mitochondrial component of the first-phase increase in fura-2 ratio is likely comprised of a combination of Ca^{2+} from another intracellular store, such as the SER, and the non-Ca^{2+} divalent cation, most likely Zn^{2+}.

The large contribution of Ca^{2+}_m to the first-phase increase in $[Ca^{2+}]_i$ in cerebellar granule cells is in contrast to the elevations of $[Ca^{2+}]_i$ in NG108-15 cells, in which the primary source of Ca^{2+}_i was the SER through the IP_3 receptor, with minimal contribution from the mitochondria (Hare and Atchison, 1995). The preference for Ca^{2+} release from mitochondria as opposed to release from another Ca^{2+} pool parallels experiments in the neuromuscular junction, in which MeHg-induced increases in spontaneous release of neurotransmitter were decreased to less than 10% of control by inhibition of Ca^{2+} release from the mitochondria and were not decreased by inhibitors of SER Ca^{2+} release (Levesque and Atchison, 1988). Motor nerve terminals may be one of the targets of MeHg during *in vivo* exposure, as a neuromuscular weakness resembling myasthenia gravis is observed (Rustam *et al.*, 1975). The heightened susceptibility of neurons exhibiting significant changes in Ca^{2+}_m suggests a correlation between MeHg interaction with mitochondria and subsequent neuronal damage. Given the relatively small mitochondrial contribution to whole-cell $[Ca^{2+}]_i$ elevations in less sensitive neuronal models, such as NG108-15 cells (Hare *et al.*, 1993; Hare and Atchison, 1995), these results suggest that disruption of mitochondrial Ca^{2+} regulation may be an important factor in determining cellular sensitivity to MeHg.

The differential effects of mitochondrial inhibitors highlight the pivotal role of Ca^{2+}_m regulation in determining the extent of whole-cell increases of $[Ca^{2+}]_i$ induced by MeHg. Mitochondrial membrane depolarization with CCCP alone did not cause significant release of Ca^{2+}_m into the cytosol. These results agree with previous experiments in granule cells (Budd and Nicholls, 1996b), in which mitochondrial membrane depolarization alone did not cause significant release of Ca^{2+}_m. Interestingly,

mitochondrial membrane depolarization alone did not alter the time-to-onset of the MeHg-induced release of Ca^{2+}_i. The smaller release of Ca^{2+}_m into the cytosol induced by CCCP alone suggests that enough Ca^{2+}_m remains to be released by MeHg.

Application of oligomycin alone resulted in partial depolarization of mitochondrial membrane and, as an ATP synthase inhibitor, maintenance of cellular ATP levels (Budd and Nicholls, 1996b). Oligomycin alone did not cause an increase in cytosolic $[Ca^{2+}]$, indicating that partial mitochondrial membrane depolarization is insufficient to empty mitochondrial Ca^{2+} stores. This is supported by the lack of effect of oligomycin on the time-to-onset of the initial increase in $[Ca^{2+}]_i$ during MeHg exposure. However, oligomycin alone did delay significantly the onset of MeHg-induced influx of Ca^{2+}_e. $[Ca^{2+}]_i$ is maintained, in part, by plasma membrane Ca^{2+} pumps which require ATP to remove Ca^{2+} from the cytosol (Werth *et al.*, 1996; Morgans *et al.*, 1998), thus depletion of cellular ATP content inhibits ATP-dependent extrusion of Ca^{2+} from the cell. This is supported by the hastening of the MeHg-induced, second-phase influx of Ca^{2+}_e caused by CCCP alone. When the mitochondrial membrane becomes depolarized, the mitochondrial ATP synthase reverses and begins hydrolyzing ATP into ADP in an attempt to maintain ψ_m (Nicholls and Scott, 1980). Experiments in cerebellar granule neurons in primary culture indicate that 5 min exposure to 2 μM CCCP rapidly depletes cellular ATP levels (Budd and Nicholls, 1996b), thus in our experiments, 8 min exposure to 5 μM CCCP would similarly be expected to deplete ATP. Under these conditions, CCCP-induced depletion of cellular ATP hastens the MeHg-induced depletion of ATP (Sarafian *et al.*, 1989) and causes an earlier onset of Ca^{2+}_e influx.

Depletion of Ca^{2+}_m with CCCP and oligomycin delays and reduces the first-phase elevation of $[Ca^{2+}]_i$ but does not prevent an eventual rise in $[Ca^{2+}]_i$. The delayed movement of rhod-2 into the cytosol by thapsigargin indicates that MeHg causes Ca^{2+} release from the SER, with this Ca^{2+} subsequently buffered by mitochondria. There is substantial evidence that release of Ca^{2+} from the SER causes a rapid, reversible rise in Ca^{2+}_m which is a normal component of intracellular signaling (Landolfi *et al.*, 1998; Pinton *et al.*, 1998; Rizzuto *et al.*, 1998). In HeLa cells transfected with a Ca^{2+}-sensitive photoprotein localized to the outer face of the inner mitochondrial membrane, Ca^{2+} released by activation of IP_3 receptors resulted in a high $[Ca^{2+}]$ within the mitochondria (Rizzuto *et al.*, 1998). This $[Ca^{2+}]$ was much higher than that observed in the bulk cytosol as a result of the close spatial relationship between mitochondria and SER in these cells (Pinton *et al.*, 1998; Rizzuto *et al.*, 1998).

These experiments also provide evidence that MeHg causes opening of the MTP, because the rhod-2 movement from punctate to diffuse staining is delayed by the MTP inhibitor CsA. Because the rhod-2 fluorescence increase associated with opening of the MTP is also delayed by thapsigargin, this suggests that release of Ca^{2+} from the SER contributes to the MeHg-induced loss of Ca^{2+}_m regulation *via* opening of the MTP. This shifting of intracellular Ca^{2+} from the SER to the mitochondria explains the large amount of Ca^{2+}_m released by MeHg in granule cells. Mitochondria are a low-affinity Ca^{2+} store which do not contain much Ca^{2+} at rest; high $[Ca^{2+}]_m$ is energetically unfavorable and interferes with the respiratory chain and the tricarboxylic acid cycle (Budd and Nicholls, 1996b; Wang and Thayer, 1996). Because mitochondria normally contain little Ca^{2+}, this movement of Ca^{2+} from the SER to the mitochondria underlies the large amplitude

release of Ca^{2+}_m during MeHg exposure. Additionally, it suggests in granule cells that the SER is in fact the initial Ca^{2+}_i target, with mitochondria being affected at a later time point. The sensitivity of mitochondria to MeHg-induced increases in Ca^{2+}_m may be dependent on the spatial relationship between SER Ca^{2+} release channels and nearby mitochondria. In NG108-15 cells, the primary source of Ca^{2+}_i during MeHg exposure originates in the IP_3-sensitive Ca^{2+} pool in the SER (Hare and Atchison, 1995). The greater proportion of Ca^{2+} from the SER in NG108-15 cells, as compared to granule cells, may be due to a lack of mitochondrial buffering rather than less release of Ca^{2+} from this store. Alternatively, the apparent sensitivity of granule cell mitochondria may be due to the Ca^{2+} uptake and release characteristics of the mitochondria themselves. Mitochondria in different brain regions have differential sensitivities to induction of the MTP in different brain regions (Friberg *et al.*, 1999). Because mitochondria are such a prominent target during MeHg exposure, the preferential sensitivity of the granule cells to MeHg-induced cell death may be related to the Ca^{2+} regulating characteristics of their mitochondria.

CHAPTER 5

ACUTE EXPOSURE TO METHYLMERCURY OPENS THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE IN RAT CEREBELLAR GRANULE CELLS

ABSTRACT

Following acute MeHg exposure, granule cells in culture undergo an increase in $[Ca^{2+}]_i$ that apparently contributes to cell death. This effect consists of several temporally and kinetically distinct phases. The initial elevation arises from release of Ca^{2+}_i stores; the second phase results from entry of Ca^{2+}_e. These experiments tested the hypothesis that release of mitochondrial Ca^{2+} through the MTP contributes to the initial release of Ca^{2+}_i. Neonatal rat cerebellar granule cells in culture and single cell microfluorimetry were used to examine MeHg-induced changes in $[Ca^{2+}]_i$ and mitochondrial membrane potential (Ψ_m). Pretreatment with the MTP inhibitor CsA (5 μM) delayed the initial phase of increased $[Ca^{2+}]_i$ induced by 0.2 and 0.5 μM MeHg, but not 1.0 μM MeHg. CsA (5 μM) also delayed the irreversible loss of Ψ_m induced by 0.5 μM MeHg. Ca^{2+}_e was not required for either effect, because the effect of CsA on the first phase increase in $[Ca^{2+}]_i$ and loss of Ψ_m was not altered in nominally Ca^{2+}-free buffer. Increasing concentrations of MeHg (0.2-2.0 μM) caused increasing incidence of cell death at 24 hr post-exposure. Treatment with CsA provided protection against cytotoxicity at lower MeHg concentrations (0.2-0.5 μM), but not at higher MeHg concentrations (1.0-2.0 μM). Thus the MTP appears to play a significant role in the cellular effects following acute exposure of cerebellar granule neurons to MeHg.

INTRODUCTION

At the cellular level, MeHg interacts with a number of processes to disrupt divalent cation homeostasis (Komulainen and Bondy, 1987; Hare *et al.*, 1993; Marty and Atchison, 1997), elevate spontaneous release of neurotransmitter (Juang, 1976; Atchison and Narahashi, 1982; Atchison *et al.*, 1986), generate reactive oxygen species (ROS) (LeBel *et al.*, 1990; Sarafian and Verity, 1991; Yee and Choi, 1996) and induce cell death (Sarafian *et al.*, 1989; Marty and Atchison, 1998). As described in previous chapters, low concentrations of MeHg (0.2-2.0 μM) disrupt the ability of rat cerebellar granule cells in primary culture to maintain $[Ca^{2+}]_i$, causing a biphasic increase in $[Ca^{2+}]_i$. The initial phase ("first phase") involves release of $Ca^{2+}{}_i$, followed several minutes later by a secondary ("second phase") influx of $Ca^{2+}{}_e$ (Marty and Atchison, 1997). MeHg-induced disruption of $[Ca^{2+}]_i$ is critical in determining neuronal survival, because agents which buffer $[Ca^{2+}]_i$ protect against MeHg-induced granule cell cytotoxicity (Marty and Atchison, 1998), and Ca^{2+} channel blockers afford some protection against MeHg-induced toxicity *in vivo* (Sakamoto *et al.*, 1996).

One possible source of the early-onset $Ca^{2+}{}_i$ elevation during MeHg exposure is the mitochondria. MeHg causes a number of effects in mitochondria, including inhibition of respiration, increased permeability of the inner membrane, and depolarization of the membrane potential (ψ_m) (Verity *et al.*, 1975; Sone *et al.*, 1977). Additionally, MeHg exposure disrupts Ca^{2+} regulation in mitochondria derived from rat brain by decreasing Ca^{2+} uptake and inducing Ca^{2+} release (Levesque and Atchison, 1991). Inhibition of mitochondrial Ca^{2+} release reduces MeHg-induced release of ACh from rat cortical synaptosomes, supporting the idea that mitochondrial Ca^{2+} release plays a key role in

MeHg-induced neurotoxicity (Levesque *et al.*, 1992). In granule neurons, mitochondria can store and release large amounts of Ca^{2+} under both physiological and pathological conditions (Budd and Nicholls, 1996b). Given that MeHg targets mitochondria in other systems, it was hypothesized that MeHg affects mitochondrial Ca^{2+} regulation in rat cerebellar granule neurons, causing release of mitochondrial Ca^{2+} into the cytosol during MeHg exposure.

One route by which mitochondria release Ca^{2+} is the MTP (Bernardi *et al.*, 1992; Petronilli *et al.*, 1993). The MTP is a megapore formed on the inner mitochondrial membrane in response to adverse conditions including oxidative stress, elevation of mitochondrial matrix Ca^{2+} and anoxia (Dubinsky and Rothman, 1991; Bernardi *et al.*, 1993). When open, the MTP allows the passage of molecules ≤1.5 KDa, including Ca^{2+}, across the usually impermeable inner mitochondrial membrane. Because MTP opening dissipates the proton gradient across the inner mitochondrial membrane, there is a corresponding loss of ψ_m which can be used as a diagnostic tool for observing a permeability transition (White and Reynolds, 1996; Trost and Lemasters, 1997). Both *in vitro* and *in vivo*, MTP induction is linked to neuronal death caused by a variety of compounds and conditions, including ischemia, stroke and glutamate excitotoxicity (Bernardi and Petronilli, 1996; Schinder *et al.*, 1996; White and Reynolds, 1996). Opening of the MTP can be inhibited by the immunosuppressant CsA, even in the presence of inducing agents (Bernardi *et al.*, 1993); (Nieminen *et al.*, 1996).

While there is evidence for induction of a permeability transition in MeHg-induced death of non-neuronal cells (InSug *et al.*, 1997; Shenker *et al.*, 1998), there is no direct experimental evidence linking MeHg to MTP induction in granule neurons.

Because MeHg creates conditions which favor opening of the MTP (generation of ROS, elevation of $[Ca^{2+}]_i$, etc.), it seemed likely that MTP induction plays a role in MeHg-induced neurotoxicity. These experiments tested the hypothesis that MeHg induces opening of the MTP (through direct or indirect action at the mitochondria), causing it to release Ca^{2+} into the cytoplasm, thereby contributing to the elevated $[Ca^{2+}]_i$ observed during the first, $Ca^{2+}{}_i$-dependent phase of MeHg neurotoxicity. MTP induction was measured by examining the ability of CsA to prevent changes in $[Ca^{2+}]_i$, ψ_m, and cell death following acute exposure to MeHg. Because CsA also inhibits the protein phosphatase calcineurin (CN) (Bernardi and Petronilli, 1996), the effects of the CN inhibitors FK506 and W7 were examined to determine if effects on CN function contributed to the observed actions of CsA.

MATERIALS AND METHODS

The materials and solutions for isolation of cerebellar granule cells and measurement of changes in $[Ca^{2+}]_i$ are described in detail in Chapter Two. The procedure for the Eukolight cell viability assay is described in detail in Chapter Three. The purchase and handling of CsA, and the use of TMRE to measure changes in Ψ_m, are described in Chapter Four. Unless specifically stated, all solutions were identical to those described in Chapter Two. The methods and solutions described below are specific to this chapter.

Materials and Experimental Solutions. Phenylarsine oxide (PhAsO) was purchased from Sigma (St. Louis, MO). FK506 and N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W7) were purchased from Calbiochem (La Jolla, CA). PhAsO was prepared as a 100 mM stock solution in 100% DMSO. FK506 was dissolved in DMSO as a 1 mM stock solution. W7 was dissolved into distilled water as a 1 mM stock solution. Control solutions contained corresponding concentrations of solvent, with all final solutions containing no more than 0.1% (v/v) solvent.

Measurement of $[Ca^{2+}]_i$ Changes Using Fura-2. To examine the role of a permeability transition in MeHg-induced changes in $[Ca^{2+}]_i$, the potent MTP inhibitor CsA was utilized (Bernardi *et al.*, 1993). Because CsA also inhibits CN function, the CN inhibitors W7 [which inhibits calmodulin (Ankarcrona *et al.*, 1996)] and FK506 [which inhibits FK506 binding protein (Marcaida *et al.*, 1996)] were also used. Granule cells

were loaded with fura-2 for 1 hr, then rinsed with HBS as described in Chapter Two. All experiments began with a 1 min baseline recording, followed by a 1 min exposure to 40 mM K^+ and a 3 min washout with HBS. For a cell to be considered viable, fura-2 fluorescence must increase transiently in response to the K^+ depolarization challenge and subsequently return to baseline during the washout period. In the first set of experiments, the K^+ depolarization was followed by a 10 min exposure to one of the inhibitors (0.25-5 μM CsA, 20 μM W7 or 1 μM FK506) or an appropriate solvent control. Next, the perfusion solution was replaced with either 0.5 μM MeHg (experiment) or HBS (control) for 45 min. In the second set of experiments, the K^+ depolarization was followed by simultaneous application of 5 μM CsA and 0.5 μM MeHg for 45 min; corresponding buffer and vehicle controls were also performed on the same day. Finally, both the 5 μM CsA pretreatment and simultaneous treatment experiments were repeated in Ca^{2+}-free buffer to verify the intracellular origins of the first Ca^{2+} phase. For the 10 min pretreatment experiments in Ca^{2+}-free buffer, the data were also analyzed for fura-2 ratio amplitude as a measure of $[Ca^{2+}]_i$ released. In this analysis, the fura-2 fluorescence ratio was normalized to the peak fluorescence increase caused by 1 min 40 mM K^+ to account for differences in dye loading.

Visualization of Changes in Ψ_m. Mitochondrial depolarization is a key feature of MTP opening; thus, prevention or attenuation of mitochondrial depolarization by CsA is indicative of MTP opening (Nieminen *et al.*, 1996; White and Reynolds, 1996; Trost and Lemasters, 1997). To measure changes in Ψ_m, cells were loaded with TMRE as described in Chapter Four. As before, all perfusion solutions contained 10 nM TMRE to

maintain dye availability for the mitochondria. Fluorescence was measured relative to an initial fluorescence of 100%. Control experiments to verify the ability of cerebellar granule cell mitochondria to undergo a permeability transition utilized the known MTP-inducing agent PhAsO (5-15 μM) (Kowaltowski and Castilho, 1997). Experiments with MeHg were performed in a similar manner to the $[Ca^{2+}]_i$ experiments, with application of 40 mM K^+ for 1 min (to verify cell viability), followed by 3 min rinse with HBS, a 10 min pretreatment with 5 μM CsA, and 45 min exposure to 0.5 μM MeHg. Similar experiments were performed in the absence of Ca^{2+}_e and in the absence of the 1 min 40 mM K^+ treatment. For all MeHg experiments using TMRE, the parameter measured was time-to-onset of the mitochondrial depolarization, determined at the point at which TMRE fluorescence dropped precipitously, indicating loss of ψ_m. For TMRE data, fluorescence was measured relative to 100% at the beginning of the experiment. The times-to-onset from all cells in an experiment were averaged to provide a mean time-to-onset of mitochondrial membrane depolarization for that dish of cells (n=1).

Cell Viability Assays. To determine whether inhibition of MTP activation would prevent MeHg-induced cell death, a series of experiments was performed which paralleled the fura-2 imaging experimental protocol. All cells were incubated for 90 min at 37°C in HBS to mimic the fura-2 incubation (1 hr) and HBS perfusion rinse (30 min) of the $[Ca^{2+}]_i$ measurement experiments. This was followed by a treatment protocol (see below), after which the cells were returned to conditioned medium (to bind excess MeHg) until the Live/Dead assay was performed (as detailed in Chapter Three).

In the first set of experiments, the effect of CsA treatment on MeHg-induced cell death was examined. Cells were pre-exposed to HBS for 90 min, followed by incubation

in 0, 1 or 5 μM CsA for 10 min prior to application of 0-2 μM MeHg at the following concentrations and durations: 0 MeHg (control) for 60 min; 0.2 μM MeHg for 76 min; 0.5 μM MeHg for 45 min; 1 μM MeHg for 38 min; or 2 μM MeHg for 30 min. These exposure times were based on the time needed for both the first and second phase $[Ca^{2+}]_i$ increases to occur in 97.5% of the cells at each concentration (Marty and Atchison, 1998). After MeHg exposure, cells were washed with conditioned medium and returned to the incubator for 24 hr. At 24 hr post-exposure, cells were washed twice with HBS, then incubated in cytotoxicity assay reagents for 30 min and assayed for fluorescence. Viability was also determined 24 hr after simultaneous exposure to 5 μM CsA and 0-2 μM MeHg for the given time periods.

In the second set of experiments, viability was determined 24 hr after simultaneous exposure to 5 μM CsA and 0-2 μM MeHg for the given time periods, using the same MeHg concentrations and durations listed above. In the final set of experiments, the effect of CN inhibition on MeHg-induced cell death was examined using 20 μM W7 and 1 μM FK506. Cells were pre-exposed to HBS for 90 min, followed by 10 min treatment with either W7 or FK506 and 45 min of 0.5 μM MeHg; viability was determined at 24 hr post-exposure. For all cytotoxicity assays, appropriate solvent and buffer controls were performed simultaneously.

Statistics. For fura-2 and TMRE experiments, comparisons of mean time-to-onset for "MeHg" *vs* the corresponding "MeHg with inhibitor" were made using Student's paired t-test. For the cell viability experiments, the percent viability data were transformed using an angular transformation and analyzed *per* experimental day using a repeated

measures one-way analysis of variance. For all experiments, a $p<0.05$ was considered to be statistically significant.

RESULTS

As noted in previous chapters, neonatal rat cerebellar granule cells in culture undergo a biphasic increase in $[Ca^{2+}]_i$ in response to acute exposure to MeHg. Exposure to CsA for 10 min immediately prior to MeHg exposure delayed the time-to-onset of both the first and second phase of $[Ca^{2+}]_i$ elevations in a concentration-dependent manner. That is, lower concentrations of CsA (0.25 and 0.5 μM) did not delay the time-to-onset of either phase, while higher concentrations (1.0 and 5.0 μM) significantly delayed the time-to-onset of the first-phase $[Ca^{2+}]_i$ elevation (Figure 5.1). The higher CsA concentrations also delayed the time-to-onset of the second phase; however, these results were not quite statistically significant (p=0.05). Interestingly, both phases were delayed to a similar extent, in that the first phase was delayed from 13.6±1.4 min (0.5 μM MeHg alone) to 26.9±5.3 min and 27.7±3.4 min for 1.0 and 5.0 μM CsA + 0.5 μM MeHg, respectively. The second phase was also delayed to a similar extent, from 21.6±2.5 min (0.5 μM MeHg alone) to 33.1±5.5 and 37.1±1.7 min for 1.0 and 5.0 μM CsA + 0.5 μM MeHg, respectively.

The next set of experiments tested the effect of a single CsA concentration on the time-to-onset of $[Ca^{2+}]_i$ elevations caused by increasing concentrations of MeHg. A 10 min pretreatment with 5 μM CsA significantly delayed the first-phase release of $Ca^{2+}{}_i$ caused by 0.2 μM MeHg (from 18.0±1.6 min to 34.5± 3.3 min) and 0.5 μM MeHg (from 12.7±1.2 min to 25.6±3.7 min), but not 1.0 μM MeHg (1.8±0.4 min for MeHg only *vs.* 1.6±0.5 min for CsA pretreatment) (Figure 5.2A). The same treatment also significantly delayed the influx of $Ca^{2+}{}_e$ caused by 0.5 μM MeHg (from 20.3±2.4 min to 33±3.9 min) and delayed, although not significantly, the influx of $Ca^{2+}{}_e$ caused by 0.2 μM MeHg

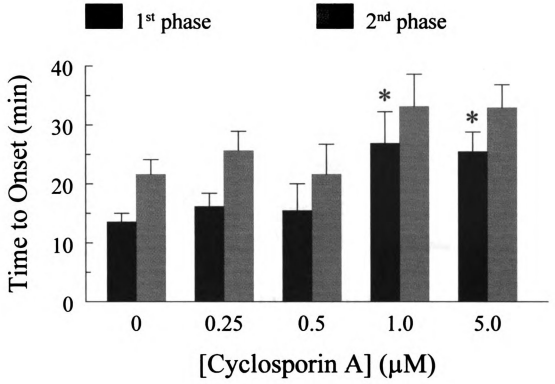

Figure 5.1. Increasing concentrations of CsA cause increasing delays in the time-to-onset of $[Ca^{2+}]_i$ elevations caused by 0.5 μM MeHg. Granule cells pretreated with increasing concentrations of CsA exhibited increasing delays in the time-to-onset of both the first and second phase increases in $[Ca^{2+}]_i$ caused by 0.5 μM MeHg. The time-to-onset was measured from the time of initial MeHg exposure. Although the times-to-onset of the second phases for pretreatment with 1 and 5 μM CsA were not considered to be statistically different from the 0 μM CsA cells, the results were remarkably close (p = 0.05 for each). Results are presented as mean ± SEM (n = 6-10). The asterisk (*) indicates a value significantly different from control ($p < 0.05$).

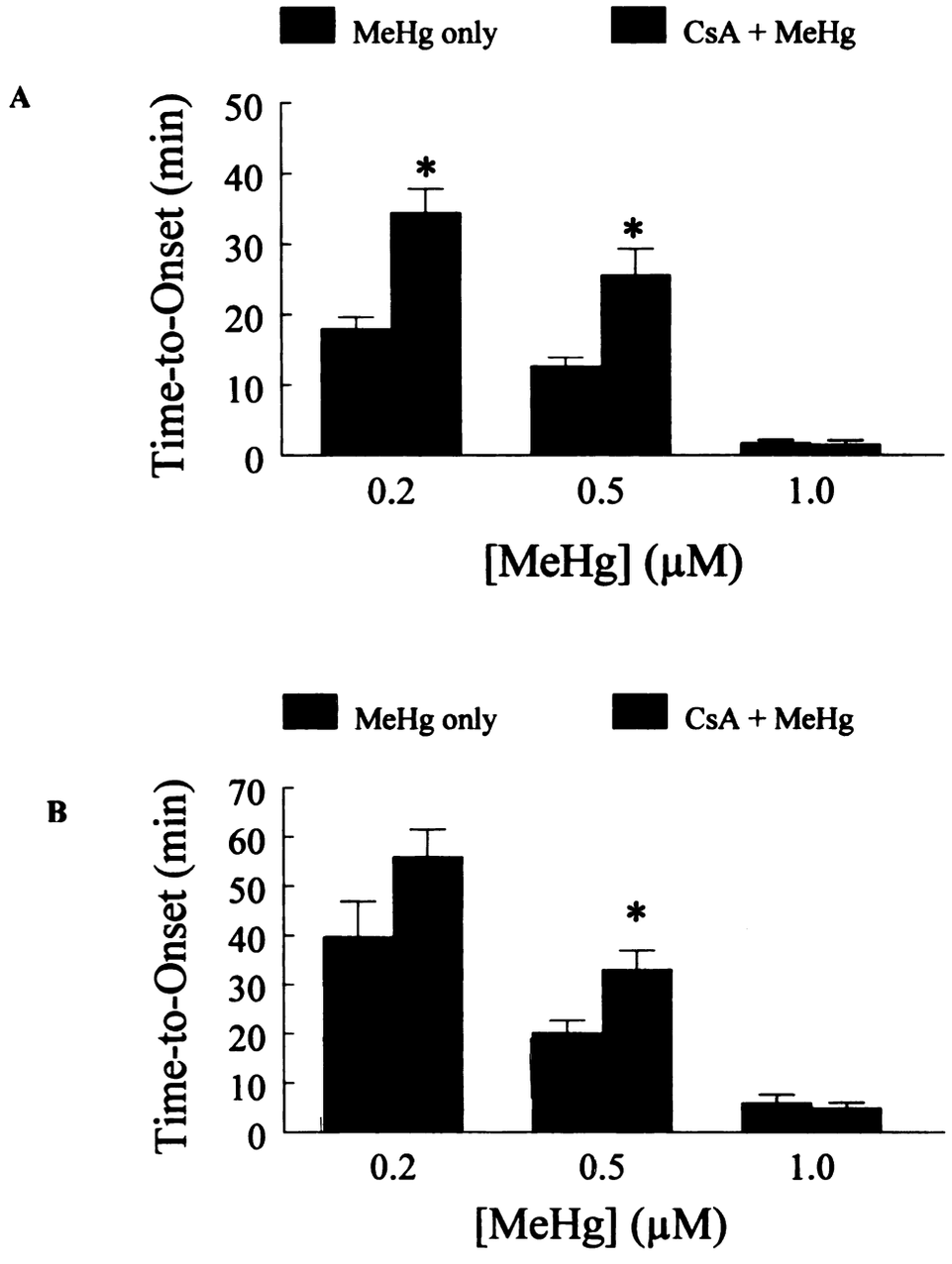

Figure 5.2. 5 μM CsA significantly delays the time-to-onset of MeHg-induced $[Ca^{2+}]_i$ increases. (A) Granule cells pretreated with 5 μM CsA exhibited delayed the time-to-onset of the MeHg-induced first-phase $[Ca^{2+}]_i$ increase caused by 0.2 and 0.5, but not 1.0 μM MeHg (n = 3-9). (B) 5 μM CsA pretreatment also delayed the time-to-onset of Ca^{2+} influx (second phase) caused by 0.2 and 0.5, but not 1.0 μM MeHg. Results are presented as mean ± SEM (n = 3-9). The asterisk (*) indicates a value significantly different from control ($p < 0.05$).

(from 39.7±7.2 min to 56±5.5 min). CsA pretreatment did not affect the Ca^{2+}_e influx caused by 1.0 μM MeHg (Figure 5.2B).

The effect of CsA is reported to be transient such that inhibition of the MTP lasts only 10-20 min (Nieminen *et al.*, 1996). As such, continuous exposure to both CsA and MeHg might increase the delay in time-to-onset of both phases to a greater extent than was seen in the pre-exposure experiments. However, there was no difference between a 10 min pretreatment with 5 μM CsA or 45 min simultaneous treatment with 5 μM CsA in terms of time-to-onset for the first and second phase $[Ca^{2+}]_i$ elevations (data not shown). Simultaneous treatment with CsA delayed the effects of MeHg for both the first phase (from 15.5±2.6 min to 27.7±3.4 min) and second phase (from 24.2±2.5 min to 37.1±1.7 min). Attempts to potentiate the effect of CsA using trifluoperazine were unsuccessful because granule cells respond to trifluoperazine alone with elevated $[Ca^{2+}]_i$ (data not shown).

In EGTA-containing buffer, pretreatment with 5 μM CsA caused similar delays in the time-to-onset of the first-phase $[Ca^{2+}]_i$ elevation as seen in Ca^{2+}-containing buffer. 5 μM CsA delayed the time-to-onset of the first phase for 0.2 μM MeHg (from 14.7±1.5 min to 24.2±1.6 min) and 0.5 μM MeHg (from 12.3±2.4 min to 22.3±3.8 min), but not for 1.0 μM MeHg (from 2.1±0.2 min to 2.6±0.7 min) (Figure 5.3). Measurement of the first phase ratio amplitude in these cells indicated that CsA did not alter the amount of Ca^{2+}_i released during MeHg exposure at any MeHg concentration examined (data not shown). Simultaneous exposure to 5 μM CsA and 0.5 μM MeHg in the EGTA buffer had a similar effect, delaying the first phase to the same extent (data not shown). These results are very similar to those obtained in Ca^{2+}-containing medium, and implicate the

contribution of an intracellular Ca^{2+} store sensitive to CsA in the first phase elevation of $[Ca^{2+}]_i$ caused by MeHg.

Several CN inhibitors were used in an experimental protocol similar to that with CsA to test whether CN contributes to the MeHg-induced changes in $[Ca^{2+}]_i$ and ψ_m. Cells were pretreated with either 20 μM W7 or 1 μM FK506 for 10 min, followed by treatment with 0.5 μM MeHg. These concentrations were chosen based on previous findings by Marcaida *et al.* (1996) and Ankarcrona *et al.* (1996). Neither FK506 nor W7 altered the time-to-onset of either the first or second MeHg-induced $[Ca^{2+}]_i$ phases (Figure 5.4), indicating that CN does not play a significant role in changes in $[Ca^{2+}]_i$ induced by MeHg.

The effect of CsA on MeHg-induced changes in ψ_m was monitored using the potentiometric dye TMRE. Control experiments using 5-15 μM of the MTP-inducing agent PhAsO (Kowaltowski and Castilho, 1997) indicated that granule cell mitochondria can undergo a permeability transition, which is visualized as a complete loss of TMRE fluorescence due to depolarization of the inner mitochondrial membrane (Figure 5.5). As seen in Figure 5.6, MeHg causes loss of TMRE fluorescence consistent with a loss of ψ_m. This is consistent with previous findings in intact monocytes and T cells (InSug *et al.*, 1997; Shenker *et al.*, 1998), as well as with mitochondria-containing synaptosomes (Hare and Atchison, 1992). Exposure to 0.5 μM MeHg alone caused loss of TMRE fluorescence at 25.0±2.5 min (Figure 5.7). The loss of ψ_m was irreversible even when the MeHg was replaced with HBS, as the TMRE fluorescence did not recover during a 60 min period following MeHg exposure (figure not shown). However, this loss of fluorescence was delayed by a 10 min pretreatment with 5 μM CsA, such that neurons

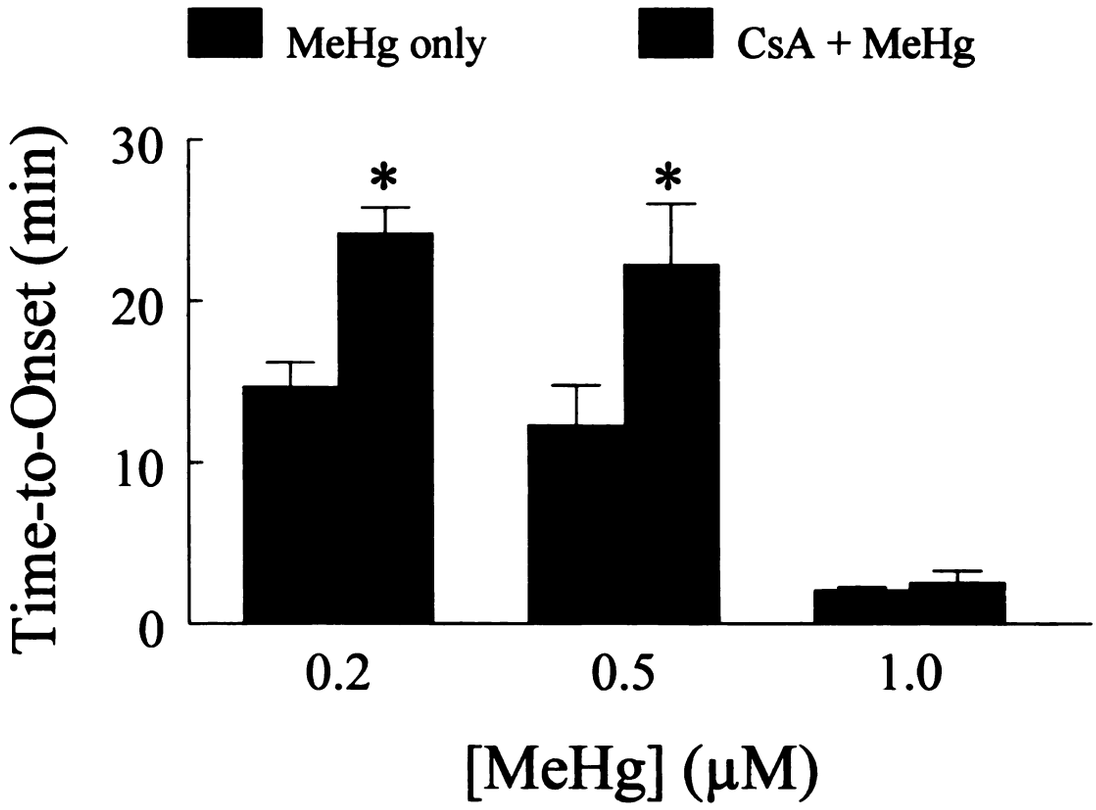

Figure 5.3. 5 μM CsA delays the time-to-onset of MeHg-induced release of Ca^{2+}_i in the absence of Ca^{2+}_e. In the absence of Ca^{2+}_e, 5 μM CsA delayed the time-to-onset of $[Ca^{2+}]_i$ elevations caused by 0.2 and 0.5, but not 1.0 μM MeHg. Results are presented as mean ± SEM (n = 3-7). The asterisk (*) indicates a value significantly different from control ($p < 0.05$).

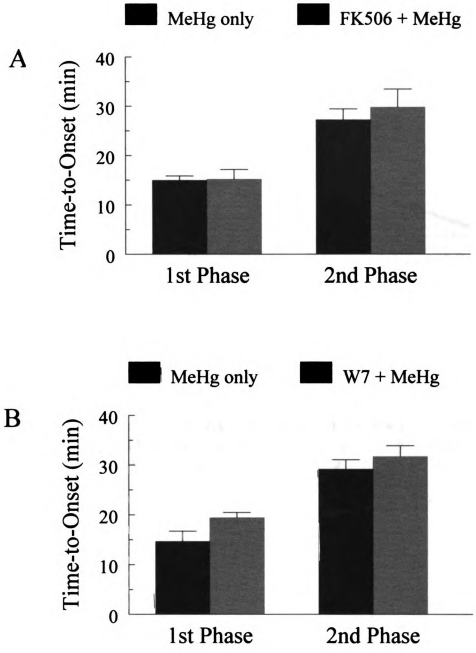

Figure 5.4. Effect of calcineurin inhibitors on MeHg-induced $[Ca^{2+}]_i$ elevations. A) A 10 min pretreatment with 1 μM FK506 did not alter the time-to-onset of $[Ca^{2+}]_i$ increases induced by 0.5 μM MeHg (n = 6). B) A 10 min pretreatment with 20 μM W7 did not alter the time-to-onset of $[Ca^{2+}]_i$ increases induced by 0.5 μM MeHg (n = 6).

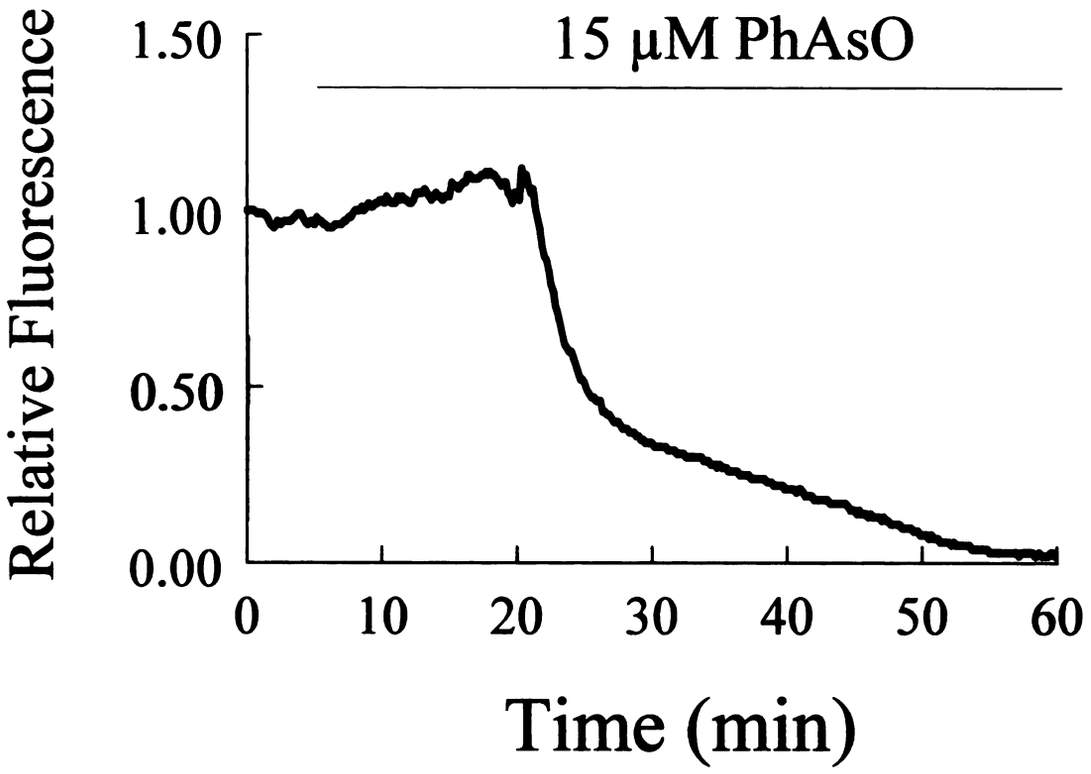

Figure 5.5. PhAsO causes a rapid decrease in TMRE fluorescence. Representative example of changes in TMRE fluorescence in a single granule cell. The known MTP-inducing compound PhAsO (15 μM) causes mitochondrial membrane depolarization, as indicated by the loss of TMRE fluorescence as measured relative to an initial fluorescence of 100%.

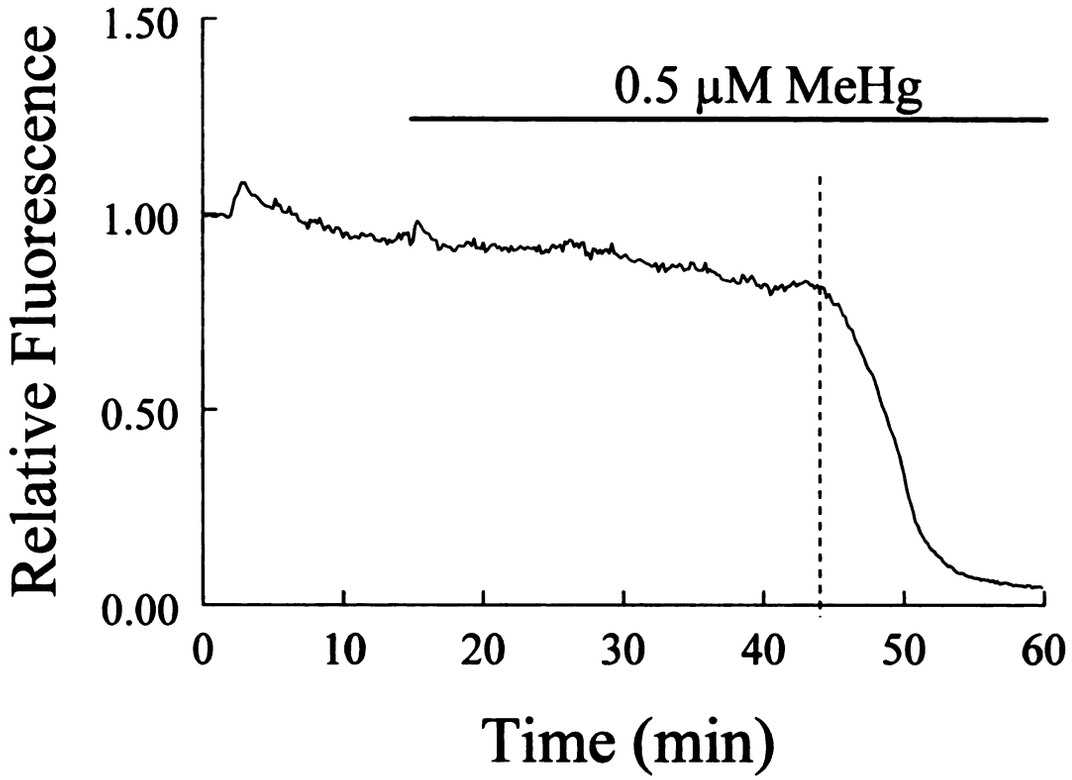

Figure 5.6. Exposure to 0.5 μM MeHg causes irreversible loss of TMRE fluorescence. In this representative example of a single granule cell loaded with TMRE, exposure to 0.5 μM MeHg causes rapid, irreversible loss of TMRE fluorescence, indicating mitochondrial membrane depolarization (dissipation of ψ_m). Time-to-onset of mitochondrial membrane depolarization is measured at the point at which TMRE fluorescence drops below baseline (dashed line). TMRE fluorescence is measured relative to an initial fluorescence of 100%.

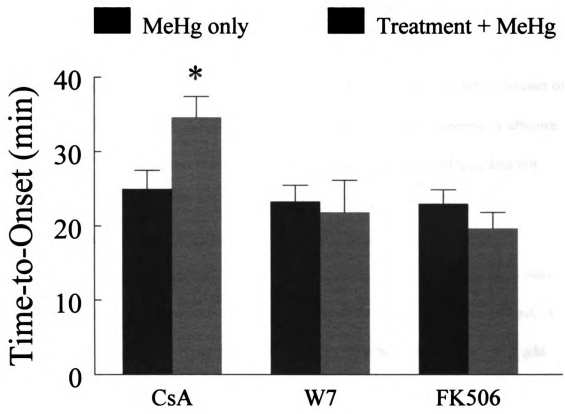

Figure 5.7. CsA, but not CN inhibitors, delay the time-to-onset of mitochondrial membrane depolarization caused by 0.5 μM MeHg. Pretreatment with 5 μM CsA significantly delayed the loss of ψ_m; however, pretreatment with 1 μM FK506 or 20 μM W7 did not. Results are presented as mean ± SEM (n = 5-9). The asterisk (*) indicates a value significantly different from the corresponding 0.5 μM MeHg data ($p < 0.05$).

receiving CsA did not lose TMRE fluorescence until 34.6±2.8 min. Mitochondrial membrane depolarization was not delayed by 10 min pretreatment with 1 μM FK506 or 20 μM W7 (Figure 5.7). Removal of Ca^{2+}_e with EGTA did not alter the time-to-onset of the depolarization caused by 0.5 μM MeHg (data not shown). The presence or absence of the initial K^+ challenge also did not affect the MeHg-induced loss of ψ_m (data not shown).

CsA prevents cell death caused by anoxia and glutamate excitotoxicity, presumably by inhibiting the MTP (Pastorino *et al.*, 1995; White and Reynolds, 1996). Because CsA altered changes in $[Ca^{2+}]_i$ and ψ_m caused by MeHg, the ability of CsA to prevent MeHg-induced cell death was examined. Pretreatment for 10 min with 1 μM CsA protected cells against subsequent exposure to 0.2 μM MeHg, but not against higher concentrations of MeHg (Figure 5.8). Increasing the CsA concentration to 5 μM was more effective, and protected against cell death caused by 0.5 μM MeHg but again, not against that caused by higher MeHg concentrations. Thus CsA is cytoprotective against acute exposure to low concentrations of MeHg.

Viability assays were also performed comparing the effect of 10 min pretreatment with 5 μM CsA *vs.* 5 μM CsA given with MeHg on the MeHg-induced cell death observed at 24 hr post-exposure (Figure 5.9). As with the comparison of the effect of pretreatment and simultaneous treatment with CsA on MeHg-induced increases in $[Ca^{2+}]_i$, there was no difference between these two protocols in their effectiveness in preventing MeHg-induced cell death.

The ability of 20 μM W7 and 1 μM FK506 to prevent 0.5 μM MeHg-induced cell death was examined using a parallel pretreatment regimen. Treatment with 1 μM FK506

for 10 min prior to 0.5 μM MeHg did not improve cell viability at 24 hr post-exposure (data not shown). Pretreatment with 20 μM W7 for 10 min significantly protected against MeHg-induced cell death, increasing the percent viability from 41.3 ± 5.6% in cells treated with 0.5 μM MeHg only, to 61.6 ± 4.6% in cells treated with W7 just prior to 0.5 μM MeHg (Figure 5.10). However, W7 was unable to reverse completely the MeHg-induced cell death and did not protect cells to the extent CsA did.

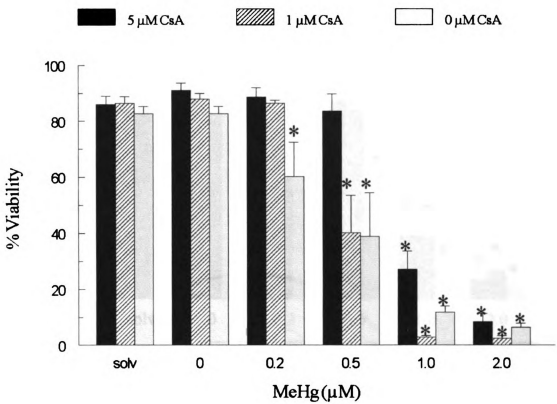

Figure 5.8. CsA provides limited protection against MeHg-induced cell death at 24 hr post-exposure. Granule cell viability following exposure to 0, 1 or 5 μM CsA and increasing concentrations of MeHg at 24 hr post-MeHg exposure. Increasing concentrations of CsA resulted in increasing protection against MeHg-induced cell death. At 1 μM CsA, cells were protected from death caused by 0.2 μM MeHg, whereas 5 μM CsA also protected cells from death from MeHg at concentrations up to 0.5 μM. However, protection was lost at higher MeHg concentrations. Results are presented as mean ± SEM (n = 4). The asterisk (*) indicates a value significantly different from the corresponding 0 μM MeHg data ($p < 0.05$).

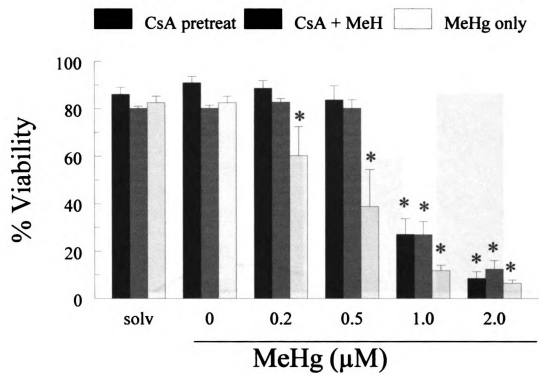

Figure 5.9. Comparison of protection against MeHg-induced cell death by pretreatment or simultaneous treatment with 5 μM CsA. Granule cell were pretreated with 5 μM CsA for 10 min ("CsA pretreat") prior to exposure to increasing concentrations of MeHg, or with 5 μM CsA simultaneously with increasing concentrations of MeHg ("CsA+MeHg"). Results are presented as mean ± SEM (n = 4). The asterisk (*) indicates a value significantly different from the corresponding 0 μM MeHg data ($p < 0.05$).

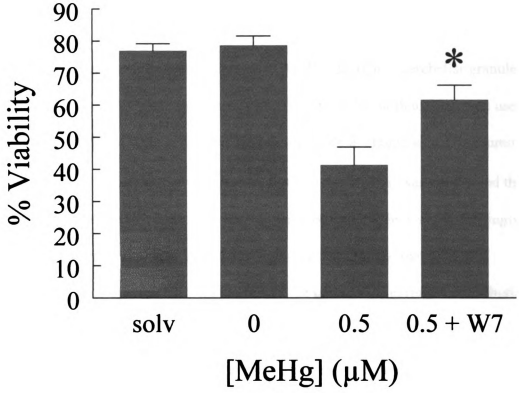

Figure 5.10. Pretreatment with 20 μM W7 significantly protects against cell death caused by 0.5 μM MeHg at 24 hr post-exposure. Results are presented as mean ± SEM (n = 4). The asterisk (*) indicates a value significantly different from the corresponding 0.5 μM MeHg data ($p < 0.05$).

DISCUSSION

These experiments examined the role of MTP induction in cerebellar granule cell neurotoxicity induced by acute exposure to MeHg. The MTP inhibitor CsA was used to define the role of the MTP in MeHg-induced changes in Ca^{2+} homeostasis. Assuming that CsA prevents mitochondrial Ca^{2+} release through the MTP, it was anticipated that the first phase increase of Ca^{2+}_i caused by MeHg would be affected by CsA; surprisingly, both the first and second phases were delayed, suggesting that the two phases of increased $[Ca^{2+}]_i$ are interrelated with the first phase perhaps triggering or contributing in some way to the onset of the second phase. The CsA-mediated delay was not dependent on Ca^{2+}_e, as indicated by the similar first phase times-to-onset in the presence and absence of Ca^{2+}_e. Pretreatment with CsA delayed, but did not prevent, MeHg-induced changes in $[Ca^{2+}]_i$, agreeing with the short-lived protection of CsA found in other systems (Nieminen *et al.*, 1996). The CN inhibitors W7 and FK506 did not change the time-to-onset of increased Ca^{2+}_i, indicating that CN does not contribute substantially to the delayed MeHg response induced by CsA. Thus, it appears that the MTP participates in the initial increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} into the cytosol upon its opening. Whether the elevated $[Ca^{2+}]_i$ is a causative factor or a reaction to a permeability transition cannot be determined from these experiments.

Previous studies indicate that the second phase of increased $[Ca^{2+}]_i$ is due to entry of Ca^{2+}_e, with a possible contribution of L, N and/or P/Q-type voltage-gated Ca^{2+} channels (Marty and Atchison, 1997). To date, no inhibitor has been able to block completely this second phase. It was hypothesized that initiation of the second phase

may be dependent on the first phase, either though pathways to replenish Ca^{2+}_i stores (depleted by release of Ca^{2+} from the SER, for example) or through another as yet undefined mechanism. These experiments provide evidence of a link between the two phases, as delay of the first phase with CsA is accompanied by delay of the second phase. Thus, induction of the MTP may link the first and second Ca^{2+} phases as well as be a critical factor in determining cell survival following MeHg exposure.

Using TMRE, it was found that mitochondria depolarize irreversibly in response to a low concentration (0.5 μM) of MeHg. Because an identical depolarization occurs in the absence of Ca^{2+}_e, it is concluded that this depolarization is dependent on an intracellular factor (such as elevated $[Ca^{2+}]_i$), on MeHg alone, or on a combination of direct damage by MeHg and other cellular factors. Neither CsA treatment nor replacement of the MeHg with normal medium reversed the loss of ψ_m, suggesting that MeHg binds at least one critical site with high affinity. MeHg exhibits high-affinity binding to intracellular organelles, especially mitochondria (Chang and Hartmann, 1972), thereby altering protein sulfhydryl groups and disrupting cellular functions, including respiration, ion transport and regulation, and protein synthesis (Verity *et al.*, 1977; Kauppinen *et al.*, 1989; Levesque and Atchison, 1991; Yee and Choi, 1996). Because MTP induction depends on critical, voltage-sensitive thiol groups (Scorrano *et al.*, 1997), it is possible that direct binding of MeHg to a site on the MTP could induce a permeability transition. MeHg binding would likely be irreversible, which could explain the irreversible mitochondrial depolarization observed with MeHg exposure. This is in contrast to other MTP-inducing agents; for example, exposure to low concentrations of NMDA in cultures of rat hippocampal neurons causes a mitochondrial depolarization

which recovers to near normal levels when the neurons also receive CsA (Schinder *et al.*, 1996). MeHg exposure, on the other hand, does not permit recovery of ψ_m. This suggests a more severe, and possibly more direct insult to the mitochondria which may be exacerbated by elevated cytosolic Ca^{2+}, thereby overwhelming the cell and leading to neuronal death.

Despite its inability to prevent mitochondrial depolarization, CsA was effective at protecting cerebellar granule cells from cell death at 24 hr following exposure to low concentrations of MeHg. Cytotoxicity assays performed at 24 hr post-exposure indicate that a significant fraction of the cultured cells undergo cell death; this fraction increases with increasing MeHg concentrations. These results agree with earlier findings by Marty and Atchison, 1998. In previous experiments, treatment with the $Ca^{2+}{}_i$ chelator BAPTA protected cerebellar granule cells from death 3.5 h after exposure to low concentrations of MeHg (Marty and Atchison, 1998). Because onset of a permeability transition is favored by elevated $Ca^{2+}{}_i$ levels, and cell death at low MeHg concentrations is attenuated by chelating $[Ca^{2+}]_i$ with BAPTA, these experiments suggest that elevated $[Ca^{2+}]_i$ contributes to a CsA-sensitive permeability transition which leads to cell death. As higher MeHg concentrations are used, less protection is provided by CsA, indicating a limit to its effectiveness. The CN inhibitor W7 also reduced the percentage of cells killed by 0.5 μM MeHg; however, W7 did not significantly alter the MeHg-induced changes in $[Ca^{2+}]_i$ or ψ_m. Thus, its effects on cell viability may not be due to inhibition of CN but rather to some other consequence of calmodulin inhibition. The inhibition of MeHg-induced cell death by CsA is interesting, as CsA delays but does not prevent the onset of the MTP in granule cells. The observed inhibition of cell death in these experiments may

simply reflect a delay in the onset of cell death in the presence of CsA, rather than actual prevention of cytotoxicity.

These experiments emphasize the importance of mitochondrial Ca^{2+} homeostasis in MeHg-induced neurotoxicity. In recent years, the importance of mitochondrial Ca^{2+} regulation has become evident not only in neuronal damage caused by a variety of agents, including glutamate excitotoxicity in cerebellar granule cells (Budd and Nicholls, 1996a), but also in regulating normal cell physiology (see Duchen, 2000, for review). Loss of mitochondrial Ca^{2+} buffering is crucial in both apoptotic and necrotic Ca^{2+}-mediated neuronal death (Kruman and Mattson, 1999). Mitochondria accumulate Ca^{2+} based on the extremely negative membrane potential within the inner mitochondrial membrane (140-180 mV, negative inside) (Gunter and Gunter, 1994), making Ca^{2+} buffering an energetically favorable process. However, Ca^{2+} buffering comes at a price: uptake of a large amount of Ca^{2+} occurs at the expense of the proton motive force, and can thus cause uncoupling of ATP synthesis from respiration. Under physiological conditions, mitochondrial Ca^{2+} buffering is important in regulating cytosolic $[Ca^{2+}]$ elevations which result from either influx of Ca^{2+}_e or release of Ca^{2+} from the SER, and may play a role in modulating cellular Ca^{2+} oscillations (Simpson and Russell, 1996; Rizzuto *et al.*, 1998). However, high $[Ca^{2+}]_m$ can become hazardous to the cell v*ia* inhibition of the electron transport chain and the mitochondrial ATPase, as well as dissipation of the proton gradient, all of which can contribute to loss of mitochondrial ATP synthesis, opening of the MTP and cell death (Gunter and Gunter, 1994; Simpson and Russell, 1998b). Loss of mitochondrial Ca^{2+} regulation appears to be a central feature of MeHg-induced loss of neuronal Ca^{2+} regulation (Levesque and Atchison, 1991). In fact, emptying of

mitochondrial Ca^{2+} stores prior to exposure to 0.5 μM MeHg decreases the amplitude of the MeHg-induced first-phase fura-2 ratio increase in granule cells by approximately 65% (see Figure 4.3B), indicating the significant contribution of mitochondrial Ca^{2+} stores to MeHg-induced $[Ca^{2+}]_i$ elevations in these highly sensitive cells.

By examining the changes which occur in cerebellar granule cells during MeHg exposure, several possible reasons for their heightened sensitivity to toxicity are revealed. For example, mitochondria are not all identical, and brain mitochondria exhibit differential sensitivity by region (Friberg *et al.*, 1999). Because these experiments indicate that MeHg induces a permeability transition in granule cells, it is possible that their mitochondria are more sensitive to conditions which favor MTP induction, such as elevated $[Ca^{2+}]_i$ and production of ROS. Cerebellar granule neurons in culture have a relatively weak capacity to up-regulate GSH levels in response to MeHg as compared to cerebellar glia; this may contribute to a heightened sensitivity to onset of the MTP (Sarafian and Verity, 1991). Further, the difference in sensitivities may be related to different Ca^{2+} buffering capacities. Granule cells lack certain Ca^{2+} buffering proteins present in neighboring cells which are less sensitive to MeHg. For example, Purkinje cells contain high concentrations of the calcium binding proteins parvalbumin and calbindin D_{-28K} (Celio, 1990). Because MTP induction relies on increased extramitochondrial Ca^{2+} levels for Ca^{2+} loading, it is possible that a lack of sufficient Ca^{2+} binding proteins puts more of a burden on mitochondria to buffer sustained increases of $[Ca^{2+}]_i$ in cerebellar granule cells.

MeHg exposure has also been linked to elevated levels of ROS, which are believed to contribute to MeHg-induced neurotoxicity (Sarafian and Verity, 1991).

However, elevated levels of ROS may not be sufficient for MeHg to be lethal. In experiments using isolated rat liver mitochondria, sensitivity of the MTP to ROS was shown to be highly dependent on the mitochondrial Ca^{2+} load (Scorrano *et al.*, 1997). Moreover, mitochondrial Ca^{2+} uptake is an early, critical event in both apoptosis and necrosis in the neuronal model PC12 cells (Kruman and Mattson, 1999). In these cells, an initial increase in cytosolic Ca^{2+} was followed by a delayed increase in mitochondrial Ca^{2+}, then accumulation of ROS and mitochondrial depolarization, caspase release and cell death. Both the mitochondrial depolarization and cell death were CsA-sensitive, implicating the permeability transition as a key event. This is remarkably similar the effect of MeHg in granule cells, in which an initial increase in $[Ca^{2+}]_i$ (resulting from release of $Ca^{2+}{}_i$ stores) is followed by mitochondrial depolarization and cell death, with all three components exhibiting sensitivity to CsA. Thus, it appears that the disruption of $[Ca^{2+}]_i$ homeostasis induced in granule neurons by MeHg is the critical factor in MeHg-induced cell death, with the MTP playing a critical role.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

This dissertation presents the results of experiments designed to examine the hypothesis that MeHg causes Ca^{2+} dysregulation in cerebellar granule cells by interacting with specific intracellular Ca^{2+} stores. Specifically, it was hypothesized that MeHg initiates Ca^{2+} release from the SER which is buffered by mitochondria; however, MeHg also impairs the ability of mitochondria to hold Ca^{2+} (by causing loss of mitochondrial membrane potential and inhibiting respiration), resulting in opening of the mitochondrial permeability transition pore and subsequent cell death. The results presented in this dissertation not only support this hypothesis but also provide additional information regarding the mechanisms underlying MeHg-induced elevations of $[Ca^{2+}]_i$ and subsequent neuronal death. The summary of the results delineated in these experiments is presented in Figure 6.1.

In the first experiments, the role of SER Ca^{2+} in MeHg-induced changes in $[Ca^{2+}]_i$ and cell viability was examined. Overall, the results suggest that the SER plays a small role in the whole-cell changes in $[Ca^{2+}]_i$ caused by MeHg in rat cerebellar granule cells. This is not surprising, given the small capacity of this Ca^{2+} store (Fohrman *et al.*, 1993; Masgrau *et al.*, 2000). The Ca^{2+} was released primarily from IP_3 receptors, with a minimal contribution of Ca^{2+} from the ryanodine receptors. Atropine delayed the time-to-onset and reduced the amplitude of MeHg-induced $[Ca^{2+}]_i$ increases, indicating that this occurred as a result of activation of muscarinic ACh receptors. However, the potential interaction between MeHg and the IP_3 receptors themselves is not ruled out by these experiments. SER store depletion does not appear to trigger Ca^{2+} influx through SOCE, as none of the inhibitors of SER Ca^{2+} release affected

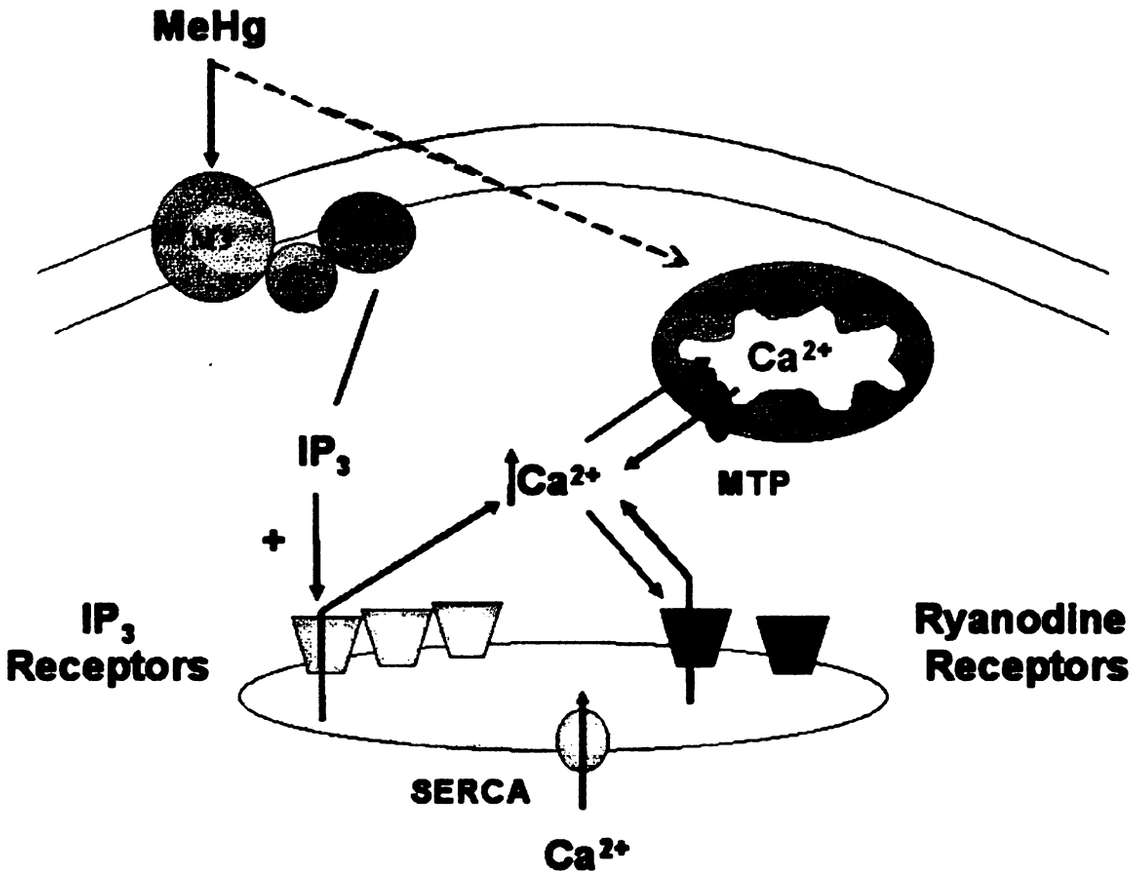

Figure 6.1. Summary of findings. MeHg binds M3 muscarinic ACh receptors, causing production of IP_3 and release of Ca^{2+} through the IP_3 receptors. This elevation of $[Ca^{2+}]_i$ triggers Ca^{2+}-induced Ca^{2+} release through the ryanodine receptors. Ca^{2+} released from the SER is buffered by mitochondria. Excess uptake of Ca^{2+} into the mitochondria, combined with direct effects of MeHg on the mitochondrial respiratory chain, leads to opening of the mitochondrial permeability transition pore (MTP) and promotion of cell death. However, there may be direct effects of MeHg on mitochondria which also contribute to the observed changes in cellular function (dashed arrow).

the MeHg-induced influx of Ca^{2+}_e.

Despite the small amount of Ca^{2+} release from the SER store, inhibition of this Ca^{2+} release was a determinant factor in later cell death caused by MeHg. Cell death was prevented by down-regulation of muscarinic and IP_3 receptors using a 24 hr treatment with BCh. This protection was likely mediated by the M3 receptor and not the nicotinic ACh receptor, as protection was not afforded by BCh when 4-DAMP or atropine were included, but was not affected by the addition of DHE. The M3 receptor is coupled to IP_3 production in granule cells (Whitham *et al.*, 1991; Fohrman *et al.*, 1993), thus inhibition of M3 receptors presumably prevents a MeHg-induced increase in $[IP_3]$. The potential contribution of the M2 receptor to MeHg-induced neurotoxicity was not examined as a result of the toxicity of methoctramine alone. However, typically activation of M2 muscarinic receptors is coupled to activation of potassium conductance (Pan and Williams, 1994), which would not be expected to facilitate Ca^{2+} release or entry. Indiscriminate release of Ca^{2+} from the SER is insufficient to contribute to MeHg-induced cell death, as indicated by the lack of protection afforded by ryanodine. Surprisingly, atropine alone and XeC were not neuroprotective, in contrast to the ability of atropine to delay MeHg-induced elevations of $[Ca^{2+}]_i$. In sum, these experiments provide evidence that the SER participates in MeHg-induced elevations of $[Ca^{2+}]_i$ and subsequent neuronal death.

In the second set of experiments, the role of mitochondrial Ca^{2+} in MeHg-induced elevations of $[Ca^{2+}]_i$ and cell death was examined. Unlike the small contribution of SER Ca^{2+}, mitochondria contribute a large portion of the $[Ca^{2+}]_i$ observed in cerebellar granule cells during exposure to MeHg. Removal of mitochondrial Ca^{2+} prior to MeHg exposure

decreased the amplitude of the Ca^{2+} release from intracellular sources by more than 50%. Observation of cells loaded with rhod-2 to monitor changes in $[Ca^{2+}]_m$ indicated that MeHg causes an early increase in $[Ca^{2+}]_m$ from Ca^{2+} originating within the cell. This increase in $[Ca^{2+}]_m$ is followed several minutes later by spreading of the dye from a punctate stain, presumably mitochondrial in origin, to more generalized whole-cell localization. The spreading of the dye beyond the mitochondria was delayed by thapsigargin, suggesting that SER Ca^{2+} contributes to the elevated $[Ca^{2+}]_m$. The inhibitor of MTP opening CsA also delayed the spreading of rhod-2 beyond the punctate pattern, suggesting that the dye movement results from opening of the MTP to allow the dye to cross rapidly through the usually impermeable inner mitochondrial membrane. The participation of the MTP in MeHg neurotoxicity is implicated further by the ability of CsA to attenuate MeHg-induced elevations of $[Ca^{2+}]_i$, loss of mitochondrial membrane potential, and cell death. Calcineurin inhibitors did not affect the MeHg-induced increase in $[Ca^{2+}]_i$ or loss of mitochondrial membrane potential. However, the calmodulin inhibitor W7 did protect against MeHg-induced cell death at 24 hr post-exposure. This is not surprising, because calmodulin is known to play a role in Ca^{2+}-mediated cell death (Orrenius and Nicotera, 1994; Urushitani *et al.*, 2001).

The impact of MeHg-induced alterations on $[Ca^{2+}]_m$ has widespread implications for the viability of affected neurons, leading to a proposed scenario in which MeHg-induced disruption of mitochondrial function is a focal point for many of the observed changes which occur in cerebellar granule neurons. As demonstrated in this dissertation, MeHg causes an early increase in $[Ca^{2+}]_m$ as a result of Ca^{2+} uptake into the mitochondrial lumen. Initially, this uptake of Ca^{2+} into the mitochondria by the Ca^{2+}

uniporter would be driven by the mitochondrial membrane potential (140-180 mV, negative inside) (Nicholls, 1974); however, as the $[Ca^{2+}]_m$ increases, the inner membrane potential becomes more positive, thereby making uptake of Ca^{2+} less favorable energetically (Nicholls and Scott, 1980). As the transmembrane potential dissipates, mitochondria buffer Ca^{2+} using ATP-dependent mechanisms (Nicholls and Scott, 1980), which diverts a portion of the neuron's ATP away from other cellular processes. The inner membrane depolarization reduces the proton motive force which drives ATP mitochondrial ATP synthesis, thus elevated $[Ca^{2+}]_m$ can effectively uncouple ATP synthesis from respiration (Hillered *et al.*, 1983). Additionally, high $[Ca^{2+}]_m$ can inhibit the electron transport chain and the mitochondrial ATP synthase, which results in inhibition of mitochondrial ATP synthesis (see Gunter and Gunter, 1994, and Simpson and Russell, 1998b, for review). In an effort to maintain ψ_m, the ATP synthase can reverse, and begin hydrolyzing ATP to ADP to produce the protons necessary to maintain the negative inner membrane potential (Leyssens *et al.*, 1996). The end result is depletion of cellular ATP, which has a wide range of effects within the cell. Loss of ATP deprives plasma membrane Ca^{2+} extrusion proteins of the energy necessary to maintain the steep $[Ca^{2+}]$ gradient across the plasma membrane, and reduces the amount of ATP available for Ca^{2+} pumps on storage organelles, such as the SERCA pump of the SER. Additionally, loss of ATP inhibits the Na^+-K^+ ATPase, which maintains the membrane potential across the plasma membrane. Inhibition of the Na^+-K^+ ATPase would lead to plasma membrane depolarization, thereby leading to activation of plasma membrane voltage-gated Ca^{2+} channels and influx of Ca^{2+}_e into a cell now compromised in its ability to extrude Ca^{2+} from the cytoplasm. ATP depletion, loss of ψ_m and elevated $[Ca^{2+}]_m$ all

contribute to induction of the MTP and promotion of cell death (Bernardi and Petronilli, 1996), and each of these actions has been shown to occur with MeHg (Levesque and Atchison, 1991; Hare and Atchison, 1992; present thesis). The far-reaching consequences of mitochondrial effects are supported by experiments presented in this dissertation and elsewhere. For example, the ability of oligomycin to delay the time-to-onset of MeHg-induced influx of Ca^{2+}_e (Figure 4.5) suggests that MeHg-induced depletion of ATP triggers influx of Ca^{2+}_e in cerebellar granule cells. This influx occurs primarily through voltage-gated Ca^{2+} channels (Marty and Atchison, 1997) and occurs at approximately the same time as the onset of plasma membrane depolarization (Stringfellow *et al.*, 2000). Thus, the mitochondria are not only affected directly by MeHg, but are also potentially responsible for a large number of the observed cellular responses which lead to neuronal damage and death.

The results presented in this dissertation support the idea that MeHg neurotoxicity results from disruption of an intricate signaling network in which disruption of one portion of the network affects the functioning of another portion such that studying each component separately does not provide an adequate picture of the mechanism underlying MeHg neurotoxicity. As suggested by results presented here, the importance of the SER as a target in MeHg neurotoxicity may lie in the effect of Ca^{2+} released from the SER on mitochondria. Under normal conditions, granule cell mitochondria do not store Ca^{2+}; however, when they are exposed to a high extra-mitochondrial [Ca^{2+}], they usually respond by buffering this Ca^{2+} *via* several uptake mechanisms (the uniporter, the rapid active mode transporter, *etc.*) (Budd and Nicholls, 1996a; Budd and Nicholls, 1996b). Experiments using specifically-targeted, Ca^{2+}-sensitive proteins demonstrate that opening

of the IP_3 receptor-activated channels results in higher $[Ca^{2+}]$ at the mitochondrial surface than in the general cytosol (Rizzuto *et al.*, 1998), suggesting a preferential signaling pathway in which Ca^{2+} stored within the SER lumen is released to communicate a pro-apoptotic signal to mitochondria. Excess mitochondrial matrix Ca^{2+} contributes to cell death by promoting release of cytochrome C, enhancing production of reactive oxygen species, inhibiting mitochondrial ATP synthesis, and opening the MTP, all of which can contribute to cellular damage and death (Bernardi and Petronilli, 1996; Szalai *et al.*, 1999). In HeLa cells, overexpression of calreticulin, an SER luminal protein which binds Ca^{2+}, causes an increased sensitivity to induction of apoptosis and increase in release of cytochrome C from the mitochondria, highlighting the role of SER-mitochondria communication in apoptosis (Nakamura *et al.*, 2000). By causing pathological release of Ca^{2+} from the SER which contributes to the mitochondrial dysfunction, MeHg causes a complicated pattern of Ca^{2+}_i changes which contribute to cell death.

In interpreting the data presented in this dissertation, there are several inconsistencies which complicate the process of delineating a precise, mechanistic model for the actions of MeHg in cerebellar granule cells. The model (as shown in Figure 6.1) focuses only on effects of MeHg on Ca^{2+} signaling systems within the cell. In this model, there is an assumption that any effects on Ca^{2+} signaling are due to a direct interaction between MeHg and the receptor signaling system being examined. In reality, MeHg-induced disruption of $[Ca^{2+}]_i$ may reflect disruption of a non-Ca^{2+} system which in turn affects $[Ca^{2+}]_i$. For example, MeHg increases free $[Zn^{2+}]$ within the cytosol in rat cortical synaptosomes (Denny *et al.*, 1993; Denny and Atchison, 1994) and possibly within NG108-15 neuroblastoma cells (Hare *et al.*, 1993) and rat cerebellar granule cells (Marty

and Atchison, 1997). MeHg also causes an elevation of ROS within rat cerebellar granule cells and human T lymphocytes (Sarafian and Verity, 1991; Oyama *et al.*, 1994; Yee and Choi, 1996; Shenker *et al.*, 1999). Because both Zn^{2+} and ROS are known inducers of the MTP (Wudarczyk *et al.*, 1999), the observed MeHg-induced elevation of $[Ca^{2+}]_i$ that is CsA-sensitive (see Chapter Five) may be an indirect result of MeHg-induced elevations of Zn^{2+} and/or ROS. Similarly, MeHg depletes cellular concentrations of ATP (Kuznetsov *et al.*, 1987; Sarafian *et al.*, 1989), which are critical for maintenance of $[Ca^{2+}]_i$ by providing energy to both intracellular Ca^{2+} pumps (such as the SERCA) and plasma membrane extrusion proteins (such as the plasma membrane Ca^{2+} pump) (Orrenius and Nicotera, 1994; Guerini *et al.*, 1998). Like Zn^{2+} and ROS, mitochondrial ATP depletion is a contributing factor to opening of the MTP (Simbula *et al.*, 1997), which could then contribute to elevation of $[Ca^{2+}]_i$ through opening of the MTP. Thus, the observed elevation of $[Ca^{2+}]_i$ in cerebellar granule cells may be symptomatic of other, non-Ca^{2+} effects of MeHg within the cell.

Additionally, the non-Ca^{2+} effects may contribute to the direct effects of MeHg on Ca^{2+} signaling pathways that are shown in Figure 6.1. For example, MeHg causes mitochondrial membrane depolarization associated with opening of the MTP (see Chapters Four and Five). However, mitochondrial depolarization may not be sufficient to cause opening of the MTP without contributing factors, such as elevations of Ca^{2+}_m or elevation of ROS. In hepatocytes, mitochondrial membrane depolarization with CCCP is not sufficient to open the MTP; however, CCCP plus the pro-oxidant t-butylhydroperoxide causes rapid opening of the MTP which is accompanied by ATP depletion and subsequent cell death (Nieminen *et al.*, 1995). This phenomenon of

mitochondrial membrane depolarization without opening of the MTP may underlie the effect of CCCP and oligomycin on MeHg-induced elevations of rhod-2 fluorescence (Figure 4.8). The combination of CCCP and oligomycin causes rapid loss of ψ_m but does not prevent the movement of rhod-2 from localized to diffuse staining when cells are exposed to 0.5 μM MeHg. This suggests that there is another factor contributing to opening of the MTP, such as production of ROS or elevation of cytosolic [Zn^{2+}], which occurs during MeHg exposure and which was not considered in the experiments undertaken for this dissertation.

Another inconsistency in this model is the assumption that MeHg has specific interactions which occur in the same temporal order during every MeHg exposure in cerebellar granule cells. The model presented in Figure 6.1 indicates an initial interaction with muscarinic receptors, followed by release of Ca^{2+} from the SER and subsequent disruption of $Ca^{2+}{}_m$ regulation. The problem with this assumption is apparent in the inconsistencies observed in the time-to-onset data for the first-phase increase of $[Ca^{2+}]_i$ caused by MeHg. For example, both atropine, which indirectly affects release of Ca^{2+} from the SER, and CsA, which inhibits opening of the MTP, delay the time-to-onset of MeHg-induced release of $[Ca^{2+}]_i$. These data contradict the proposed model because they suggest that MeHg is targeting both the SER and mitochondria simultaneously rather than sequentially, as suggested by the thapsigargin-sensitive nature of MeHg-induced increases of $Ca^{2+}{}_m$. Another discrepancy is apparent in the lack of effect of thapsigargin on the time-to-onset of MeHg-induced elevations of $[Ca^{2+}]_i$. Both BCh and atropine changed the time-to-onset, but thapsigargin had no effect on the timing of the $[Ca^{2+}]_i$ increase. While this can be explained by the presence of a thapsigargin-insensitive store

in cerebellar granule cells, as has been suggested by others (Masgrau *et al.*, 2000), this hypothesis was not tested. Thus, while the data presented in this dissertation provide strong evidence for participation of M3 muscarinic receptors, IP_3 receptors and the MTP in MeHg-induced elevations of $[Ca^{2+}]_i$ and subsequent neuronal death, they do not yet provide a clear picture of the temporal order of the events which take place during the initial exposure period in cerebellar granule cells.

Regardless of the source(s) of the initial increase of $[Ca^{2+}]_i$, in all experiments there is a latent period between exposure to MeHg and the increase in $[Ca^{2+}]_i$ which has been observed in this dissertation and elsewhere (Hare *et al.*, 1993; Marty and Atchison, 1997). This latent period decreases as the concentration of MeHg increases, suggesting a threshold for the Ca^{2+} response. The physiological basis for this threshold is unknown. Hypothetically, the threshold could depend on saturation of binding of MeHg to proteins not directly involved in the observed elevation of $[Ca^{2+}]_i$. For example, MeHg binds thiols contained in cytosolic GSH (Sarafian and Verity, 1991; Sanfeliu *et al.*, 2001). Saturation of intracellular GSH would not only increase the concentration of non-bound MeHg in the cytosol but would also deplete the GSH reserve, thereby interfering with the cell's ability to manage oxidative stress (Sanfeliu *et al.*, 2001). As discussed above, oxidative stress contributes to opening of the MTP, thus MeHg-induced elevations of $[Ca^{2+}]_i$ could potentially depend on depletion of GSH. The latent period could also result from saturation of extracellular, non-specific binding sites. Many plasma membrane proteins, including muscarinic and nicotinic ACh receptors, as well as NMDA receptors, contain cysteine residues which regulate receptor activity and which could potentially participate in non-specific binding of MeHg (Sato *et al.*, 1976; Janaky *et al.*, 1993; Zeng

et al., 1999). Whereas none of these possibilities was tested directly in experiments presented in this dissertation, MeHg has been shown to impair function of neuronal nicotinic and muscarinic receptor-mediated responses in N1E-115 neuroblastoma cells (Quandt *et al.*, 1982) and of glutamate receptors (Yuan and Atchison, 1997).

The model presented in this dissertation is based on pharmacological manipulations which did not provide sufficient evidence to answer unequivocally the question of whether MeHg interacts with specific, potentially unique molecular targets in cerebellar granule cells. An alternative approach which could potentially provide this answer would be based on genetic manipulation of biological systems to add and/or remove specific proteins from cells and, when possible, experimental animals. Theoretically, a better approach would use transgenic methodology to create, for example, IP_3 knockout mice to remove the IP_3 receptor from mouse cells and observe whether its removal alters the effects of MeHg in the whole animal and/or cells derived from these animals. However, experiments of this nature are limited by cost, the time needed to engineer genetically-altered animals, and the limited viability of some strains of knockout mice. Additionally, animals often compensate by substituting a different isoform of the receptor, which can complicate interpretation of data gathered from this type of experiment. Thus, genetic manipulation provides a potentially enlightening experimental approach to determining the specific molecular target(s) of MeHg, but as observed with the pharmacological approaches, is also constrained by potential problems.

In conclusion, this dissertation provides evidence that MeHg alters $[Ca^{2+}]_i$ regulation by causing early, irreversible release of Ca^{2+} from both the SER and mitochondria to cause cell death in cerebellar granule cells. In cerebellar granule cells,

MeHg-induced neuronal death is prevented by inhibition of opening of the MTP or by inhibition of Ca^{2+} release through the IP_3 receptor, with protection falling off as the MeHg concentration exceeds 1 μM. It is interesting to note that, while MeHg has many effects which can be harmful (*i.e.*, generation of reactive oxygen species, disruption of protein and ATP synthesis, *etc.*), the highly sensitive granule cells can be protected by interfering with the MeHg-induced release of Ca^{2+} from intracellular stores (Sarafian *et al.*, 1984; Sarafian, 1999). Because there is great diversity in the location and abundance of intracellular Ca^{2+}-release channels, particularly the IP_3 receptor, and the location of their upstream receptors, such as the muscarinic receptors, within different areas of the nervous system, this may contribute to the selective neurotoxicity of MeHg within the cerebellum.

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