Effects of Methylmercury on Human Neuronal L-Type Calcium Channels Transiently Expressed in Human Embryonic Kidney Cells (HEK-293)

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ABSTRACT
Methylmercury (MeHg) disrupts the function of native, high voltage-activated neuronal Ca\(^{2+}\) channels in several types of cells. However, the effects of MeHg on isolated Ca\(^{2+}\) channel phenotypes have not been examined. The aim of the present study was to examine the action of MeHg on recombinant, neuronal L-type voltage-sensitive Ca\(^{2+}\) channels. Human embryonic kidney cells (HEK-293) were transfected with human neuronal cDNA clones of the alpha(2b) and beta(3a) Ca\(^{2+}\) channel subunits and the reporter jellyfish green fluorescent protein for transient expression. Current from expressed channels (I\(_{\text{Ba}}\)) and their response to MeHg applied acutely were measured using whole-cell voltage-clamp recording techniques and Ba\(^{2+}\) (5 mM) as charge carrier. Amplitude of I\(_{\text{Ba}}\) in these cells was reduced by the dihydropyridine (DHP), nimodipine, and enhanced by Bay K8644 [S(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3 pyridine carboxylic acid methyl ester]. MeHg (0.125–5.0 \(\mu\)M) caused a time- and concentration-dependent reduction in amplitude of the peak and sustained current through these channels. However, even at the highest concentration of MeHg tested, reduction of current amplitude by MeHg was incomplete. Washing with MeHg-free solution could not reverse its effects. The steady-state inactivation curve was unaltered by MeHg. Increasing the stimulation frequency or the extracellular Ba\(^{2+}\) concentration each attenuated slightly the reduction in amplitude of I\(_{\text{Ba}}\) by MeHg. In the presence of MeHg (5.0 \(\mu\)M), Bay K8644 still increased the remaining current, and nimodipine (10 \(\mu\)M) reduced residual current that was resistant to MeHg. Thus, although MeHg reduces the amplitude of recombinant, heterologously expressed L-type channel current, a portion of current is resistant to reduction by MeHg. Furthermore, DHP agonists and antagonists retain their ability to affect L-type Ca\(^{2+}\) channel current even in the presence of MeHg.

Voltage-sensitive Ca\(^{2+}\) channels play critical roles in a number of cellular functions such as synaptic transmission, muscle contraction, and regulation of gene expression (Catterall, 1998). Several distinct subtypes of “high voltage-activated” Ca\(^{2+}\) channels (L, N, P/Q, and R) have been identified based on their biophysical and pharmacological properties (Tsien et al., 1995). L-type channels, the subject of the present study, are present in various forms in both neurons and other kinds of cells. Ca\(^{2+}\) channels in general are affected by the actions of a number of toxicants (Kiss and Osipenko, 1994; Sirois and Atchison, 1996). Because of the crucial roles these proteins play in key cellular functions, toxicant effects on Ca\(^{2+}\) channels could have significant deleterious consequences on cell function (Audesirk et al., 2001).

Methylmercury (MeHg) is an important environmental contaminant that has been responsible for episodes of mass environmental neurotoxicity in Minamata, Japan (Takeuchi, 1982) and Iraq (Bakir et al., 1973). MeHg neurotoxicity remains a contemporary concern, especially for populations with a high component of fish in their diet (Kosatsky and Dumont 1991; Grandjean et al., 1997; Hansen and Danscher, 1997; Renzoni et al., 1998). The mechanisms underlying MeHg neurotoxicity are unclear, and multiple sites of action probably contribute to the ultimate expression of toxicity (Atchison and Hare, 1994). Among the many cellular targets associated with effects of MeHg, voltage-gated Ca\(^{2+}\) channels including, apparently, the


ABBREVIATIONS: MeHg, methylmercury; HEK, human embryonic kidney; DHP, dihydropyridine; Bay K8644, S(-)-1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3 pyridine carboxylic acid methyl ester; GFP, green fluorescent protein; I_{Ba}, Ba\(^{2+}\) current; PC12, pheochromocytoma; [Ba\(^{2+}\)]_w, extracellular barium concentration.
L-type, are affected rapidly and at low micromolar concentrations in isolated systems. The actions of MeHg on Ca\(^{2+}\) channels are conflicting, depending upon the type of measurement being made. Like divalent heavy metals such as Pb\(^{2+}\), Cd\(^{2+}\), and inorganic Hg\(^{2+}\), acute application of MeHg to cells in culture causes a marked reduction in amplitude of native currents carried through Ca\(^{2+}\) channels (Shafer and Atchison, 1991; Sirois and Atchison, 2000). Similarly, reduced influx of \(45\text{Ca}^{2+}\) during KCl-induced depolarization results from extremely brief (1 s) exposures to MeHg in rat cortical synaptosomes (Atchison et al., 1986; Shafer and Atchison, 1989; Shafer et al., 1990; Hewett and Atchison, 1992). However, in functional assays of effects of MeHg on regulation of intracellular free Ca\(^{2+}\) using measurements of Fura-2 acetoxyethyl ester fluorescence, continuous exposure to MeHg (0.1–5 \(\mu\text{M}\)) for periods of up to an hour induces an increase in intracellular Ca\(^{2+}\), part of which occurs by entry of Ca\(^{2+}\) from the extracellular medium (Denny et al., 1993; Hare et al., 1993). In both NG108–15 cells and primary cultures of cerebellar granule cells, nifedipine, a dihydropyridine (DHP)-type blocker of L-type Ca\(^{2+}\) channels, delays the onset of this MeHg-mediated influx of extracellular Ca\(^{2+}\) (Hare and Atchison 1995; Marty and Atchison 1997), and partially protects granule cells in culture from MeHg-induced cytotoxicity (Marty and Atchison, 1998). Moreover, in vivo administration of nifedipine and other Ca\(^{2+}\) channel blockers protects rats from MeHg-induced neurotoxicity (Sakamoto et al., 1996). These observations suggest that at least some of the effects of MeHg are mediated by L-type Ca\(^{2+}\) channels or, alternatively, that MeHg, like Pb\(^{2+}\) (Simons and Pocock, 1987), may enter neurons by means of L-type Ca\(^{2+}\) channels.

To begin to reconcile these seemingly contradictory effects of MeHg and hence understand more clearly the actions of MeHg on voltage-gated Ca\(^{2+}\) channels, we undertook the first study of actions of MeHg on a homogeneous population of a single defined phenotype of Ca\(^{2+}\) channels. One problem with existing studies using native cell currents is that multiple types of Ca\(^{2+}\) channels occur in these preparations (Sirois and Atchison, 2000), and pharmacological methods for isolating a single phenotype are incomplete. Thus, theoretically, MeHg could act differentially on different subtypes of Ca\(^{2+}\) channels, perhaps impeding current flow through one subtype, while not affecting, or actually facilitating, current flow through another. Because of the protective effects of L-type Ca\(^{2+}\) channel antagonists against MeHg-induced neurotoxicity in several experimental paradigms, we chose to focus initially on this phenotype in isolation using a heterologous expression system. Specifically, the goal of the present study was to determine whether current through L-type voltage-sensitive Ca\(^{2+}\) channels was reduced by acute administration of MeHg and, if so, what were the characteristics of reduction of channel function by MeHg.

To do this, we used recombinant clones of human neuronal L-type Ca\(^{2+}\) channels expressed using cDNA copies of their genes transferred into human embryonic kidney cells (HEK-293). These nonexcitable cells are commonly used for heterologous expression of membrane proteins including voltage-dependent Ca\(^{2+}\) channels (Williams et al., 1994; Perez-Garcia et al., 1995; Querfurth et al., 1998). Whole-cell voltage-clamp methods were used to characterize the currents expressed in these cells, and then to examine the actions of MeHg on these currents.

**Experimental Procedures**

**Materials.** Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were purchased from the American Type Culture Collection (Manassas, VA). All reagents were pure or ultra-pure laboratory grade unless otherwise noted. S(-)-Bay K8644, (−)-nimodipine, ATP-Mg, cAMP, HEPES, EGTA, and tetrodotoxin were obtained from Sigma-Aldrich (St. Louis, MO). Bay K8644 and nimodipine were made up as stock solutions in absolute ethanol to give final concentrations in solutions, which limited the ethanol concentration to less than 0.1% (v/v). Stock solutions (5 mM) of methylmercuric chloride (MeHg) (ICN Pharmaceuticals, Costa Mesa, CA) were prepared weekly in double distilled water, from which test solutions were prepared daily in extracellular solution. Expression cDNA clone plasmids of the human neuronal Ca\(^{2+}\) channel subunits used in the study were all generously provided by Dr. Kenneth A. Stauderman of SIBIA Neurosciences (San Diego, CA), now Merck Research Laboratories. \(\alpha_{1C}\) and \(\beta_{2}\) cDNA were isolated from hippocampus (Mark Williams, Merck Research, personal communication); \(\alpha_{2b}\) was isolated from cerebellum (Williams et al., 1992).

**Cell Culture and Transfection.** HEK-293 cells were grown at 37°C in Eagle’s minimal essential medium fortified with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM l-glutamine, 1.5 g/l sodium bicarbonate, 10% fetal bovine serum, and penicillin/streptomycin/ampicillin B (Invitrogen, Carlsbad, CA) in a 5% CO\(_2\) environment. One day before gene transfer, cells were plated at a density of 5 \(\times\) 10\(^4\) on 35-mm culture dishes. Cells were transfected with a mixture of plasmids containing \(\alpha_{1C-1}, \alpha_{2b}\), and \(\beta_{3a}\) cDNA subunits and a jellyfish green fluorescent protein (GFP) cDNA sequence using Fugene 6 (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions. Reactions contained a total of 3 \(\mu\)l of Fugene 6 and 1 \(\mu\)l of plasmid DNA containing the three subunits in a 1:1:1 molar ratio, with GFP plasmid at 20% of the total DNA. Two days were allowed for optimal transient expression of proteins, at which time the cells were examined for GFP expression. Cells from dishes with a reasonable number of green fluorescent cells (usually 20%) were replated at a lower density to facilitate recording. Recordings were typically made using cells from a minimum of three independent transfections.

**Ca\(^{2+}\) Channel Current Recording.** Prior to recording, culture medium was removed, and cells were rinsed twice with extracellular solution and then replenished with 1 ml of extracellular recording bath solution. The extracellular solution contained 150 mM tetrathylammonium chloride, 5 mM BaCl\(_2\), 1 mM MgCl\(_2\), 25 mM d-glucose, 10 mM HEPES, 0.001 mM tetrodotoxin, pH adjusted to 7.2 at room temperature (23–25°C) with tetrathylammonium hydroxide. The osmolality of solution was 310 mOsm. In some experiments, [BaCl\(_2\)] was increased to 20 mM; the tetrathylammonium chloride concentration was simultaneously reduced to 117 mM to maintain iso-osmolarity. Patch-clamp pipettes with resistances between 3 and 5 MΩ were prepared from 1.5-mm-i.d. glass capillaries (World Precision Instruments, Sarasota, FL) using a PP-830 two-stage micro-electrode puller (Narishige, Tokyo, Japan) and fire-polished using an MF-830 microforge (Narishige). The intracellular (pipette) solution contained 140 mM CsCl, 10 mM EGTA, 10 mM HEPES, 2 mM ATP-Mg, and 1 mM CAMP; pH was adjusted to 7.2 at room temperature with CsOH.

The tight-seal, whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used on fluorescent green cells to record Ba\(^{2+}\) currents (\(I_{\text{Ba}}\)) through transiently expressed Ca\(^{2+}\) channels and study the effects of MeHg or other pharmacological agents on them. Whole-cell currents were recorded using an Axopatch-1D amplifier (Axon Instruments, Union City, CA), sampled at 10 kHz, filtered at 2 kHz (−3 dB, four-pole Bessel filter; Axon Instruments) and acquired online using the pClamp6 program (Axon Instruments).
ments). Pipette and cell capacitance were compensated online in all experiments. Series resistance was also compensated online in the range of 60 to 80%. Extracellular media were exchanged using a gravity-fed bath perfusion system (BP5-4; ALA Scientific Instruments, Westbury, NY); the flow rate was 5 × 10⁻³ ml/s. The distance of the flow pipette from the cell remained approximately 150 μm and the pipette tip diameter was 300 μm. All experiments were carried out at room temperature (~23–25°C).

Except when noted otherwise, a pulse protocol was used to examine the effects of MeHg on membrane currents. A hyperpolarizing pulse with one-quarter of the test pulse magnitude was applied to measure the leak current, followed by a depolarizing pulse to elicit inward current. Linear components of leak and capacitive current were not subtracted online from these records. Thus, effects of MeHg on inward current, leak current, and capacitive current could be examined in consecutive current traces. This pulse protocol was repeated once every 10 s. Leak subtraction was performed offline, subtracting the scaled current observed with the P/N protocol. For some experiments, a ramp protocol was used. The duration of the ramp was 150 ms and the interval between ramps was 30 s.

**Statistical Analysis.** Origin (MicroCal, Northampton, MA) and pClamp (Axon Instruments) software suites were used to perform linear and nonlinear fit of data. Statistical comparisons were performed using paired Student’s t test. Results are expressed as mean ± standard error of the mean, and p < 0.05 was considered to be statistically significant. Asterisks indicate statistically significant differences: *p < 0.05, **p < 0.01, and ***p < 0.001.

**Results**

**Characteristics of Current Expressed by Recombinant α₁C, α₃, and β Subunits of Ca²⁺ Channels in HEK-293 Cells.** Expression of cloned Ca²⁺ channels in HEK-293 cells, using the above-mentioned protocol, produced I⁢Ba with biophysical and pharmacological similarities to the neuronal L-type Ca²⁺ channel expressed in its native environment. Representative I⁢Ba results elicited from these cells along with their current-voltage relationship are shown in Fig. 1, A and B. No significant inward current was observed until the depolarizing step reached −40 mV. Currents reached maximum amplitude at approximately 0 mV, and the reversal potential was approximately +50 mV. The peak current shows voltage dependence and inactivated only slowly.

Figure 1C depicts the voltage dependence of activation, or steady-state activation (mₛ), of these Ca²⁺ channels, where mₛ was determined from the relative amplitudes of currents (IIₘₐₓ) elicited at different test membrane potentials. The voltage for half-maximal activation (V₁/₂) was −18.8 mV. The isochronal inactivation (hₛ) was determined using an 8-s inactivating prepulse. The inactivation curve is shown with slope factor (K) of 13.3 mV and V₁/₂ of −53.0 mV.

Figure 2 demonstrates DHP antagonist and agonist sensitivity of I⁢Ba elicited from these recombinant Ca²⁺ channels. Application of the agonist Bay K8644 (10 μM) caused a 100% increase of peak current (Fig. 2, A and C), whereas addition of the DHP antagonist nimodipine (10 μM) reduced the current by more than 90% (Fig. 2, B and C).

**Characteristics of Reduction of I⁢Ba by MeHg.** As shown in representative traces (Fig. 3A), and in composite data from a number of recordings (Fig. 3B), continuous superfusion with MeHg caused a time-dependent reduction of I⁢Ba in transfected cells. The degree of reduction of current appeared to be approximately the same for both peak and sustained components of current (Fig. 3A). Thus, inactivation did not appear to be affected by MeHg under these conditions. The time course of effect on I⁢Ba of different concentrations of MeHg (0.125–5.0 μM) is shown in Fig. 3B. MeHg was applied after at least 5 min of stabilization of the control current. In comparison with control cells, which underwent 10% rundown of current during 9 min of continuous recording, MeHg treatment caused an apparent concentration-de-
pending upon the concentration of MeHg used, the rapid
initially rapid, and the other, gradual and progressive. De-
by MeHg appeared to follow two distinct phases: one that was

tude (results not shown). The reduction in current amplitude
pared with paired control values (p < 0.001). Values shown
are the mean ± S.E.M. of four to five different cells. Cells were depolar-
ed from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current
responses were filtered at 2 kHz and leak current was subtracted.

Fig. 2. Effect of dihydropyridines on peak L-type I_{Ba} in transfected HEK-293 cells. A, increase of current amplitude by 10.0 μM Bay K8644. B, reduction of current amplitude by 1.0 and 10.0 μM nimodipine (Nim). C, amplitude of inward Ba^{2+} current recorded before and after a 2-min exposure to Bay K8644 and nimodipine (Nim). The asterisk (*) indicates a significant change in current amplitude in the presence of dihydropy-
ridines compared with paired control values (p < 0.001). Values shown
are the mean ± S.E.M. of four to five different cells. Cells were depolar-
ized from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current
responses were filtered at 2 kHz and leak current was subtracted.

phase was completed between 100 and 150 s after beginning
treatment. Lower concentrations of MeHg (0.125–1.25 μM)
caused a more gradual decline in current amplitude. Figure 4
depicts the inhibition of peak current by various concentra-
tions of MeHg after a 2-min exposure.

Reduction by MeHg of current through putative L-type Ca^{2+} channels in differentiated PC12 cells has been shown
previously to be irreversible (Shafer and Atchison, 1991). Therefore, we tested whether this phenomenon was also seen
in recombinant expressed channels of similar phenotype. A
4-min wash with MeHg-free solution after 5 min of exposure
to 5 μM MeHg did not reverse the reduction of I_{Ba} current
(Fig. 5).

Effect of DHPs on Reduction of I_{Ba} by MeHg. Current
carried through these recombinant Ca^{2+} channels expressed in
HEK-293 cells was sensitive to DHP-type drugs. MeHg
has been reported to decrease the affinity of binding for
[^3H]nitrendipine to cortical synaptosomes (Shafer et al.,
1990). Thus, we sought to determine whether MeHg caused its inhibitory effect by acting at sites that might overlap with
those used by DHP-type Ca^{2+} channel antagonists or ago-
nists. Consequently, nimodipine or Bay K8644 was applied in
the continued presence of MeHg. As seen in Fig. 6A, 10 μM nimodipine was able to elicit a further decline in the current remaining, even after a 5-min exposure to 5 μM MeHg. Furthermore, after a similar reduction by MeHg, 10 μM Bay K8644 was able to cause a slight increase in the residual current (Fig. 6B).

Voltage Dependence of I_{Ba} Reduction by MeHg. To
determine whether reduction by MeHg of the heterologously expressed L-type Ca^{2+} channel-mediated current is voltage-
dependent, we applied a ramp protocol to obtain the current-
voltage relationships for I_{Ba}. Inactivation properties were
studied by varying the holding potential for 8 s before applying
a test pulse to 0 mV in the presence or absence of MeHg. Figure 7A shows the current-voltage relationships for I_{Ba}
before and after addition of 5.0 μM MeHg at different times
of exposure. MeHg decreased the peak current amplitude at all potentials that activated current, but did not alter either the threshold of activation of I_{Ba} or the reversal potential. There were slight changes in the potential at which the maximum current was elicited; for example, there appeared to be a slight shift in the hyperpolarizing direction at 3 min
(Fig. 7A). However, there was no apparent consistent pattern
evident in these changes. MeHg had no effect on the inactiva-
tion curve (Fig. 7B).

Frequency Dependence of I_{Ba} Reduction by MeHg. Some chemicals block ion channels in a use-dependent man-
ner (Hille, 1992). During this process, the inhibitory effect is
enhanced because of repetitive opening of the channels by
increasing the stimulation frequency. The premise is that
these inhibitors act either at sites within the channel or
intracellularly. Shafer and Atchison (1991) and Sirois and
Atchison (2000) demonstrated that the effect of MeHg on
Ca^{2+} channels expressed in differentiated PC12 and cerebellar gran-
ule cells, respectively. We examined whether this type of
reduction could also occur in recombinant Ca^{2+} channels by
stimulating transfected cells at frequencies of 0.1, 0.2, and
0.4 Hz. No frequency-dependent enhancement of MeHg-in-
duced reduction of current amplitude was observed. Instead,
the effect of MeHg on peak currents was either unchanged or
actually reduced at higher stimulation frequencies (0.2 and

Fig. 2. Effect of dihydropyridines on peak L-type I_{Ba} in transfected HEK-293 cells. A, increase of current amplitude by 10.0 μM Bay K8644. B, reduction of current amplitude by 1.0 and 10.0 μM nimodipine (Nim). C, amplitude of inward Ba^{2+} current recorded before and after a 2-min exposure to Bay K8644 and nimodipine (Nim). The asterisk (*) indicates a significant change in current amplitude in the presence of dihydropy-
ridines compared with paired control values (p < 0.001). Values shown
are the mean ± S.E.M. of four to five different cells. Cells were depolar-
ed from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current
responses were filtered at 2 kHz and leak current was subtracted.
0.4 Hz; Fig. 8A), depending upon the duration of exposure to MeHg. At a stimulation frequency of 0.1 Hz, 5 μM MeHg reduced peak current by 67 ± 7% and 83 ± 3% after a 4-min and a 9-min exposure, respectively. When the stimulation frequency was increased, initially, the rate of decline of current amplitude appeared identical to that at 0.1 Hz. At both 0.2 and 0.4 Hz, there was a transient reversal of block by MeHg that occurred at ~150 s. In each case this reversal lasted for about 75 s and caused an ~33% increase in current amplitude despite the continued presence of MeHg. However, by a little over 200 s, current amplitude again began to decline. Although the rates of decline were not measured mathematically, visual inspection suggested that the rate of decline after this transient reversal was approximately equivalent to that prior to the reversal. At 0.2 Hz, the reduction of peak current induced by 5 μM MeHg was decreased to 43 ± 6% (p < 0.05) and 69 ± 2% (p < 0.05) after 4-min and 9-min exposures, respectively. At a stimulation frequency of 0.4 Hz, 5 μM MeHg induced an even smaller reduction than

Fig. 3. Effect of MeHg on L-type $I_{\text{Ba}}$ in HEK-293 cells. A, representative current traces depict the effect of 5.0 μM MeHg at different lengths of exposure. Leak current was not subtracted. B, time course of reduction of peak current amplitude by different concentrations of MeHg [●, 0 μM (control); ♦, 0.125 μM; ○, 1.25 μM; □, 2.5 μM; ●, 5.0 μM]. Values shown are the mean of five to seven different cells. Cells were depolarized from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current responses were filtered at 2 kHz and leak current was subtracted.

Fig. 4. Amplitude of inward $\text{Ba}^{2+}$ current recorded before and after a 2-min exposure to different concentrations of MeHg in HEK-293 cells transfected with $\alpha_{\text{NC}}$ subunit along with the $\alpha_{\text{L}}$ and $\beta$ subunits of human neuronal $\text{Ca}^{2+}$ channels. Cells were depolarized from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current responses were filtered at 2 kHz and leak current was subtracted. The asterisk (●) indicates a significant change in peak current amplitude in the presence of MeHg when compared with control values (p < 0.001). Values shown are the mean of five to seven different cells and were obtained from panel B of Fig. 3.

Fig. 5. Irreversible reduction of peak L-type $I_{\text{Ba}}$ by MeHg in HEK-293 cells. Peak current through expressed channels was reduced by 5.0 μM MeHg during exposure for 5 min; washing with MeHg-free extracellular solution did not reverse the reduction in current amplitude induced by MeHg. Cells were depolarized from −70 mV to 0 mV at a stimulus frequency of 0.1 Hz. Current responses were filtered at 2 kHz and leak current was subtracted. Values are the mean of five cells. Representative standard error bars are indicated.
it did at 0.1 Hz during 4 min of exposure (36 ± 1%, p < 0.05). After a 9-min exposure, the reduction of peak current at 0.4 Hz (82 ± 3%) was not significantly different from that at 0.1 Hz. A similar reduction in inhibition was observed with 1.25 μM MeHg. At a stimulation frequency of 0.1 Hz, peak current amplitude was reduced by 70 ± 6% after 9 min of exposure to MeHg. After the stimulation frequency was increased to 0.2 Hz, peak current amplitude was reduced by only 50 ± 3% (Fig. 8B).

Increasing [Ba²⁺]e Reduces the Effectiveness of Block of I_{Na} by MeHg. If the mechanism by which a divalent cation acts involves direct occlusion of the channel within the pore itself, the effectiveness of reduction in current would depend on the concentration of charge carrier. Increasing [Ca²⁺]i counters the magnitude of reduction of ⁴⁵Ca²⁺ influx in synaptosomes by MeHg (Atchison et al., 1986; Shafer and Atchison, 1989), and increasing [Ba²⁺]e slightly reduces the negative effect of MeHg on I_{Na} in PC12 cells (Shafer and Atchison, 1991). As such, we sought to determine whether this action was associated with recombinant L-type Ca²⁺ channels. Indeed, the degree to which 5.0 μM MeHg reduced the peak current through these channels was dependent on the concentration of MeHg (Fig. 8B).

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Fig. 6. Effect of dihydropyridines on reduction of peak L-type I_{Na} by MeHg in HEK-293 cells. A, peak current was reduced by ~60% within 5 min by 5.0 μM MeHg; addition of 10 μM nimodipine further reduced the residual I_{Na} left after the peak effect of MeHg. B, reduction of Ba²⁺ currents caused by 5.0 μM MeHg was partly restored by 10 μM Bay K8644. Cells were depolarized from −70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current responses were filtered at 2 kHz and leak current was subtracted. Values are the mean of five to seven cells. Representative standard error bars are indicated.

Fig. 7. Effect of MeHg on current-voltage relationships and steady-state inactivation of L-type I_{Ba} in HEK-293 cells. A, current-voltage relationships for I_{Ba} recorded before and after addition of 5 μM MeHg for different exposure times. Test pulses were delivered by a ramp protocol of 150 ms duration and 30-s intervals between ramps as shown at the top of panel A; test potentials ranged from −60 mV to +60 mV with a holding potential of −70 mV. B, MeHg has no effect on voltage dependence of steady-state inactivation. The peak currents during a 150-ms test pulse from −70 mV to 0 mV were plotted against voltage with an 8-s conditioning prepulse. The peak currents before (control, solid circles) and after a 3-min exposure to 5.0 μM MeHg (MeHg, open circles) were normalized by the largest current recorded after conditioning prepulses ranging from −100 mV to 0 mV. The fitted curves are plotted using a Boltzmann function, I/I_{max} = [1 + exp((V - V_{1/2})/K)]⁻¹, with control V_{1/2} = −54.3 mV, K = 12.9 mV, and a 3-min exposure to MeHg V_{1/2} = −56.9 mV, K = 13.4 mV. Values in panel B are the mean ± S.E.M. of seven cells.

Fig. 8. Effect of different rates of stimulation on MeHg-induced reduction of peak L-type I_{Na} in HEK-293 cells. Panel A, effect of 5.0 μM MeHg at stimulation frequencies of 0.1, 0.2, and 0.4 Hz. B, effect of 1.25 μM MeHg at stimulation frequencies of 0.1 and 0.2 Hz. Cells were depolarized from −70 mV to 0 mV. Current responses were filtered at 2 kHz and leak current was subtracted. Values are the mean of five to six cells. Representative standard error bars are indicated.
reduction of peak current only reached 40 ± 6% (p < 0.05; Fig. 9). This suggests that increasing [Ba\(^{2+}\)] in extracellular solution on current reduction induced by 5.0 \(\mu\)M MeHg in HEK-293 cells expressing L-type human neuronal Ca\(^{2+}\) channels. A, the time course of current reduction produced by 5.0 \(\mu\)M MeHg with 5 mM and 20 mM Ba\(^{2+}\) as charge carrier. Values are the mean time courses of 7 to 11 cells. B, amplitude of \(I_{\text{Ba}}\) current recorded before and after a 3-min exposure to 5.0 \(\mu\)M MeHg. The asterisk (*) indicates a significant change in peak current amplitude in the presence of MeHg when compared with control values (p < 0.001). The number sign (#) indicates a significant change in peak current amplitude when compared with 5 mM Ba\(^{2+}\) as a charge carrier in the presence of MeHg (p < 0.05). Cells were depolarized from -70 mV to 0 mV at a stimulation frequency of 0.1 Hz. Current responses were filtered at 2 kHz and leak current was subtracted. Values shown are the mean ± S.E.M. of 7 to 11 cells.

**Discussion**

The present study is the first to examine the inhibitory actions of MeHg on a single identified phenotype of voltage-sensitive Ca\(^{2+}\) channels. While corroborating several aspects of previous studies examining the effects of MeHg on Ca\(^{2+}\) channel function in native cells, our study extends them in several important ways. First, MeHg exerts a concentration-dependent depressant effect on function of neuronal L-type high voltage-activated Ca\(^{2+}\) channels that was not readily reversible by washing with MeHg-free solution. Second, unlike the situation seen for mixed populations of Ca\(^{2+}\) channels in their native environment, reduction of current carried through these recombinant L-type Ca\(^{2+}\) channels by MeHg is not total and apparently is not mediated by action at the DHP binding site. Third, concentration of charge carrier can modulate the inhibitory effects of MeHg, due possibly to steric hindrance or overlap. The overall shape of the current recordings as well as the voltage and time dependence of the current recorded from cells transfected with recombinant Ca\(^{2+}\) channels comprising \(\alpha_{1C-1}\), \(\alpha_{2b}\), and \(\beta_{2a}\) Ca\(^{2+}\) channel subunits resemble those of native L-type Ca\(^{2+}\) channels. Furthermore, the \(I_{\text{Ba}}\) from these recombinant Ca\(^{2+}\) channels exhibits the pharmacological sensitivities to DHPs in the manner and concentration range reported in numerous studies on native DHP-sensitive L-type Ca\(^{2+}\) channels.

MeHg disrupts normal Ca\(^{2+}\) channel function in a variety of preparations as seen using synaptosome 45Ca\(^{2+}\) influx, ligand binding, and electrophysiological techniques (Shafer and Atchison, 1989; Shafer et al., 1990; Hewett and Atchison, 1992; Leonhardt et al., 1996; Shafer, 1998). Siros and Atchison (2000) demonstrated that MeHg affects multiple subtypes of Ca\(^{2+}\) channels including L-type in rat cerebellar granule cells. However, there are no previous studies on the discrete effects of MeHg on identified subtypes of Ca\(^{2+}\) channels in isolation, mainly because of the multiplicity of Ca\(^{2+}\) channel types that coexist in most primary cultures of neurons, such as cerebellar granule cells, and the lack of completely specific pharmacological probes. Our results show that low concentrations of MeHg (0.125–5.0 \(\mu\)M) rapidly reduced peak \(I_{\text{Ba}}\) in a time- and concentration-dependent manner through recombinant, heterologously expressed L-type Ca\(^{2+}\) channels, a phenotype expressed in several kinds of neuronal as well as non-neuronal cells. Currents through recombinant L-type channels were affected at lower concentrations of MeHg than were ostensibly similar native L-type currents in rat PC12 cells (Shafer and Atchison, 1991; Shafer, 1998) and dorsal root ganglion cells (Leonhardt et al., 1996), but the lower concentration of charge carrier used in our present study as compared with other studies may have contributed to this effect. Current from recombinant channels was similar in sensitivity to MeHg to native currents in rat cerebellar granule cells (Sirois and Atchison, 2000) using the same [Ba\(^{2+}\)].

Other subtle differences are also apparent between our results and those from native channels. For example, in differentiated PC12 cells, MeHg (10–20 \(\mu\)M) rapidly and completely blocked whole-cell current through presumed L-type Ca\(^{2+}\) channels (Shafer and Atchison, 1991). In contrast, the recombinant L-type Ca\(^{2+}\) channel current expressed in HEK-293 cells could be blocked only incompletely by MeHg. This difference in pharmacological sensitivity between native and cloned, heterologously expressed channels has also been reported for calciludine reduction of L-type (Stotz et al., 2000), \(\omega\)-agatoxin IVA reduction of P/Q-type (Bourinet et al., 1999), and peptide spider toxin DW13.3 reduction of N-type (Sutton et al., 1998) currents. One possible reason for this apparent difference in our experiments could be the use of a \(\beta\) subunit with properties different from the native \(\beta\) subunits that associate with the corresponding native L-type Ca\(^{2+}\) channels; \(\beta\) subunits are known to modulate channel
kinetics (Hofmann et al., 1999). Different β subunit isoforms cause shifts in the kinetics and voltage dependence of gating and thus result in substantially altered α, subunit function. In the *Xenopus laevis* oocyte transfection system, inactivation and recovery from inactivation of “L-type” channels depend not only on the nature of the β subunit, but also on the splice-variant of L-type channel expressed (Soldatov et al., 1997).

In nerve growth factor-differentiated PC12 cells, which express L-type Ca\(^{2+}\) channels (Plummer et al., 1989; Usowicz et al., 1990), MeHg appears to reduce current amplitude in a voltage-dependent manner (Shafer and Atchison, 1991). In synaptosomes, reduction of \(^{45}\text{Ca}^{2+}\) influx induced by MeHg also appears to be voltage-dependent (Shafer et al., 1990). However, in rat cerebellar granule cells, which possess multiple types of Ca\(^{2+}\) channels (L, N, P/Q, and R) (Randall and Tsien, 1995), reduction of Ca\(^{2+}\) current amplitudes by 0.25 to 1.0 \(\mu\)M MeHg was not voltage-dependent (Sirois and Atchison, 2000). In the experiments presented here, the reduction of peak current by 5.0 \(\mu\)M MeHg occurs at all membrane potentials and does not alter either the threshold of activation of \(I_{\text{BA}}\) or the reversal potential. MeHg did cause some slight changes in the potential at which maximum current is elicited. However, these effects were not consistent, nor was the steady-state inactivation curve altered.

If reduction of current amplitude by MeHg occurs through binding within the pore of the channels or intracellularly, it should exhibit “use-dependence”. In PC12 cells the effect of 5.0 \(\mu\)M MeHg was facilitated by increasing stimulation frequency from 0.1 to 0.2 or 0.4 Hz (Shafer and Atchison, 1991). In primary cultures of cerebellar granule cells, increasing the stimulation rate hastened the onset and increased the magnitude of reduction at both 0.25 and 0.5 \(\mu\)M MeHg, but not at 1 \(\mu\)M (Sirois and Atchison, 2000). In contrast, in our experiments increasing stimulation does not facilitate the effect of 5.0 \(\mu\)M MeHg and, in fact, decreases it to some extent. It is difficult to explain definitively the reason for the brief reversal of MeHg effect (Fig. 8A) at the higher rates of stimulation. We have also seen a similar phenomenon of temporary reversal under certain conditions of blocking action of MeHg on Ca\(^{2+}\)-dependent synaptic transmission in isolated brain slices (Yuan and Atchison, 1993) and motor end plates (Traxinger and Atchison, 1987). Perhaps a subset of Ca\(^{2+}\) channels remains closed during the lower stimulation rate but opens up during the faster stimulations, causing current flow for a limited time before the effect of MeHg sets in. As such, there may be a frequency- and time-dependent “reversing” of additional channels that become available to conduct current before they too are affected by the MeHg.

Increasing the extracellular concentration of Ba\(^{2+}\) from 5 mM to 20 mM partially attenuated the reduction of current by MeHg. This observation is consistent with effects on whole cell Ca\(^{2+}\) currents previously reported in PC12 cells (Shafer et al., 1990), effects on synaptosomal \(^{45}\text{Ca}^{2+}\) influx (Shafer and Atchison, 1989), and effects at the neuromuscular junction (Traxinger and Atchison, 1987). It suggests that MeHg may compete with Ba\(^{2+}\) for the same active sites within the channel (steric overlap) or there may be a steric hindrance due to close proximity of their binding sites.

The original aim of this study was to begin to reconcile differences in action of MeHg between results of acute electrophysiological-type studies with those of functional studies in which antagonists of L-type Ca\(^{2+}\) channels delay or protect against MeHg-induced neurotoxicity. One possibility was that L-type channels were not blocked by MeHg and perhaps even had current flow enhanced by the metal. However, MeHg reduces amplitude of currents carried through recombinant L-type channels, albeit not completely. A second possibility was that short-term exposures to MeHg impeded Ca\(^{2+}\) channel function, whereas longer exposures might facilitate current flow. The present study did not address this possibility directly; however, Shafer et al. (2002) find that exposure of PC12 cells to micromolar concentrations of MeHg for up to 24 h still impeded current flow. The most parsimonious explanation for this dichotomy is that MeHg itself enters cells through Ca\(^{2+}\) channels of various phenotypes—including L-type. Such a phenomenon is reported to occur for other neurotoxic metals such as Pb\(^{2+}\) (Simons and Pocock, 1987) and Cd\(^{2+}\) (Hinkle et al., 1987, 1992; Hinkle and Osborne, 1994) and is consistent with results from in situ preparations in which KCl- or veratridine-induced depolarizations hastened the onset of increased miniature end-plate potential frequency with MeHg, even in the absence of extracellular Ca\(^{2+}\) (Atchison, 1986, 1987). Thus, the ability of L-type Ca\(^{2+}\) channel blockers to protect against actions of MeHg may then reflect their ability to prevent its entry into the cell prior to producing intracellular actions. The question, then, is how does MeHg enter the channel if the blocking action was neither voltage-dependent nor use-dependent? Both of these factors should enhance entry of MeHg into the channel.

In conclusion, MeHg alters function of heterologously expressed, recombinant human neuronal L-type Ca\(^{2+}\) channels at low micromolar concentrations, concentrations well within the range associated with MeHg neurotoxicity in vivo. The mechanism by which this occurs and the consequences of this action are not yet clear. However, this action is temporally among the earliest reported for MeHg and, thus, would be expected to contribute ultimately to the neurotoxic effects.

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References


