Evidence for Interactions between Intracellular Calcium Stores during Methylmercury-Induced Intracellular Calcium Dysregulation in Rat Cerebellar Granule Neurons

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ABSTRACT

Acute exposure to methylmercury (MeHg) causes severe disruption of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) regulation, which apparently contributes to neuronal death. Activation of the mitochondrial permeability transition pore (MTP) evidently contributes to this effect. We examined in more detail the contribution of mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_m\)) to elevations of [Ca\(^{2+}\)]\(_i\), caused by acute exposure to a low concentration of MeHg in primary cultures of rat cerebellar granule neurons. In particular, we sought to determine whether interactions occurred between Ca\(^{2+}\) pools in response to MeHg. Prior depletion of Ca\(^{2+}\)\(_m\) using carbonyl cyanide m-chlorophenylhydrazone (CCCP) and oligomycin significantly decreased the amplitude of [Ca\(^{2+}\)]\(_i\) release from intracellular stores, and delayed the onset of whole-cell [Ca\(^{2+}\)]\(_i\) elevations, caused by 0.5 \(\mu\)M MeHg. CCCP alone hastened the MeHg-induced release of Ca\(^{2+}\) within the cell, whereas oligomycin alone delayed the MeHg-induced influx of extracellular Ca\(^{2+}\). In granule cells loaded with rhod-2 acetoxymethylester to measure changes in [Ca\(^{2+}\)]\(_i\), MeHg exposure caused a biphasic increase in fluorescence. The initial increase in fluorescence occurred in the absence of extracellular Ca\(^{2+}\) and was abolished by mitochondrial depolarization. The secondary increase was associated with spreading of the dye from punctate staining to whole-cell distribution, and was delayed significantly by the MTP inhibitor cyclosporin A and the smooth endoplasmic reticulum Ca\(^{2+}\) ATPase inhibitor thapsigargin. We conclude that MeHg causes release of Ca\(^{2+}\) from the mitochondria through opening of the MTP, which contributes to the bulk of the elevated [Ca\(^{2+}\)]\(_i\) observed during MeHg neurotoxicity. Additionally, the Ca\(^{2+}\) that enters the mitochondria seems to originate in the smooth endoplasmic reticulum, providing a mechanism for the observed mitochondrial Ca\(^{2+}\) overload.

Methylmercury (MeHg) is an environmental neurotoxicant that preferentially damages specific neurons within the central nervous system (Hunter and Russell, 1954). One area affected is the granule cell layer of the cerebellar cortex, which undergoes preferential degeneration in response to MeHg exposure in humans and experimental animals (Hunter and Russell, 1954; Leyshon-Sorland and Morgan, 1991). Specific loss of granule cells occurs in vivo despite granule cells receiving equal exposure as neighboring neurons that are relatively unaffected (Hunter and Russell, 1954; Leyshon-Sorland and Morgan, 1991). This heightened susceptibility of granule cells is surprising considering the relatively nonspecific interactions of MeHg with cellular proteins as a result of its high lipophilicity and strong interactions with thiol groups (Roberts et al., 1980). However, MeHg consistently causes loss of Ca\(^{2+}\) homeostasis in cells ranging from neurons and neuroblastoma cells to T lymphocytes (Hare et al., 1993; Tan et al., 1993; Marty and Atchison, 1997). MeHg-induced cell death in cerebellar granule cells is linked to an early, severe loss of intracellular Ca\(^{2+}\) (Ca\(^{2+}\)\(_m\)) homeostasis observed in vitro (Marty and Atchison, 1997, 1998). In these neurons and NG108-15 neuroblastoma cells, MeHg causes a characteristic biphasic increase in [Ca\(^{2+}\)]\(_i\), that consists of an initial (“first-phase”) release of Ca\(^{2+}\) from one or more intracellular stores into the cytosol, and a secondary (“second-
phase”) influx of extracellular Ca\(^{2+}\) (\(\text{Ca}^{2+}\_\text{e}\)) (Hare et al., 1993; Marty and Atchison, 1997). The elevation of \([\text{Ca}^{2+}]_{\text{m}}\) occurs at much lower MeHg concentrations in cerebellar granule cells than in NG108-15 cells, which parallels the heightened sensitivity of granule cells to the toxic effects of MeHg (Hare et al., 1993; Marty and Atchison, 1997, 1998). Recent evidence suggests that mitochondria contribute significantly to both the first-phase \([\text{Ca}^{2+}]_{\text{m}}\), increase and subsequent cell death through opening of the mitochondrial permeability transition pore (MTP) (Limke and Atchison, 2002); however, the relative contribution of mitochondrial \(\text{Ca}^{2+}\) (\(\text{Ca}^{2+}\_\text{m}\)) to the first-phase \([\text{Ca}^{2+}]_{\text{m}}\), increase and the mechanism underlying the opening of the MTP has not yet been examined.

MeHg has the potential to affect \(\text{Ca}^{2+}\_\text{m}\) regulation through at least two mechanisms. The first is through direct interaction with mitochondrial proteins, which alters the ability of mitochondria to function normally. MeHg inhibits mitochondrial nucleare aid synthesis (Frenkel and Harrington, 1983) and interferes with the electron transport chain via direct interaction with respiratory proteins (Sone et al., 1977). MeHg causes loss of inner mitochondrial membrane potential in isolated mitochondria (Bondy and McKee, 1991), human monocytes (Insug et al., 1997), and rat cerebellar granule cells (Limke and Atchison, 2002), suggesting that the observed opening of the MTP could result from inner mitochondrial membrane depolarization. However, a second potential mechanism is an indirect inhibition of mitochondrial function via excessive uptake of \(\text{Ca}^{2+}\_\text{e}\) into the mitochondria. Elevated \([\text{Ca}^{2+}]_{\text{m}}\) depolarizes the inner mitochondrial membrane (Loew et al., 1994), inhibits the tricarboxylic acid cycle and the mitochondrial ATPase, dissipates the mitochondrial proton gradient (Gunter and Gunter, 1994; Simpson and Russell, 1998b), and promotes opening of the MTP (Petronilli et al., 1993; Byrne et al., 1999). Recent experiments suggest that \([\text{Ca}^{2+}]_{\text{m}}\) is elevated after release of \(\text{Ca}^{2+}\) from the smooth endoplasmic reticulum (SER), through ryanodine receptors (Szlai et al., 2000) and/or inositol-1,4,5-triphosphate (IP\(_3\)) receptors (Rizzuto et al., 1998; Szlai et al., 1999).

Based on experiments in NG108-15 neuroblastoma cells (Hare and Atchison, 1995) and cerebellar granule cells (Bearss et al., 2001), MeHg causes \(\text{Ca}^{2+}\) release from the SER, primarily through the IP\(_3\) receptor (Hare and Atchison, 1995). Thus, a potential mechanism for the observed opening of the MTP in cerebellar granule cells is excessive uptake of \(\text{Ca}^{2+}\_\text{m}\) after release of \(\text{Ca}^{2+}\) from the SER, followed by opening of the MTP and release of \(\text{Ca}^{2+}\_\text{m}\) from the mitochondrial lumen into the cytosol.

In this study, we examine the contribution of \(\text{Ca}^{2+}\_\text{m}\) to MeHg-induced elevations of \([\text{Ca}^{2+}]_{\text{e}}\), and examine whether the SER contributes to MeHg-induced changes in \([\text{Ca}^{2+}]_{\text{m}}\). Our results suggest that MeHg causes an early increase of \([\text{Ca}^{2+}]_{\text{m}}\) followed by release of \(\text{Ca}^{2+}\_\text{m}\) into the cytosol via opening of the MTP. The observed elevation of \([\text{Ca}^{2+}]_{\text{m}}\) is affected by SER \(\text{Ca}^{2+}\) such that depletion of SER \(\text{Ca}^{2+}\) reduces the MeHg-induced increase of \([\text{Ca}^{2+}]_{\text{m}}\). Furthermore, these experiments indicate that \(\text{Ca}^{2+}\_\text{m}\) contributes the bulk of the cytosolic \([\text{Ca}^{2+}]_{\text{m}}\), observed during MeHg exposure, highlighting the importance of disruption of \(\text{Ca}^{2+}\_\text{m}\) during MeHg neurotoxicity and outlining a temporal order of events in MeHg-induced loss of \(\text{Ca}^{2+}\) homeostasis.

### Materials and Methods

#### Materials and Solutions

- Fura-2 acetoxymethylster (fura-2 AM), rhod-2 acetoxymethylster (rhod-2 AM), tetramethylrhodamine ethyl ester (TMRE) and MitoTracker Green were purchased from Molecular Probes (Eugene, OR).
- Cyclosporin A (CsA), carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), oligomycin, thapsigargin, EGTA, and cystine-β-arabinofuranoside were purchased from Sigma-Aldrich (St. Louis, MO).
- Methyl mercuric chloride (MeHg) was purchased from ICN Pharmaceuticals Biochemicals Division (Aurora, OH), and made up as a 5 mM stock solution in deionized water on a weekly basis.

**Unless otherwise noted,** the standard buffer used for experimental solutions was HEPES-buffered saline (HBS) which contained 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 20 mM d-glucose, and 20 mM HEPES (free acid) (pH 7.3). The 400 mM K\(^+\) solution contained the same components as HBS except that the NaCl was reduced to 115.4 mM to maintain isosmolarity. The EGTA-containing buffer (EGTA-HBS) had the same components as HBS minus CaCl\(_2\) and plus 0.02 mM EGTA (final \([\text{Ca}^{2+}]_{\text{m}}\) = 60 nM; Marty and Atchison 1997). For all experiments, pharmacological agents were dissolved in either HBS or EGTA-HBS, with appropriate controls for any additional solvents used (dimethyl sulfoxide or ethanol, with maximum solvent of 0.01% (v/v) for all experiments). In a cell-free system, none of the pharmacological agents used altered fura-2 fluorescence (data not shown).

#### Isolation of Rat Cerebellar Granule Cells

Cerebellar granule cells were isolated from 7-day-old Sprague-Dawley rats (Harlan, Verona, WI) of both sexes as described previously (Marty and Atchison, 1997). Cells were plated at a density of 2.0 to 2.2 × 10\(^4\) cells/35-mm dish on 25-mm glass coverslips coated with 0.1 mg/ml poly-D-lysine. After 24 h, 10 μM cystine-β-arabinofuranoside was added to inhibit glial proliferation. Cells used in all experiments were maintained for 6 to 8 days in vitro to allow for cell maturation.

**Measurement of \([\text{Ca}^{2+}]_{\text{m}}\)**: To measure changes in \([\text{Ca}^{2+}]_{\text{m}}\), cells were loaded with 3 to 4 μM fura-2 AM in HBS for 1 h at 37°C, followed by perfusion with HBS for 30 min. Imaging experiments were then performed as described previously (Hare et al., 1993; Marty and Atchison, 1997). Digital fluorescent images were obtained using a Diaphot microscope (Nikon, Tokyo, Japan) or an IX-70 microscope (Olympus, Tokyo, Japan) coupled to an IonOptix (Milton, MA) system with a heated perfusion system (37°C, 3 ml/min). In each experiment, changes in fluorescence at excitation wavelengths of 340 and 380 nm were monitored simultaneously in multiple soma (3–8) within the same microscopic field every 6 s. Cells were exposed continually to MeHg, which was applied by bath superfusion at 0.5 μM. This concentration of MeHg was chosen based on previous work in granule cells (Marty and Atchison, 1997, 1998).

At 0.5 μM, MeHg causes a reproducible biphasic increase in \([\text{Ca}^{2+}]_{\text{m}}\), within 30 min of exposure to MeHg, but is minimally cytotoxic 24 h postexposure. This concentration is ~40× less than the concentration associated with cerebellar-based ataxia during acute in vivo exposures (Bakir et al., 1973) as occurred in Iraq (see Atchison, 1986, for further explanation of dosage relevance). After exposure to MeHg, the times-to-onset of the first and second phases of increased \([\text{Ca}^{2+}]_{\text{m}}\), were determined, with onset of MeHg application being time 0. The onset of the first phase (due to release of \([\text{Ca}^{2+}]_{\text{e}}\)) from an extracellular source was measured manually from the point at which the ratioed fluorescence rose irreversibly above the peak \([\text{Ca}^{2+}]_{\text{m}}\), level during baseline. The second phase (influx of \(\text{Ca}^{2+}\_\text{m}\)) began at the point at which the slope of the 380 line (in the normalized data) changed abruptly by inspection, indicating a large, rapid increase in \([\text{Ca}^{2+}]_{\text{m}}\). This response has been shown previously to be extracellular in origin (Hare et al., 1993; Marty and Atchison, 1997).

In experiments performed in the nominally \(\text{Ca}^{2+}\)-free buffer (EGTA-HBS), the amplitude of \([\text{Ca}^{2+}]_{\text{m}}\), 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 the peak
response to the K⁺-induced depolarization. The fluorescence ratio \( F_{440/380} \) indicates the relative \([\text{Ca}^{2+}]_i\); however, the data were not converted to actual \([\text{Ca}^{2+}]_i\), due to interactions of fura-2 with other divalent cations, such as \(\text{Zn}^{2+}\), a cation known to play a role in responses to MeHg (Hare et al., 1993; Denny and Atchison, 1994). For both time-to-onset and normalized fluorescence ratio, the data from the multiple cells monitored during each trial were averaged to provide a mean time-to-onset or mean normalized ratio for that dish of cells \( n \). To minimize differences between cell isolates, experiments using MeHg alone and MeHg with a given pharmacological agent were performed on the same day, and experiments using the same agents were replicated using at least two separate cell isolates.

**Measurement of \([\text{Ca}^{2+}]_m\)** To measure changes in \([\text{Ca}^{2+}]_m\), cells were loaded with 1 μM rhod-2 AM for 1 h at room temperature (23–25°C), followed by overnight incubation in normal culture medium (18–24 h) (Trost and Lemasters, 1997; Peng et al., 1998; Simpson and Russell, 1998a). Because rhod-2 can fluoresce in both the cytosol and mitochondria, this protocol was used to ensure that mitochondrial loading occurred and to provide time for the cytosolic dye to leak out of the cell. Multiple cells were imaged simultaneously using the IonOptix system, using an excitation wavelength of 450 nm and an emission wavelength of 590 nm. Efficiency of mitochondrial loading was confirmed daily by using an 8-min exposure to 5 μM CCCP, which uncouples oxidative phosphorylation, and 10 μM oligomycin, which dissipates \( \psi_m \) (Budd and Nicholls, 1996). Thus, mitochondrial uptake of \([\text{Ca}^{2+}]_m\) during \([\text{Ca}^{2+}]_m\) influx caused by K⁺ depolarization (1 min, 40 mM) was prevented using CCCP and oligomycin. Like fura-2, rhod-2 data were 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. The secondary elevation (from release of rhod-2 into the cytosol) was measured as the point at which there was 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 \( n = 1 \). Mitochondrial localization of rhod-2 was examined using granule cells loaded with rhod-2, and then loaded with 500 nM MitoTracker Green for 30 min. Images of cells loaded with MitoTracker Green and/or rhod-2 were obtained using excitation wavelengths of 488 and 543 nm, respectively, on a Leica DM LFSA laser scanning confocall microscope using 40 to 63× objectives.

**Measurement of \( \psi_m \).** To measure changes in \( \psi_m \), cells were loaded with 100 nM TMRE in HBS for 30 min at 37°C, and then rinsed in 10 nM TMRE in HBS for 30 min (White and Reynolds, 1996; Trost and Lemasters, 1997). Cellular fluorescence was then measured continuously using the IonOptix system. In the 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 h of observation (data not shown).

**Statistics.** Comparisons of mean time-to-onset for MeHg versus 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**

To examine the contribution of the mitochondrial \([\text{Ca}^{2+}]_m\) store to MeHg-induced elevations of \([\text{Ca}^{2+}]_i\), CCCP and oligomycin were used separately and in combination to produce differing effects on mitochondrial function and cellular ATP levels. CCCP is an uncoupler of oxidative phosphorylation that depolarizes the inner mitochondrial membrane, whereas oligomycin inhibits the mitochondrial ATP synthase, thereby preventing hydrolysis of ATP caused by reversal of the ATP synthase during mitochondrial membrane depolarization (Budd and Nicholls, 1996). Thus, the combination of CCCP and oligomycin dissipates \( \psi_m \) while preserving cellular ATP (Budd and Nicholls, 1996). Because mitochondria accumulate \([\text{Ca}^{2+}]_i\) only when their inner membranes are sufficiently hyperpolarized, mitochondrial depolarization causes release of \([\text{Ca}^{2+}]_m\) and prevents further \([\text{Ca}^{2+}]_m\) uptake (Budd and Nicholls, 1996). We first confirmed that exposure to 5 μM CCCP and 10 μM oligomycin for 8 min was sufficient to depolarize completely mitochondria, as measured using TMRE, and cause a large increase in \([\text{Ca}^{2+}]_i\), as measured using fura-2, in rat cerebellar granule cells. After exposure to CCCP plus oligomycin, granule cells loaded with either TMRE or fura-2 did not respond to another application of CCCP plus oligomycin, indicating the irreversible loss of mitochondrial membrane potential, and successful depletion of \([\text{Ca}^{2+}]_m\), with this protocol (data not shown). Oligomycin alone did not cause an increase in \([\text{Ca}^{2+}]_i\), whereas CCCP alone caused a much smaller increase in cytosolic \([\text{Ca}^{2+}]_i\) (Fig. 1A) than that caused by CCCP plus oligomycin (Fig. 1B), despite the rapid, irreversible loss of mitochondrial membrane potential caused by CCCP alone.

We then examined the effect of depleting mitochondrial \([\text{Ca}^{2+}]_m\) stores on MeHg-induced elevations of \([\text{Ca}^{2+}]_i\), in cerebellar granule cells. In granule cells loaded with fura-2, 0.5 μM MeHg causes a biphasic increase in \([\text{Ca}^{2+}]_i\), that consists of a first-phase release of \([\text{Ca}^{2+}]_m\) from at least one intracellular source, and a second-phase influx of \([\text{Ca}^{2+}]_m\) (Fig. 2A). Depletion of \([\text{Ca}^{2+}]_m\) using CCCP and oligomycin before application of MeHg delayed the time-to-onset of increases in \([\text{Ca}^{2+}]_i\), caused by 0.5 μM MeHg (from 12.6 ± 2.5 to 37.7 ± 3.0 min) (Fig. 2B). The time-to-onset of the first-phase increase in \([\text{Ca}^{2+}]_i\), was not significantly altered by CCCP alone or oligomycin alone (Fig. 2B). When granule cells were exposed to MeHg in \([\text{Ca}^{2+}]_m\)-free buffer, the first-phase increase in \([\text{Ca}^{2+}]_i\) was evident, whereas the second-phase increase was absent (Fig. 2C). Emptying the mitochondria with CCCP and oligomycin before exposure to 0.5 μM MeHg in \([\text{Ca}^{2+}]_m\)-free buffer revealed a similar delay of the onset of the first-phase increase in \([\text{Ca}^{2+}]_i\), as that observed in \([\text{Ca}^{2+}]_m\)-containing medium (from 15.3 ± 3.2 to 45.4 ± 3.9 min) (data not shown). Additionally, CCCP and oligomycin treatment significantly reduced the amplitude of the MeHg-induced increase in fura-2 ratio from a normalized value of 1.25 ± 0.26 to 0.41 ± 0.13, representing a 67% decrease in the ratio amplitude (Fig. 2D). Thus, \([\text{Ca}^{2+}]_m\) contributed the majority of the first-phase increase of \([\text{Ca}^{2+}]_i\), observed during exposure to a low concentration of MeHg in granule cells.

The mitochondrial inhibitors had differing effects on the time-to-onset of second-phase influx of \([\text{Ca}^{2+}]_i\), caused by 0.5 μM MeHg. Mitochondrial membrane depolarization without maintenance of cellular ATP levels using CCCP alone hastened the influx of \([\text{Ca}^{2+}]_m\) induced by MeHg, from 32.7 ± 3.3 min to 22.0 ± 3.3 min to 15.6 ± 2.7 min (Fig. 3). This effect was not seen with CCCP in the absence of MeHg, which did not cause an \([\text{Ca}^{2+}]_m\)-dependent increase in \([\text{Ca}^{2+}]_i\) (data not shown). Oligomycin alone had the opposite effect, delaying the time-to-onset of the MeHg-induced \([\text{Ca}^{2+}]_m\) influx (Fig. 3), suggesting that MeHg-induced depletion of ATP triggered the onset of the second-phase increase in \([\text{Ca}^{2+}]_i\). Application of CCCP plus oligomycin caused a similar delay in the time-to-onset of the MeHg-induced \([\text{Ca}^{2+}]_m\) influx as that caused by oligomycin.
alone, from 22 ± 3.4 to 36.3 ± 8.3 min (Fig. 3). Thus, MeHg-induced influx of Ca\(^{2+}\) seemed 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\(^{2+}\)\(_{\text{m}}\) from the mitochondria into the cytosol. Mitochondrial localization of rhod-2 was confirmed by the colocalization of rhod-2 and MitoTracker green fluorescence (Fig. 4A). To test whether changes in rhod-2 fluorescence reflected changes in [Ca\(^{2+}\)\(_{\text{m}}\)], cells were first loaded with fura-2 and exposed to increasing concentrations of K\(^{+}\) (10–100 mM) for 1 min each, to demonstrate that this protocol caused reversible increases in [Ca\(^{2+}\)\(_{\text{m}}\)] as the [K\(^{+}\)] increases (Fig. 4B). Granule cells were then loaded with rhod-2 to monitor changes in [Ca\(^{2+}\)\(_{\text{m}}\)]. Control experiments using 1-min depolarizations with increasing concentrations of K\(^{+}\) (10–100 mM) indicated that rhod-2 fluorescence increased during an increase in [Ca\(^{2+}\)\(_{\text{m}}\)] and returned to baseline after removal of the K\(^{+}\) solution (Fig. 4C). Furthermore, this increase in fluorescence was due to a change in Ca\(^{2+}\)\(_{\text{m}}\) 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 (Fig. 4C).

As seen in Fig. 5, granule cells loaded with rhod-2 exhibited a biphasic increase in rhod-2 fluorescence during exposure to 0.5 \(\mu\)M MeHg. Initially, rhod-2 fluorescence increased to a similar intensity as that caused by 1-min depolarization with 40 mM K\(^{+}\) (Fig. 5A). As shown in Fig. 5B as a representative tracing, and as composite data in Fig. 6A, this initial increase was abolished by 8-min exposure to CCCP and oligomycin, indicating that the initial increase in fluorescence was due to an increase in Ca\(^{2+}\)\(_{\text{m}}\). Comparison of the time-to-onset of the initial increase in rhod-2 fluorescence with the time-to-onset of the initial increase in fura-2 fluorescence indicated that the elevation of Ca\(^{2+}\)\(_{\text{m}}\) occurred at a significantly earlier time point (7.9 ± 1.5 min, \(n = 4\)) than the increase in cytosolic Ca\(^{2+}\) (12.6 ± 2.5 min, \(n = 5\)). The secondary increase in fluorescence was associated with a spreading of the dye from the mitochondria to the cytosol, which was apparent in the digital image recorded by the IonOptix software (data not shown). Confocal imaging confirmed that exposure to 0.5 \(\mu\)M MeHg caused rhod-2 to fluoresce throughout the cell rather than maintaining mito-
chondrial localization, confirming that MeHg caused rhod-2 to move from the mitochondria into the cytosol (Fig. 5C).

The dye movement observed using the IonOptix system 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 mM K+ -induced depolarization. Interestingly, treatment with CCCP and oligomycin abolished the initial elevation of fluorescence but did not alter the time-to-onset of the secondary fluorescence increase associated with the dye spreading throughout the cell. Because the initial increase of fluorescence did not occur until the spreading of dye throughout the cell occurred, the first-phase increase in rhod-2 fluorescence occurred simultaneously with the second-phase release of dye into the cytosol (Fig. 6A). When granule cells were exposed to 0.5 μM MeHg in the nominally Ca2+-free EGTA-HBS, the pattern and time-to-onset of rhod-2 fluorescence changes were similar, because MeHg again caused an initial fluorescence increase that was abolished by CCCP and oligomycin, and a secondary fluorescence increase from dye movement into the cytosol (data not shown).

![Fig. 2](image-url)

**Fig. 2.** A, representative changes in fura-2 fluorescence in an individual granule cell during exposure to 0.5 μM MeHg. MeHg alone causes a biphasic increase in [Ca2+]i, comprised of an initial release of Ca2+ from within the cell (1st) and a temporally and kinetically distinct influx of Ca2+ (2nd). B, effect of mitochondrial inhibitors on time-to-onset of first-phase increase in [Ca2+]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 before 0.5 μM MeHg. Time-to-onset of the increase in [Ca2+]i was compared between MeHg alone and MeHg plus inhibitor for each treatment. Results are presented as mean ± S.E.M. (n = 5–8). The asterisk (*) indicates a value significantly different from MeHg-only control (p < 0.05). C, representative changes in fura-2 fluorescence in an individual granule cell during exposure to 0.5 μM MeHg in the absence of Ca2+. Without Ca2+, the first-phase Ca2+ elevation (1st) is evident, whereas the second phase is absent. D, measurement of the first-phase ratio amplitude in the absence of Ca2+. The amplitude of the first-phase ratio increase was normalized to the peak fura-2 response to the 1-min depolarization with 40 mM K+ (at the beginning of each experiment). Results are presented as mean ± S.E.M. (n = 5). The asterisk (*) indicates a value significantly different from MeHg-only control (p < 0.05).

![Fig. 3](image-url)

**Fig. 3.** Comparison of time-to-onset of Ca2+ 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 before 0.5 μM MeHg. Comparisons are made between MeHg alone and MeHg plus inhibitor for each treatment. Results are presented as mean ± S.E.M. (n = 5–8). The asterisk (*) indicates a value significantly different from MeHg-only control (p < 0.05).
Because MeHg caused an increase in \( \text{Ca}^{2+}_m \) in the absence of \( \text{Ca}^{2+}_e \), we hypothesized that mitochondria were buffering \( \text{Ca}^{2+}_e \) released from another intracellular \( \text{Ca}^{2+} \) store, possibly the SER; thus, the next experiment examined the effect of SER \( \text{Ca}^{2+} \) depletion on MeHg-induced elevations of \( \text{Ca}^{2+}_m \) using the smooth endoplasmic reticulum \( \text{Ca}^{2+} \) ATPase inhibitor thapsigargin. As shown for the representative experiment (\( n = 3 \)) in Fig. 6B, treatment with 10 \( \mu \text{M} \) thapsigargin for 5 min immediately before exposure to 0.5 \( \mu \text{M} \) MeHg did not affect the time-to-onset of the initial rhod-2 fluorescence increase, but did significantly delay the secondary dye movement into the cytosol.

Finally, we examined whether release of rhod-2 from the mitochondria into the cytosol was due to opening of the MTP. Previous experiments indicated that the MTP inhibitor CsA delays the MeHg-induced increase in [\( \text{Ca}^{2+} \)], caused by 0.2 and 0.5, but not 1.0 \( \mu \text{M} \) MeHg (Limke and Atchison, 2002). In granule cells loaded with rhod-2, application of 5 \( \mu \text{M} \) CsA for 10 min immediately before 0.5 \( \mu \text{M} \) MeHg did not alter the time-to-onset of the initial fluorescence increase but significantly delayed the increase of fluorescence associated with spreading of rhod-2 from punctate to whole-cell localization (Fig. 6C).

**Discussion**

Disruption of [\( \text{Ca}^{2+} \)], is an early step in MeHg neurotoxicity that apparently contributes to MeHg-induced cell death in rat cerebellar granule neurons (Marty and Atchison, 1998). Results from this study indicate that the bulk of this [\( \text{Ca}^{2+} \)], originates in the mitochondria. When the mitochondrial \( \text{Ca}^{2+} \) store is emptied after mitochondrial membrane depolarization with CCCP plus oligomycin, the amplitude of the whole-cell [\( \text{Ca}^{2+} \)], elevation caused by MeHg is reduced by approximately 65%. However, removal of \( \text{Ca}^{2+}_m \) does not abolish the MeHg-induced release of [\( \text{Ca}^{2+} \)], indicating the
participation of more than one intracellular Ca\textsuperscript{2+} source. The contribution of more than one intracellular store agrees with results of experiments in T lymphocytes, in which MeHg caused release of Ca\textsuperscript{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\textsuperscript{2+}, because 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\textsuperscript{2+} by \textsuperscript{19}F-NMR (Denny and Atchison, 1994). Zn\textsuperscript{2+} has recently been identified as a toxic cation capable of opening the MTP during neurotoxic insult (Wudarczyk et al., 1999). Although non-Ca\textsuperscript{2+} divalent cations do not seem to contribute to MeHg-induced cell death in granule cells (Marty and Atchison, 1997), elevated Zn\textsuperscript{2+} could contribute to the opening of the MTP observed in this and other studies (Limke and Atchison, 2002). Thus, the nonmitochondrial component of the first-phase increase in fura-2 ratio is likely comprised of a combination of Ca\textsuperscript{2+} from another intracellular store, such as the SER, and the non-Ca\textsuperscript{2+} divalent cation, most likely Zn\textsuperscript{2+}, whose origin is as yet unclear.

The large contribution of Ca\textsuperscript{2+}\textsubscript{m} to the first-phase increase in [Ca\textsuperscript{2+}]\textsubscript{i} in cerebellar granule cells is in contrast to the elevations of [Ca\textsuperscript{2+}]\textsubscript{i} in NG108-15 cells, in which the primary source of Ca\textsuperscript{2+}\textsubscript{i} was the SER through the IP\textsubscript{3} receptor, with minimal contribution from the mitochondria (Hare and Atchison, 1995). The preference for Ca\textsuperscript{2+} release from mitochondria as opposed to release from another Ca\textsuperscript{2+} pool parallels experiments at 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\textsuperscript{2+} release from the mitochondria and were not decreased by inhibitors of SER Ca\textsuperscript{2+} release (Levesque and Atchison, 1988). The heightened susceptibility of neu-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5}
\caption{A, changes in rhod-2 fluorescence in a single cerebellar granule cell exposed to 0.5 \textmu M MeHg. Cells were exposed initially to 40 mM K\textsuperscript{+} for 1 min to verify viability (K\textsuperscript{+}). During MeHg exposure, there is a biphasic increase in rhod-2 fluorescence; the initial rise (1st) plateaus at a similar fluorescence intensity as that caused by the K\textsuperscript{+} depolarization; the second increase (2nd) is associated with the dye spreading throughout the entire cytosol. B, 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. Granule cells were pretreated with 5 \textmu M CCCP plus 10 \textmu M oligomycin (8 min) immediately before 0.5 \textmu M MeHg. C, confocal images comparing rhod-2 localization in the absence (HBS) or presence (MeHg) of 0.5 \textmu M MeHg for 0 to 60 min. Cells exposed to HBS alone did not exhibit a change in dye localization for 60 min (top). Exposure to 0.5 \textmu M MeHg for 60 min resulted in a gradual spreading of the fluorescence from mitochondrial localization (Fig. 4A) to whole-cell fluorescence (bottom) (40\times). Each panel depicts representative experiments that were replicated three to four times.}
\end{figure}
Fig. 6. A, 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. Granule cells were pretreated with 5 μM CCCP plus 10 μM oligomycin (8 min) immediately before 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. Treatment with CCCP and oligomycin abolished the gradual increase of rhod-2 fluorescence observed in MeHg treated cells, thus the first phase and second phase increase in fluorescence occurred simultaneously. Results are quantitation of data shown for the single representative experiment in Fig. 4B and are presented as mean ± S.E.M. (n = 4). The asterisk (*) indicates a value significantly different from the corresponding MeHg-only data (p < 0.05). B, 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 before 0.5 μM MeHg. Results are presented as mean ± S.E.M. (n = 5). The asterisk (*) indicates a value significantly different from MeHg-only control (p < 0.05). C, 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 before 0.5 μM MeHg. Results are presented as mean ± S.E.M. (n = 7). The asterisk (*) indicates a value significantly different from MeHg-only control (p < 0.05).
Thayer, 1996). Based on experiments presented here and elsewhere (Hare and Atchison, 1995; Bearss et al., 2001), the nonmitochondrial Ca$^{2+}$ likely originates in the SER. Because mitochondria normally contain little Ca$^{2+}$, movement of Ca$^{2+}$ from the SER to the mitochondria would underlie the large-amplitude release of Ca$^{2+}$ during MeHg exposure. The sensitivity of mitochondria to MeHg-induced increases in Ca$^{2+}$ 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$^{2+}$ during MeHg exposure originates in the IP$_3$-sensitive Ca$^{2+}$ pool in the SER (Hare and Atchison, 1995). In these cells, loss of Ca$^{2+}$ from the SER is followed by mitochondrial membrane depolarization, suggesting that mitochondrial function can be compromised in cells in which the mitochondria are not the initial target (Hare and Atchison, 1995). The greater proportion of Ca$^{2+}$ from the SER in NG108-15 cells, compared with granule cells, may reflect a lack of mitochondrial buffering rather than less release of Ca$^{2+}$ from this store. In fact, when disruption of SER Ca$^{2+}$ content leads to cell death, this apoptotic signal is probably transduced through mitochondrial effects (Szalai et al., 1999). Experiments using specifically-targeted, Ca$^{2+}$-sensitive proteins demonstrate that opening of the IP$_3$ receptors 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 proapoptotic signal to nearby mitochondria. 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 proapoptotic cytochrome c into the cytosol (Szalai et al., 1999). Thus, MeHg-induced Ca$^{2+}$ release from the SER could translate into a cell death signal through alteration of mitochondrial function.

In summary, this study suggests a model of the temporal order of events during the initial stages of MeHg intoxication in rat cerebellar granule neurons, and suggests that mitochondrial play a central role in both the release of Ca$^{2+}$ and influx of Ca$^{2+}$.$\omega$. It is now known that MeHg causes an early, severe loss of Ca$^{2+}$.$\omega$ regulation that involves initial Ca$^{2+}$ release from the SER (Hare and Atchison, 1995; Bearss et al., 2001), which is buffered by mitochondria (this article). When the mitochondria become loaded with excess Ca$^{2+}$, the MTP opens (Limke and Atchison, 2002; this article), resulting in the first-phase increase of cytosolic Ca$^{2+}$. This is followed several minutes later by an influx of Ca$^{2+}$.$\omega$, which is sensitive to mitochondrial inhibitors, suggesting that the mitochondria somehow triggers this Ca$^{2+}$.$\omega$ influx, possibly through depletion of cellular ATP (Sarafian et al., 1989). The ability of MeHg to cause a consistent pattern of changes in spite of its ability to bind thiol groups indiscriminately suggests a preference for binding sites in the cell, which may explain the heightened susceptibility of cerebellar granule cells to MeHg-induced cell death.

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References


Szalai G, Cserda G, Hantash BM, Thomas AP, and Hajnoczy G (2000) Calcium...

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