Methylmercury Induces a Spontaneous, Transient Slow Inward Chloride Current in Purkinje Cells of Rat Cerebellar Slices

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Abbreviations: ACSF, artificial cerebrospinal fluid; APV, amino-5-phosphonopentanoic acid; BAPTA/AM, 1,2 bis(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; E_Cl, Nernst or Equilibrium potential for Cl'; HP, holding potential; MeHg, methylmercury; mIPSCs, miniature inhibitory postsynaptic currents; sIPSCs, spontaneous inhibitory postsynaptic currents; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine
Methylmercury (MeHg, 10 - 100 µM) induced a spontaneous, transient, slow inward current in Purkinje cells in rat cerebellar slices. Insensitivity of this current to tetrodotoxin suggests that its generation is not related to presynaptic firing. The present study was designed to attempt to identify the ionic origin of this current. Neither Gd³⁺, a nonspecific cation channel blocker, nor tetrakis(2-pyridylmethyl)ethylethylenediamine which chelates Zn²⁺, could prevent this current. Following dialysis of cells with a low [Cl⁻] pipette solution, the giant currents were inducible only when the cells were held at potentials more positive than 0 mV, but not at potentials more negative than -60 mV. Also, no giant currents were observed when cells were held at 0 mV under symmetrical [Cl⁻] conditions. Thus, this current appears to be mediated by Cl⁻. However, it was insensitive to the glycine receptor antagonist strychnine. The anion channel blockers 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) or niflumic acid suppressed GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents. Niflumic acid also prevented appearance of this giant current; DIDS was only effective at more positive membrane potentials. Thus, this current appears to be carried by a voltage-dependent Cl⁻ channel. Reducing extracellular Ca²⁺ concentration and/or intracellular application of the Ca²⁺ chelator BAPTA appeared to be ineffective at preventing appearance of this current. Thus, these data do not appear to support the conclusion that this current is mediated by a Ca²⁺-activated Cl⁻ channel. The role which this current plays in MeHg-induced neurotoxicity is unknown.
INTRODUCTION

The environmental neurotoxicant methylmercury (MeHg), interacts with several membrane voltage-gated and ligand-gated ion channels, albeit with differential sensitivity (Shrivastav et al., 1976; Quandt et al., 1982; Shafer and Atchison 1991; Sirois and Atchison 2000). These interactions may contribute to MeHg-induced neurological disorders in the peripheral and/or central nervous systems following acute and chronic exposure.

Among the effects described for MeHg on ion channel function, in dorsal root ganglion neurons and cerebellar granule cells in culture, MeHg induced a slowly developing but steady-state inward current (Arakawa et al., 1991; Xu and Atchison, 1998). This current was not blocked by the GABA<sub>A</sub> receptor antagonists bicuculline, picrotoxin or La<sup>3+</sup>, and was independent of extracellular [Ca<sup>2+</sup>], [Na<sup>+</sup>] and [K<sup>+</sup>], suggesting that MeHg might also affect nonspecific cation channels. This MeHg-induced slowly developing steady-state inward current was also observed in Purkinje and granule cells in cerebellar slices of rats (Yuan and Atchison, 2003). However the identity of this very slow current remains unknown, as does the possible role that it plays in neurons exposed to MeHg. Interestingly, we recently observed another type of MeHg-induced slow inward current in cerebellar granule, and particularly Purkinje cells, during the course of studying effects of MeHg on inhibitory synaptic transmission in cerebellar slices (Yuan and Atchison, 2003). Apparently distinct from the time course of the slowly developing, steady-state
inward current, this slow inward current was transient and had amplitude varying from a few hundred pA to several nA. It occurred spontaneously and randomly without a regular rhythm. The time of onset and frequency of occurrence of this current varied considerably from cell to cell; in some cells it never appeared or did so occasionally, whereas in other cells it occurred repeatedly—several or even more than 10 times in a given cell. However, it behaved like a MeHg exposure-related, time- and voltage-dependent response. The time to appearance of this current was MeHg concentration-dependent. Direction of the current flow could be reversed by changing the membrane holding potential. Reducing the intracellular Cl⁻ concentration ([Cl⁻]ᵢ) eliminated the inwardly-, but not the outwardly-directed giant slow currents at more positive membrane potentials, suggesting that this giant slow inward current might be a Cl⁻-mediated response. However, it does not appear to be a GABAergic response because the GABAₐ receptor antagonists bicuculline and picrotoxin failed to block it (Yuan and Atchison, 2003). Thus, the ionic origin of this current remains as yet unclear.

Since this current was seen most frequently in Purkinje cells, the present study was designed specifically to attempt to identify what ion(s) or possible channels is (are) responsible for mediation of this transient giant slow inward current in cerebellar Purkinje cells. Specifically, we sought to determine (1) if nonspecific cation channels are involved in generating this giant inward current; (2) if Cl⁻ channels mediate this current and if so, (3) what type of Cl⁻ channels or receptors may be involved. However, because of the nature of low frequency, random onset and
variable amplitude of this current (variations in amplitude and duration of this response exist even in a given individual cell), it is very difficult to characterize quantitatively this slow inward current (including obtaining a current-voltage relationship curve). Thus, data in this paper are presented principally in a descriptive manner.
MATERIALS and METHODS

Preparation of cerebellar slices.

All animal procedures complied with the National Institutes of Health guidelines on animal care and use and were approved by Michigan State University Animal Use and Care Committee. Cerebellar slices were prepared as described previously (Yuan and Atchison, 1999). In brief, the cerebellum was removed quickly from the brain of a young Sprague-Dawley rat (10 - 21 days postnatal, either gender) (Harlan Industries, Verona, WI) and immersed immediately in cold, oxygenated “slicing” solution which contained (in mM): 125, NaCl; 2.5, KCl; 4, MgCl2; 1.25, KH2PO4; 26, NaHCO3; 1, CaCl2 and 25, D-glucose (pH 7.35 - 7.4 when saturated with 95% O2 /5% CO2 at room temperature). A portion of vermis isolated by two cuts, was glued onto the tissue pedestal of an OTS-3000-05 Automatic Oscillating Slicer (FHC, Inc. Brunswick, ME) with cyanoacrylate glue. The tissue block was then transected sagittally to produce 5 - 8 slices with a thickness of approximately 150 - 200 μm. Slices were then transferred to a home-made holding chamber aerated continuously with 95% O2 /5% CO2, incubated at 35 °C for 60 min and then at room temperature of 22 - 25 °C until use.

Whole cell recording in cerebellar slices.

One slice was transferred to a modified RC-26 recording chamber (Warner Instruments Corp., Hamden, CT) assembled with an SS-3 slice support, and mechanically fixed using a “U” shaped...
anchor made of a platinum wire frame with nylon mesh. The slice was superfused (2 - 4 ml/min) continuously with modified artificial cerebrospinal fluid (ACSF) consisting of (in mM): 125, NaCl; 2.5, KCl; 1, MgCl₂; 1.25, KH₂PO₄; 26, NaHCO₃; 2, CaCl₂ and 25, D-glucose (pH 7.35 - 7.4 saturated with 95% O₂/5% CO₂ at room temperature) by gravity force. In experiments involving use of low extracellular Ca²⁺ concentration ([Ca²⁺]ₑ), 2 mM Ca²⁺ in the ACSF was replaced with equimolar or 4 mM Mg²⁺ as in the “slicing solution” (see above). Purkinje cells in slices were identified based on their characteristic electrophysiological properties and visually by their size, shape and location using a Nikon E600FN upright microscope (Nikon Optics, Tokyo, Japan) equipped with Nomarski optics (x 40 water immersion objective) and Sony IR-1000 infrared CCD video camera system (DAGE MTI, Michigan City, IN). Recording electrodes were pulled from 7052 glass capillaries (outer diameter = 1.5 mm, inner diameter = 1.0 mm, Garner Glass Co., Claremont, CA) using a P-97 puller (Sutter Instrument Company, Novato, CA). These were subsequently coated with Sylgard resin 184 (Dow Corning, Midland, MI) and fire-polished to a resistance of 2 - 3 MΩ when filled with the standard pipette solution consisting of (in mM): 140, CsCl; 4, NaCl; 0.5, CaCl₂; 10, HEPES; 5, EGTA, 2, Mg-ATP and 0.4, GTP (pH 7.3 adjusted with CsOH). In experiments to characterize the giant slow inward currents, a low [Cl⁻]-containing pipette solution was used; CsCl of the standard pipette solution was replaced with equimolar cesium gluconate in the low (~5 mM) [Cl⁻]-containing pipette solution and was prepared freshly just before recording. For experiments to determine if intracellular calcium concentration ([Ca²⁺]ᵢ) played a role in
inducing this giant current, the pipette solution consisted of (in mM): 110, CsCl; 4, NaCl; 0.5, CaCl$_2$; 10, HEPES; 10, 1,2-bis (2-aminophenoxy)ethane-$N,N',N''$-tetra-acetic acid (BAPTA-Cs$_4$); 2, Mg-ATP and 0.4, GTP (pH 7.3 adjusted with CsOH). Because the giant slow inward currents were initially observed during recordings of spontaneous synaptic inhibitory currents (sIPSCs), we maintained all recording procedures the same as those for recording sIPSCs described previously (Yuan and Atchison, 2003). Whole cell recordings were made using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and standard “blow and seal” techniques. The sIPSCs were recorded continuously at a holding potential of -60 or -70 mV in the presence of 10 $\mu$M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 50 - 100 $\mu$M amino-5-phosphonopentanoic acid (APV) in the external solution to block glutamate receptor-mediated excitatory postsynaptic currents. Miniature IPSCs (mIPSCs) were isolated under similar recording conditions but in the presence of 0.5 $\mu$M tetrodotoxin (TTX) in addition to CNQX and APV in the external solution to block the presynaptic, action potential-evoked release of GABA. For experiments involving the photo-sensitive chemical 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS), DIDS stock solution was prepared freshly just before experiments, and recordings were carried out under dark conditions. After capacitance and 60 - 80% series resistance compensation, series resistances were usually less than 10 M$\Omega$ for Purkinje cells and were monitored throughout the experiments to ensure their constancy. The liquid junction potential was estimated to be less than 4 mV and was ignored. All experiments were carried out at room temperature of 22 - 25 °C. Only one slice per rat was used for any given experiment and
only one concentration of MeHg was applied per slice.

Data acquisition and analysis.

Data were acquired using a PC-compatible computer equipped with a Digidata 1200B interface and pClamp8.1 software (Axon Instruments, Union City, CA). Whole cell currents were filtered at 1 - 5 kHz with an 8-pole low-pass Bessel filter and digitized at 10 - 20 kHz for later off-line analysis using pClamp8.1 or 9.0 program.

Data were collected continuously before and during application of MeHg and analyzed statistically using Student’s paired $t$ test or one-way analysis of variance (ANOVA) for time-dependent measures, unless otherwise specified. Dunnett’s procedure was used for post hoc comparisons. Values were considered statistically significant at $p \leq 0.05$. The data are presented as mean ± standard error of the mean (SEM), unless otherwise specified. Each experiment was replicated a minimum of 3 times; the actual number of replicates for each experiment is listed in the corresponding figure legend.

Chemicals.

Methylmercuric chloride, purchased from ICN Biomedical, Inc (Costa, CA), was dissolved in deionized water to a final concentration of 10 mM to serve as stock solution. The applied solutions (10 - 100 $\mu$M) were diluted with ACSF just before perfusion. Three different MeHg
concentrations (10 µM, 20 µM and 100 µM) were used in the present studies. Inasmuch as all applications were acute, these studies model most accurately effects of acute exposure to MeHg, or early onset effects in cases of chronic exposure. Clinical studies of MeHg-induced neurotoxicity following the relatively acute exposure in Iraq in the 1970s indicate that generalized weakness of the extremities progressing to cerebellar ataxia occurred in humans with a frequency of incidence of 70 - 100% at body burdens of 200 - 312 mg of Hg (Bakir et al., 1973) or 0.997 - 1.56 mmoles of Hg. Taking the upper range in an 80-kg adult male and assuming an approximate tissue density of 1.0 kg/liter, this converts to 19.5 µM. It was also shown that patients would develop symptoms of chronic MeHg poisoning when the blood and brain MeHg level reached 4 - 80 µM (Skerfving, 1972). Thus the concentrations of MeHg used in the present study are consistent with the pattern and exposure seen in this epidemic of acute poisoning. Our previous *in vitro* studies showed that onset of effects of MeHg on central synaptic transmission were MeHg concentration- and exposure time-dependent (Yuan and Atchison, 1993, 1995, 2003). The patterns of effects induced by 4 µM and 500 µM MeHg were generally similar to those reported at the concentration range used in this study, except that a much longer latency to onset of the effect of MeHg was required for the lower concentration of MeHg (Yuan and Atchison, 1993) and a very short latency occurred at 500 µM. In addition, as shown in our previous report (Yuan and Atchison, 2003), time to onset of the giant currents usually developed slowly, sometimes requiring up to 70 - 80 min after exposure to 10 µM MeHg to observe the appearance of giant currents. Much longer latencies would be expected if the
lower concentration of MeHg was used. Continuous whole cell recording epochs of 70 - 90 min in slice were seldom attained. Thus, relatively higher concentrations of MeHg were applied in this study. As mentioned above, the concentrations of 10 µM and 20 µM MeHg are within the range of those reported to be found in the blood of patients poisoned with MeHg in Iraq. The higher concentration of MeHg, 100 µM, was used to shorten the time course of effect of MeHg.

BAPTA acetoxyethyl ester (BAPTA/AM) and BAPTA free acid were purchased from Molecular Probes, (Eugene, OR) CNQX, APV, (-)-bicuculline methobromide, DIDS, strychnine and niflumic acid were all purchased from Sigma Chemical Co. (St Louis, MO). TTX was purchased from either Sigma or Alomone Labs LTD (Jerusalem, Israel).
RESULTS

MeHg-induced transient slow inward current is independent of activity of presynaptic action potentials.

The first set of experiments was designed to determine if the presence of a presynaptic action potential plays a role in generation of this slow inward current. Effects of MeHg on action potential independent mIPSCs in Purkinje cells were examined in cerebellar slices by application of TTX (0.5 \( \mu \)M) to block the presynaptic action potential-mediated release of neurotransmitter.

In the absence of MeHg, the frequency and amplitude of mIPSCs in normal Purkinje cells varied significantly from cell to cell. The averaged amplitudes of mIPSCs ranged from 20.4 - 106.8 pA with a mean of 43.7 ± 4.0 pA (n = 42). The average control frequency of mIPSCs in individual cells also varied considerably from 0.3 - 9.4 Hz with a mean of 2.7 ± 0.4 Hz (n = 42). This result is consistent with those seen previously for sIPSCs recorded from Purkinje cells in slice under similar control conditions (Yuan and Atchison, 2003). Bath application of 10 - 100 \( \mu \)M MeHg caused an initial increase then decrease in frequency and amplitude of mIPSCs prior to their complete block and induced a transient giant slow inward current during the late stage of MeHg exposure. The times of onset and cessation of these responses were inversely proportional to the MeHg concentrations (Figure 1). As shown for a representative experiment in Figure 1A, in this Purkinje cell, the mean amplitude of 728 mIPSCs over a period of 2 min
(6.1 Hz) just before application of 20 μM MeHg (Figure 1Aa and Ba) was 70.8 ± 58.1 pA (mean ± SD). After 20 min of exposure to MeHg, amplitudes of mIPSCs were increased to the maximum mean amplitude of 81.6 ± 61.8 pA (mean ± SD of 955 events) (Figure 1Ab, Bb) and subsequently started to decline. At the same time, the frequency of mIPSCs, mostly with small amplitudes, continued to increase and did not reach the peak level (14.7 Hz) until 60 min later (Figure 1Ac and Bc). As the amplitude and frequency of mIPSCs continued to decline, the transient, giant, slowly-decaying inward currents emerged during the late stage of MeHg exposure (Figure 1Ad, Ae, Af, Bd, Be and Bf). Eventually, all events were blocked completely by MeHg (Figure 1Ah and Bh). This giant, slow inward current appeared in 26 of 38 Purkinje cells tested. In contrast, as shown in Table 1 (control), no such slow giant inward currents were ever observed in recordings lasting for 60 - 90 min in cells from six slices which were not treated with MeHg. In addition, the slowly developing steady-state inward currents were also observed in all cells exposed to 10 - 100 μM MeHg. This steady-state inward current can be seen as a continuous change in baseline in Figure 1A (the actual change in baseline would be more apparent if the traces were shown continuously without interruption). Steady-state inward current was similarly never observed in control slices (results not shown). Figure 1C depicts changes in the frequency and amplitude of mIPSCs, the times to onset of giant currents and time to block of mIPSCs in three individual Purkinje cells after exposure to 100 μM (Figure 1Ca), 20 μM (Figure 1Cb) and 10 μM MeHg (Figure 1Cc), respectively. In all three cells, MeHg caused an early increase in the frequency and amplitude of mIPSCs. On average (n = 5 - 17), 100 μM,
20 μM and 10 μM MeHg initially increased the mean frequency from 2.3 ± 0.8 (range 0.3 - 9.4), 2.7 ± 0.5 (0.7 - 6.9) and 4.2 ± 1.2 (0.3 - 6.9) Hz in the pre-MeHg treatment control to 11.9 ± 2.8, 9.4 ± 2.2 and 9.8 ± 0.2 Hz ($p < 0.05$, Student’s paired $t$ test), respectively, and increased the mean amplitude of mIPSCs from 47.0 ± 12.2, 44.7 ± 7.1 and 49.4 ± 7.2 pA in the pre-MeHg treatment control to 103.1 ± 34.0, 72.6 ± 11.5 and 65 ± 10 pA ($p < 0.05$, Student’s paired $t$ test), respectively. As noted above, the mean frequency of mIPSCs in MeHg-free conditions varied considerably. This can be seen in comparing the results for controls at 10 μM and 20 μM MeHg. Consistent with our previous observation in sIPSC recordings (Yuan and Atchison, 2003), the times to onset of MeHg-induced maximum increase in frequency and amplitude of mIPSCs did not correlate very well. That is, they appeared to be independent events. The time to maximum increase in amplitude usually occurred earlier than did the increase in the frequency of mIPSCs (Figure 1 and Table 1). However, both times to onset of MeHg-induced maximum increase in the frequency and amplitude of mIPSCs were MeHg concentration-dependent. Similarly, as shown in Figure 1 and Table 1, times to appearance of the giant, slow inward current and to block mIPSCs were also MeHg concentration-dependent. All these values are consistent with those obtained from sIPSC recordings in Purkinje cells (Yuan and Atchison, 2003). Thus, these data suggest that induction by MeHg of the giant inward currents is independent of activity of presynaptic action potentials.

MeHg-induced giant slow inward currents are not mediated by nonspecific cation
channels nor by changes in Zn²⁺ permeability.

Nonspecific cation channels have been postulated to be responsible for both inorganic mercury- and MeHg-induced, slowly developing steady-state inward currents in dorsal root ganglion (Arakawa et al., 1991) or cerebellar granule cells in culture (Xu and Atchison, 1998). To determine if nonspecific cation currents play a role in generation of this giant inward current, Purkinje cells in cerebellar slices were pretreated for 5 - 10 min with 100 µM Gd³⁺ (Volk et al., 1995; Staruschenko and Vedernikova, 2002), a blocker of nonspecific cation channels, and then with 20 µM MeHg plus 100 µM Gd³⁺ until all postsynaptic events disappeared. As shown in Figure 2 (Left), treatment of cells with 100 µM Gd³⁺ alone did not induce a giant slow inward current in any of the cells tested (Gd³⁺). However, after 10 - 30 min exposure to 20 µM MeHg, by which time apparent increases in both frequency and amplitude of mIPSCs had occurred, MeHg still induced giant slow inward currents in five of six cells in the presence of 100 µM Gd³⁺. Thus, nonspecific cation channels do not appear to contribute to generation of this transient giant inward current.

MeHg also causes an increase in intracellular Zn²⁺, an ion that is able to permeate a number of cation channels (Denny et al., 1993; Denny and Atchison, 1994). The divalent cation Zn²⁺ is present throughout the mammalian CNS and concentrated in nerve terminals. Interestingly, Zn²⁺ also induced spontaneous giant depolarizing potentials in CA1 and CA3 hippocampal neurons (Xie and Smart, 1991, 1993). Thus, it is possible that a MeHg-induced increase in intracellular
Zn$^{2+}$ plays a role in generating this giant slow inward current. To test this possibility, Purkinje cells were pretreated for 10 – 20 min with 40 µM tetrakis(2-pyridylmethyl)ethylethlenediamine (TPEN), a membrane permeable Zn$^{2+}$ chelator, which does not chelate MeHg, and then were exposed to MeHg plus TPEN until all events were blocked. As shown in Figure 2 (Right), pretreatment of this Purkinje cell for 25 min with 40 µM TPEN in addition to 10 µM CNQX, 100 µM APV and 0.5 µM TTX did not induce any giant slow inward current (TPEN). Exposure of the cell for 15 min to 20 µM MeHg caused both frequency and amplitude of mIPSCs to increase significantly. Coincidently, the giant, slowly-decaying inward currents also began to appear at 23 min after exposure to MeHg (Figure 2, Right). Switching the holding potential from -60 mV to +60 mV at 70 min was still able to reverse the direction of current flow (see inset), reconfirming that the current flow direction of this response was voltage-dependent. As exposure to MeHg continued, all events eventually disappeared at 85 min. In 9 of 11 cells pretreated with TPEN, MeHg still effectively induced these giant slow inward currents. Thus, MeHg-induced release of intracellular Zn$^{2+}$ does not appear to be a major factor in induction of the giant currents.

The MeHg-induced giant slow inward current appears to be mediated by Cl$^-$ channels. Our previous study (Yuan and Atchison, 2003) suggested that the MeHg-induced transient giant slow inward current might be a Cl$^-$-mediated response, because the giant inward currents disappeared when cells were held at potentials more negative than -60 mV with pipette solution.
containing a low (~5 mM) [Cl\(^{-}\)] (in which Cl\(^{-}\) was replaced with equimolar membrane-impermeable gluconate - the predicted equilibrium potential (E\(_{Cl}\)) for Cl\(^{-}\) is about -82 mV). Conversely, in cells which were held at potentials more positive than 0 mV, MeHg still induced this type of giant slow current but with outward direction (Yuan and Atchison, 2003). Because of cell- to cell-variation, however, some cells (20 - 30%) never exhibited MeHg-induced giant inward current. Thus, it is possible that those cells in which MeHg failed to induce the giant slow inward current at holding potentials more negative than -60 mV might be incapable of generating this response. To rule out of this possibility, we used a protocol that alternatively switched the membrane holding potentials between 0, +20 or +60 mV and -60, -70, -80 or -100 mV during recording in the same individual cell dialyzed with the low (5 mM) [Cl\(^{-}\)] pipette solution. To do this, after establishment of the whole cell recording configuration, cells were first voltage-clamped at 0, +20 or +60 mV while exposing them continuously to a given concentration of MeHg (20 – 100 µM). Once the outwardly-directed giant slow currents appeared, the membrane holding potential was then switched to a potential more negative than -60 mV and maintained at this potential for a period of at least 5 min before it was switched back to 0, +20 or +60 mV. Occasionally cells were held at -100 mV, but typically the recording was very “noisy” when holding potentials more negative than -80 mV were used. As shown in Figure 3 for a representative Purkinje cell, only outward sIPSCs were observed when the cell was held at 0 mV before MeHg exposure. After exposure of the cell to 20 µM MeHg for 28 - 37 min, several giant slow outward currents appeared. At this point, the membrane holding
potential was switched immediately from 0 mV to -70 mV and maintained at -70 mV for 5 min, during which time no giant slow inward current was observed (Figure 3b). After returning the holding potential back to 0 mV at 44 min, however, the outward giant slow currents reappeared at 48 and 49 min, respectively (Figure 3c,d). Repeating this procedure once at 50 (-70 mV) and 56 min (0 mV), respectively, yielded a similar result. In all five cells tested by this method, no giant currents were ever elicited at a holding potential of -70 mV. These data suggest that the inwardly-directed giant currents are due to efflux of intracellular Cl⁻.

The Cl⁻-dependence of this giant slow inward current was verified by holding Purkinje cells at 0 mV, which is near the E_{Cl} predicted by the Nernst equation when the standard ([Cl⁻] = 145 mM) pipette solution was used. We reasoned that if this transient giant slow current is a Cl⁻-mediated response, it should not be observable when the cell is voltage-clamped at a membrane potential close to E_{Cl}. Figure 4 depicts a representative recording from a Purkinje cell under approximately symmetrical [Cl⁻]ᵢ conditions. When the cell was held at -70 mV, typical downward sIPSCs were observed (Control, -70 mV). After switching the holding potential to 0 mV (Control, 0 mV), only some tiny upward sIPSCs could be seen. Under these conditions, exposure of the cell to 20 µM MeHg for 65 min did not induce any giant current. Switching occasionally the holding potential to -70 mV at 51 and 64 min in the presence of MeHg, sIPSCs could still be seen, indicating that the cell was still able to respond at those points during MeHg exposure. In four cells examined at a holding potential of 0 mV under such recording...
conditions, MeHg failed to induce any giant current. Thus, the induction of this giant slow inward current is evidently both voltage- and Cl\(^{-}\)-dependent.

Previously, this giant slow current was shown not to be a GABAergic response because of its insensitivity to block by the GABA\(_{A}\) receptor antagonists bicuculline and picrotoxin (Yuan and Atchison, 2003). However, it is possible that Cl\(^{-}\)-permeable glycine receptor channels play a role in generating this current. To test this, Purkinje cells in cerebellar slices were pretreated with 1 – 100 \(\mu\)M strychnine, a glycine receptor antagonist. In the presence of strychnine, both the frequency and amplitude of sIPSCs were suppressed significantly or disappeared almost completely at higher concentrations (50 – 100 \(\mu\)M) of strychnine, suggesting a possible nonspecific action of strychnine with GABA\(_{A}\) receptors at higher concentrations, or a contribution of glycine receptors to the sIPSCs observed. Nevertheless, MeHg still could induce a giant slow inward current even in the presence of these relatively high concentrations of strychnine. Figure 5 depicts a representative experiment in the presence of 20 \(\mu\)M strychnine. After treatment of this slice with 20 \(\mu\)M strychnine for 5 min, sIPSCs disappeared completely. However, exposure of this cell to 100 \(\mu\)M MeHg induced reappearance of small amplitude sIPSCs (at 4 - 6 min) and onset of the giant slow inward currents at 6, 8 and 10 min, respectively. This slow inward current occurred in 4 of 6 cells tested in the presence of strychnine. Thus, glycine receptors also seem unlikely to play a major role in generation of this giant slow current.
Inasmuch as the current appeared to be mediated by Cl\(^-\) and not to be associated with typical Cl\(^-\) permeable ligand-gated channels, we next tested if voltage-gated Cl\(^-\) channels are responsible for generating this giant slow inward current. Purkinje cells were pretreated with anion channel blockers DIDS or niflumic acid. Consistent with previous reports (Woodward et al., 1994; Raiteri et al., 2001; Sinkkonen et al., 2003), both DIDS and niflumic acid suppressed, and subsequently blocked GABA\(\alpha\) receptor-mediated sIPSCs or mIPSCs in a concentration-dependent manner (data not shown). Surprisingly, pretreatment of cells with 200 – 300 µM DIDS at a holding potential of -60 to -70 mV was not as effective at preventing appearance of MeHg-induced giant slow inward currents. As shown in Figure 6A, in the presence of 300 µM DIDS, the frequency of sIPSCs for this cell recorded at a holding potential of -60 mV was reduced significantly compared with the pre-DIDS treatment control. However, exposure of this cell to 100 µM MeHg still induced the giant slow inward currents at 6, 17, 20 and 22 min, respectively. Four out of nine Purkinje cells pretreated with DIDS at this holding potential expressed the MeHg-induced giant slow inward currents, although the incidence of occurrence was slightly lower than for cells treated with MeHg alone (26 out of 38 cells). To test further if the failure of DIDS in preventing appearance of the giant currents in cells was due to a voltage-dependent effect of DIDS, we did another set of experiments to test if DIDS could prevent appearance of the giant currents in cells held at +60 mV. Because recordings usually became unstable when a given cell was held at a very positive potential for an extended period of time,
most of these experiments were done with 100 µM MeHg to shorten the time course required for completion of such experiments. As shown in Figure 6B, upon switching the holding potential from -70 mV to +60 mV, the downward sIPSCs became upward sIPSCs (Control, +60 mV). After exposure of this cell to 200 µM DIDS for 5 min, all sIPSCs disappeared. Subsequent application of 100 µM MeHg plus 200 µM DIDS for up to 20 min did not induce any giant current. In five cells treated with 100 µM MeHg and three cells treated with 20 µM MeHg under these conditions, no giant currents were ever observed in the presence of DIDS. Thus, it appeared that block of this giant current by DIDS is voltage-dependent. In contrast, as shown in Figure 7, pretreatment of the Purkinje cell with 200 µM niflumic acid, not only blocked sIPSCs completely, but also prevented appearance of MeHg-induced giant inward current, even at a holding potential of -60 mV. In seven cells pretreated with 200 - 300 µM niflumic acid, none exhibited MeHg-induced giant inward currents. Thus, niflumic acid appears to be an effective blocker of this giant slow inward current as well. This further suggests that the giant slow inward current is a response mediated by voltage-dependent Cl⁻ channels. However, this Cl⁻ current could also be mediated by a nonspecific anion channel or receptor. As such, several strategies were used in attempts to confirm unequivocally the Cl⁻ dependence on of the giant currents. Anion substitution experiments in which the Cl⁻ in the external solution was replaced with equimolar I⁻ were attempted to determine if MeHg could also induce an I⁻-mediated giant inward current. However, replacement of Cl⁻ with I⁻ in the ACSF produced an offset current varying from 600 -1000 pA. Because the I⁻ likely interacts with Ag/AgCl silver wire in the
pipette, we attempted to use an agar bridge ground method to reduce the liquid junction potential. However, switching the external solution from regular ACSF to I–-based external solution still caused a continuous drop of baseline which could not be reversed by washing with ACSF, suggesting that I– may interact with either the agar bridge and/or the pipette solution. Consequently, we did not pursue these experiments further. Thus we cannot exclude the possibility that this giant Cl– current is mediated by a nonspecific anion channel.

Are Ca2+-activated Cl– channels responsible for generation of this MeHg-induced slow giant current?

Several types of smooth muscle cells (Hashitani and Edwards, 1999; Toland et al., 2000; Sergeant et al., 2001) express a giant, spontaneous transient depolarizing potential (current-clamp recordings) or inward current (voltage-clamp recordings). This giant spontaneous transient depolarizing potential or inward current was shown to be mediated by Ca2+-activated Cl– channels.

Exposure to MeHg disrupts intracellular Ca2+ homeostasis and causes intracellular Ca2+ concentration ([Ca2+]i) to increase due to extracellular Ca2+ influx or/and intracellular store Ca2+ release in a variety of cell types (Hare et al., 1993; Sarafian, 1993; Denny et al., 1993). It is possible that an increase in [Ca2+]i induces current flow through Ca2+-activated Cl– channels, which in turn generates the giant slow inward currents. If this is true, then perhaps preventing
an increase in \([\text{Ca}^{2+}]_i\) will block or delay induction by MeHg of giant slow inward currents. Thus, to ascertain the \(\text{Ca}^{2+}\)-dependency of this giant slow current, three sets of experiments were designed. First, extracellular \(\text{Ca}^{2+}\) was removed to test if reducing influx of extracellular \(\text{Ca}^{2+}\) could prevent or delay onset of MeHg-induced giant slow inward current. As shown in Figure 8A, in the low \([\text{Ca}^{2+}]\) bath solution, exposure of this cell to 20 \(\mu\text{M}\) MeHg still induced a giant inward current at 35 min and subsequent time points (not all are shown in Figure 8A). In four cells tested using this protocol, all showed induction by MeHg of giant slow inward currents. Perhaps reducing \(\text{Ca}^{2+}\) influx alone is not sufficient to prevent a MeHg-induced increase in \([\text{Ca}^{2+}]_i\) because MeHg also induces release of \(\text{Ca}^{2+}\) from intracellular stores (Hare and Atchison, 1995; Limke and Atchison, 2002; Limke et al., 2003). Thus, the next set of experiments involved dialyzing cells with an intracellular solution containing 10 mM BAPTA, a \(\text{Ca}^{2+}\) chelator, to attempt to prevent a MeHg-induced increase in \([\text{Ca}^{2+}]_i\). However, this treatment was also ineffective at preventing or significantly delaying appearance of the MeHg-induced giant inward current. Seven out of eleven cells dialyzed with BAPTA-containing pipette solution still exhibited giant slow inward currents. The mean times to onset of this giant slow current induced by 100 \(\mu\text{M}\) and 20 \(\mu\text{M}\) MeHg are 12.5 ± 1.7 (n = 4) and 26.7 ± 6.0 min (n = 3), respectively. In the Purkinje cell shown in Figure 8B, after dialysis with the BAPTA-containing pipette solution, giant slow inward currents appeared at 30, 35, 40, 43 and 58 min after exposure to 20 \(\mu\text{M}\) MeHg, respectively (only one trace at 43 min is shown in Figure 8B). In considering whether the continuous accumulation of intracellular \(\text{Ca}^{2+}\) might eventually deplete the BAPTA,
in some recordings with BAPTA-containing pipette solution, BAPTA/AM, a membrane permeable form of BAPTA, was added to the bath solution to reinforce the Ca$^{2+}$ buffering capacity of pipette-applied BAPTA. As shown in Figure 8C, after establishing the whole cell recording configuration and dialyzing this Purkinje cell with 10 mM BAPTA-containing pipette solution, the cell was incubated with ACSF containing 50 µM BAPTA/AM for another 30 min. The cell was then exposed to 20 µM MeHg plus 50 µM BAPTA/AM. At 22 min after MeHg exposure, the giant slow inward currents remained inducible. In six cells treated with this combination, four cells exhibited giant inward currents. Also, the mean time to onset of this MeHg-induced giant inward current was 28.4 ± 4.5 min (n = 4) at 20 µM MeHg. This was not significantly different from that (Table 1) observed in recordings in the absence of BAPTA treatments (p > 0.05, Student t test). Thus, these data suggest that BAPTA treatment alone is also ineffective at preventing the MeHg-induced giant inward current. Subsequently, a combination of reduced extracellular [Ca$^{2+}$] and intracellular dialysis of the cell with BAPTA-containing pipette solution was used to test if MeHg exposure was still able to induce giant slow inward current. As shown in Figure 8D, the combined treatment of this cell with low [Ca$^{2+}$]$_{\text{e}}$ bath solution and BAPTA-containing pipette solution also failed to prevent MeHg-induced giant inward current. Additional experiments in which slices were pretreated with 5 - 100 µM cyclopiazonic acid (an inhibitor of Ca$^{2+}$-ATPase), 5 mM caffeine (an inducer of release of Ca$^{2+}$ from intracellular store), or 100 µM ruthenium red (an inhibitor of uptake of Ca$^{2+}$ by mitochondria and inducer of release of Ca$^{2+}$ from mitochondria, respectively) all failed to
prevent onset of this slow inward current (data not shown). Thus, evidence to date does not support the hypothesis that the MeHg-induced giant slow inward current is a Ca\textsuperscript{2+}-activated or Ca\textsuperscript{2+}-dependent response.

Figure 9 summarizes and compares the incidence of occurrence (number of positive cells) of the giant slow inward current after different treatments. Clearly, pretreatment of cells with Gd\textsuperscript{3+}, TPEN, strychnine or BAPTA before MeHg exposure did not significantly alter the incidence of occurrence of MeHg-induced giant slow inward current in cerebellar Purkinje cells. In contrast, both anion channel blockers, niflumic acid and DIDS, effectively blocked appearance of this giant current.
DISCUSSION

The primary goal of the present study was to follow up at the mechanistic level on our previous observations and attempt to identify the ionic origin of the transient giant slow inward current induced by MeHg in Purkinje cells in cerebellar slices. Consistent with our previous findings (Yuan and Atchison, 2003), acute bath application of 10 – 100 MeHg induced a transient, giant slow inward current in cerebellar Purkinje cells in a concentration- and time-dependent manner. This giant slow inward current was insensitive to TTX, to the nonspecific cation channel blocker Gd$^{3+}$ and the Zn$^{2+}$ chelator TPEN. It was not inducible when cells were held at a holding potential near the equilibrium potentials for Cl$^-$ under either low [Cl$^-$]$_i$ or symmetrical [Cl$^-$] conditions. The glycine receptor antagonist strychnine, like the GABA$\alpha$ receptor antagonists tested in our previous study (Yuan and Atchison, 2003), was ineffective at preventing appearance of this MeHg-induced giant current. This giant slow inward current was sensitive to the Cl$^-$ channel blocker niflumic acid and DIDS in a voltage-dependent manner. Reducing [Ca$^{2+}$]$_e$ or intracellular dialysis of cells with the Ca$^{2+}$ chelator BAPTA failed to prevent induction by MeHg of this giant slow inward current.

In dorsal root ganglion and cerebellar granule cells in culture, MeHg induced a slowly, but continuously developing inward current (Arakawa et al., 1991; Xu and Atchison, 1998). This steady-state inward current also occurred in cerebellar slices, and was independent of the
transient currents described in this study. It was thought to be a nonspecific cation-mediated response. Clearly, the time course and kinetics of the MeHg-induced transient giant slow inward currents observed in cerebellar Purkinje cells in the present studies differ from those of the slowly developing steady-state inward current in cells in culture. This may imply that the transient giant slow inward current is mediated by a different ion. Failure of Gd³⁺ in preventing induction by MeHg of the transient giant inward current suggests further that this response is not mediated by nonspecific cation channels. Although Zn²⁺ has been shown to induce a spontaneous giant depolarizing potential in CA1 and CA3 hippocampal neurons (Xie and Smart, 1991, 1993), MeHg-induced increase in intracellular Zn²⁺ does not appear to be responsible for generation of this giant slow current in cerebellar Purkinje cells, because pretreatment of cells with the Zn²⁺ chelator TPEN failed to prevent appearance of the giant inward currents.

In smooth muscles cells from a number of preparations (Hashitani and Edwards, 1999; Toland et al., 2000), giant spontaneous transient depolarizing potentials or inward currents (STICs) are mediated by Cl⁻. Thus, we hypothesized that this MeHg-induced giant inward current in Purkinje cells was also mediated by anion(s). Consistent with this notion are our previous findings that reducing [Cl⁻], and holding the membrane potentials at -60 to -100 mV eliminated the transient, giant slow inward current (the holding potentials of -60 to -100 mV are close to E_Cl under these conditions), whereas when the membrane potentials were held at 0 to +60 mV, the outwardly-directed, giant slow currents remained inducible (Yuan and Atchison, 2003). These
data suggest that the transient giant inward current is mediated by Cl\(^-\). However, because of variation among individual cells, certain cells never expressed this type of giant inward current after MeHg exposure. In those recordings, any given individual cell was held at only a single given membrane potential - either at a potential more negative than -60 mV or at a potential more positive than 0 mV. Hence, it was still possible, although unlikely, that the cells held at potentials more negative than -60 mV happened to be those cells which were incapable of generating the giant slow inward currents. To rule out this possibility and to verify further that this giant current is a Cl\(^-\)-mediated response, we carried out two sets of experiments with different strategies in the present studies. In the first set of experiments, similar standard extracellular and low [Cl\(^-\)]\(_i\) (Cl\(^-\) was replaced with equimolar membrane-impermeable cesium gluconate) intracellular solutions were used as described previously (Yuan and Atchison, 2003). However, a different protocol was adopted in which we switched the membrane holding potentials in any given cell alternatively between 0, +20 or +60 mV and -60, -70, -80 or -100 mV. The purpose of this design was to ensure that the cell under testing was capable of generating the transient giant current by first holding the membrane potential at values equal to, or more positive than, 0 mV. Once outward giant currents were observed, the holding potential was then immediately switched to -60 mV or more negative and was maintained at this potential for at least 5 min to allow sufficient time to generate the giant current. To assure that lack of giant inward currents at the hyperpolarized membrane potentials was not due to complete block by MeHg of all responses, the same procedure was repeated at least twice in the same individual
cell. In none of the five cells tested, was a giant inward current ever elicited at holding potentials more negative than -60 mV, although the outward giant currents were seen routinely at holding potentials of 0, +20 and +60 mV. Thus clearly, the lack of any giant inward current at a holding potential more negative then -60 mV when using a low [Cl\(^{-}\)]\(_{i}\) solution was not due to cell variation, but due to reduced [Cl\(^{-}\)]\(_{i}\) and altered driving force for Cl\(^{-}\). This was supported further by the second set of experiments, which took advantage of the principle that at a holding potential near \(E_{\text{Cl}}\), no net Cl\(^{-}\)-mediated currents should be observed. In these experiments, cells were voltage-clamped at 0 mV which was close to \(E_{\text{Cl}}\) under the symmetrical [Cl\(^{-}\)] conditions that we used. As expected, no giant slow inward currents were observed in any of the four cells tested, implying that this giant current is a Cl\(^{-}\)-mediated response.

Among Cl\(^{-}\)-mediated currents, there are several obvious receptor-operated Cl\(^{-}\) permeable channels that could conceivably carry this giant current. As our previous study in which we discovered this current, was designed to examine effects of MeHg on GABAergic synaptic function we demonstrated that the GABA\(_{A}\) receptor did not appear to be responsible for generating this giant current (Yuan and Atchison, 2003). In the present study, failure of the glycine receptor antagonist strychnine over a wide range of concentrations to prevent appearance of MeHg-induced giant inward current also ruled out the likely involvement of glycine receptors in generating this giant inward current.
The next most likely candidates responsible for generation of this giant inward current are voltage- or Ca\(^{2+}\)-activated Cl\(^{-}\) channels. Spontaneous transient depolarizing potentials or inward currents in smooth muscle or interstitial cells are the result of release of Ca\(^{2+}\) from intracellular stores and subsequent activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (Sergeant et al., 2001; Karkanis et al., 2003; Craven et al., 2004). Elevation of [Ca\(^{2+}\)]\(_i\) is seen with MeHg in many types of neurons including cerebellar granule and Purkinje cells (Marty and Atchison, 1997; Limke et al., 2003; Edwards et al., 2005). Increased [Ca\(^{2+}\)]\(_i\) could, in turn, activate Ca\(^{2+}\)-dependent Cl\(^{-}\) channels to generate this giant current. In fura-2 microfluorometric studies of [Ca\(^{2+}\)]\(_i\), MeHg requires time to induce increases in fura-2 fluorescence. This latent period is inversely related to the concentration of MeHg used (Hare et al., 1993; Marty and Atchison, 1997; Limke et al., 2003). Thus, onset of this giant current at the late stage of MeHg exposure might be related to the time required to build-up [Ca\(^{2+}\)]\(_i\). Consistent with this prediction is the observation that application of niflumic acid, a voltage-dependent and Ca\(^{2+}\)-activated Cl\(^{-}\) channel blocker, effectively prevented induction by MeHg of the giant inward currents. DIDS, another voltage-dependent and Ca\(^{2+}\)-activated Cl\(^{-}\) channel blocker was unable to block appearance of the MeHg-induced giant currents in cells held at a negative potential (-60 to -70 mV), but appeared to be an effective blocker of this current when cells were held at a more positive holding potential. Thus, these data again suggest that this giant current appears to be mediated by a voltage-dependent Cl\(^{-}\) channel. However, neither reducing extracellular Ca\(^{2+}\), increasing intracellular Ca\(^{2+}\) buffering capacity, nor a combination of the two effectively blocked or significantly
delayed onset of induction by MeHg of the giant inward current in Purkinje cells under our present experimental conditions. Thus, these data do not appear to be consistent with the notion that the MeHg-induced giant inward current is mediated by Ca\(^{2+}\)-activated Cl\(^-\) channels. Perhaps, this MeHg-induced spontaneous giant slow current in Purkinje cells is mediated by a type of Cl\(^-\) channel that differs from those in smooth muscle cells. In fact, unlike the spontaneous depolarizing potentials or inward currents in smooth muscle and interstitial cells, which occurred with a regular rhythm and amplitude, the MeHg-induced giant spontaneous responses in Purkinje cells occurred randomly and with an irregular rhythm and variable amplitudes. The differences between these two types of spontaneous events suggest that they are probably generated by different mechanisms. However, we still cannot eliminate conclusively the possibility that the ineffectiveness at preventing appearance of this giant current in experiments using BAPTA to buffer [Ca\(^{2+}\)]\(_i\) changes is due simply to insufficient loading of BAPTA, particularly in the remote dendritic regions of Purkinje cells, to buffer against MeHg-induced changes in [Ca\(^{2+}\)]\(_i\). Similarly, we cannot exclude the possibility that this current is a nonspecific anion channel.

In immature rat hippocampal CA3 neurons, spontaneous giant depolarizing potentials are network driven synaptic events and mediated by synaptic release of GABA and presynaptically controlled by NMDA receptors (Ben-Ari et al., 1989). It is not yet clear whether this giant slow current is pre- or postsynaptically generated in Purkinje cells, although it is independent of
presynaptic action potential generation. However, the MeHg-induced giant spontaneous response in Purkinje cells differs from that in hippocampal CA3 neurons reported by Ben-Ari et al. (1989), because the spontaneous current in Purkinje cells is insensitive to both GABA\textsubscript{A} and glutamate (including NMDA) receptor antagonists. As this giant current displayed a relatively slow time-course, it might be mediated by some unidentified substance such as a neuropeptide. These substances could be released from presynaptic fibers upon MeHg exposure and act at the postsynaptic neurons to induce this slow giant current via a second message pathway. Further studies in acutely isolated or primary cultures of Purkinje cells should be helpful in identifying whether generation of this slow giant current is a pre- or postsynaptic event.

Large spontaneous depolarizing potentials or inward currents in immature hippocampal CA1 and CA3 neurons, as well as some types of smooth muscle cells, are thought to be related to pacemaker or tonic activity (Sergeant et al., 2001; Karkanis et al., 2003; Craven et al., 2004). As this giant slow inward current has not been described previously, nothing is known about its function in cerebellar Purkinje cells. These cells are pacemaker neurons, which display an intrinsic tonic, spontaneous firing or bursting activity. Because of the nature of spontaneous occurrence of this giant inward current, it could be related to synchronized bursting firing activity of cerebellar Purkinje cells. In fact, we have previously shown that MeHg exposure changed the pattern of repetitive firing of Purkinje cells in cerebellar slices (Yuan and Atchison, 1999). However, insensitivity of this giant spontaneous current to TTX and BAPTA treatments
distiguishes it from the intrinsic tonic, rhythmic spontaneous firing and complex Ca^{2+} spike activity in Purkinje cells. Moreover, because the giant current typically occurred at the later stages of MeHg exposure, it is possible that appearance of this giant current is an indicator of loss of membrane integrity or changes in membrane ionic permeability. MeHg causes membrane depolarization in hippocampal CA1 neurons (Yuan and Atchison, 1995) and cerebellar Purkinje cells (Yuan and Atchison, 1999), and the time to onset of the giant current appears to be consistent with the time-course of MeHg-induced membrane depolarization. Thus, this giant current could be a voltage-dependent response that senses and regulates membrane potential changes by modification of \([\text{Cl}^{-}]_i\) concentration via compensatory mechanism. Under experimental symmetric \([\text{Cl}^{-}]\) conditions, activation of GABA_A receptors at a holding potential of -60 or -70 mV will cause efflux of Cl^-. Inhibition by MeHg of GABA_A receptors will reduce efflux of Cl^- and possibly result in accumulation of Cl^- within the cells. The increased \([\text{Cl}^{-}]_i\), may in turn, somehow activate a voltage- and Cl^- dependent response that attempts to regulate or relieve the build-up of \([\text{Cl}^{-}]_i\). One possible set of candidates for doing this are the cation-Cl^- co-transporters. The K^+-Cl co-transporter (KCC2) plays an important role in extruding both K^+ and Cl^- from cells (Rivera et al., 1999; Delpire, 2000). Accumulation of \([\text{Cl}^{-}]_i\) induced by MeHg could activate KCC2 to extrude Cl^- from the cells. Under normal physiological conditions, the \([\text{Cl}^{-}]_i\) concentration of neurons in the brain is lower than that outside. Thus, activation of GABA_A receptors normally causes influx of Cl^- into the cells, resulting in hyperpolarization. Once GABA_A receptor-mediated responses are inhibited by
MeHg, influx of Cl⁻ into the cells via GABA_A receptors should be reduced. In this case, theoretically, [Cl⁻]_i concentration should be reduced rather than increased. If the giant inward current is a compensatory response which somehow resulted from regulation of [Cl⁻]_i under the symmetric experimental conditions, then this mechanism should be also able to regulate the reduced [Cl⁻]_i under physiological [Cl⁻]_i conditions. Consequently, a MeHg-induced giant outward current should be observed under physiological [Cl⁻]_i conditions. In fact, even under symmetric [Cl⁻]_i conditions, holding the cell membrane at more positive potentials could still induce an outward giant current, suggesting that a sudden influx of Cl⁻ into the cells occurred. Thus, this mechanism appears to allow flow of Cl⁻ across membrane in both directions depending on the net driving force for Cl⁻ across the membrane. In ether case, the key factor is that changes occur in [Cl⁻]_i. Thus, further experiments to monitor [Cl⁻]_i changes with a Cl⁻ -sensitive dye or using gramicidin-perforated patch-clamp recordings will be required to decipher these possibilities.

Although onset of the giant current appears to be related to MeHg exposure, it does not necessarily mean that generation of this giant current is a MeHg-induced specific effect. Instead, this response could result from a secondary effect of MeHg on other cellular systems. Spontaneous slow inward currents were observed in the present study in two Purkinje cells (separate slices) which were exposed accidently and briefly to hypoxic or ischemic insults (accidental termination of oxygenization or discontinued perfusion of oxygenized ACSF). Thus
perhaps MeHg induced a hypoxic or ischemic response which causes these currents. It has been shown that *in vitro* ischemia induced by oxygen-glucose deprivation of adult hippocampal slices also caused the accumulation of [Cl\(^{-}\)]\(_i\) (Galeffi et al., 2004). If the MeHg-induced giant inward current was due to accumulation of [Cl\(^{-}\)]\(_i\) and subsequent sudden efflux of Cl\(^{-}\) from cells, theoretically, the ischemia-induced accumulation of intracellular Cl\(^{-}\) should be also able to generate this type of slow giant inward current. This might indicate why slow giant inward currents were also seen in the two slices which were accidently exposed to ischemic insults. Thus, it is possible that generation of this current in cells treated with MeHg may be related to a secondary effect of MeHg-induced changes in mitochondrial energy metabolism (such as ATP depletion). However, to the best of our knowledge, no report has yet shown that ischemia can induce a similar type of spontaneous giant inward current. Even if this current is indeed a secondary response due to MeHg-induced ischemic effects, MeHg is probably not a good candidate for a tool to study this response, due to the infrequency with which the response occurs and the fact that the current is superimposed on numerous other cellular effects of MeHg.

Compared with cerebellar granule cells, pathologically, Purkinje cells are much more resistant to MeHg, and thus they may be not the primary target of MeHg neurotoxicity *in vivo*. Pathological damage to Purkinje cells following MeHg neurotoxicity *in vivo* is much less than granule cells. However, cerebellar based ataxia is a primary effect of MeHg neurotoxicity. Purkinje cells
receive synaptic inputs from both climbing fibers and parallel fibers (axons of granule cells) and the axons of Purkinje cells are the only output from the cerebellar cortex. Thus any cerebellar cortical neuronal (including granule cells) dysfunction must be ultimately expressed in or through Purkinje cells. This is particularly true for synaptic transmission between the parallel fibers and Purkinje cells. Giant slow inward currents were most frequently observed in Purkinje cells. Perhaps this current is a compensatory response that is related to the relative resistance of Purkinje cells to MeHg. There are no reports of studies of effects of MeHg on Purkinje cell membrane function in cells in culture, so whether or not this effect is a general response of Purkinje cells to MeHg is unclear.

Direct comparison of the effective concentrations of MeHg used for experiments in cell culture (Marty and Atchison, 1997; Sirois and Atchison, 2000; Edwards et al., 2005) and those used for experiments in brain slices is difficult. Higher concentrations of MeHg are required for the brain slice experiments. There are several reasons for this. First, there are a large number of nonspecific binding sites provided by the slice tissue as compared to cells in low density monolayer culture. Second, typically the cells under recording in brain slices are not those on the surface of tissues, but in the subsurface of tissues providing a greater diffusion barrier to MeHg. Third, the presence of support cells in the slice provides a better or more integrated buffering or protective system against actions of MeHg than for neurons in monolayer cultures for which astrocytes have been removed.
The slowly developing steady-state inward current, shown as a continuous baseline drop, is observed in not only cerebellar Purkinje cells, but also granule cells (Yuan and Atchison, 2003) and in cultures of dorsal root ganglion cells exposed to MeHg (Arakawa et al., 1991). This current is apparently related to MeHg-induced cell membrane depolarization. In contrast, the giant transient slow inward current was not observed in every cell examined. The giant transient inward currents were often superimposed on the continuously developing steady-state inward current. Thus the events appear to be extinct.

In conclusion, the MeHg-induced transient giant inward current appears to be a Cl–-mediated response. Evidence presented in this study suggests that this response is mediated by a voltage-dependent Cl– channel. However, whether or not MeHg-induced increase in [Ca2+]i plays a role in its generation through Ca2+-activated Cl– channels or this current is mediated by a nonspecific anion channel remains in question, as does the role which this giant current plays, if any, in MeHg-mediated toxicity.
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FOOTNOTES

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2 Address all correspondence including reprint requests to: Dr. Bill Atchison, Department of Pharmacology & Toxicology, Michigan State University, B-331 Life Sciences Bldg., East Lansing MI, 48824-1317, or by email to atchiso1@msu.edu
Figure 1. MeHg-induced a biphasic effect on amplitude and frequency of mIPSCs in cerebellar Purkinje cells in slice. A, time course of effects of 10 µM MeHg on spontaneous currents recorded from a representative Purkinje cell in a slice at a holding potential (HP) of -60 mV. Note: traces were sampled before and after MeHg exposure at 10 min intervals and are shown over a 2 min period. All recordings were made in the presence of 10 µM CNQX, 100 µM APV and 0.5 µM TTX in the external solution to block glutamatergic synaptic responses and presynaptic action potential firing. The lower case letters (a – h) indicate specific changes in spontaneous events before and during MeHg exposure. B, the same sampling points in A are shown on an expanded time scale: a, control; d, e and f, MeHg-induced giant slow inward currents; b and c, MeHg-induced initial peak increase in amplitude and frequency of mIPSCs; g, spontaneous repetitive firing of Purkinje cell; h, complete block of whole cell currents. C, times to onset of peak increases in amplitude and frequency of mIPSCs and appearance of giant inward currents were MeHg concentration-dependent. Data were collected before (control) and at different time points after exposure to 100 (a), 20 (b) and 10 mM MeHg (c). Each trace is a representative depiction of 4 – 17 individual experiments.

Figure 2. Neither nonspecific cation channels nor Zn\(^{2+}\) is responsible for generation of the MeHg-induced giant current. mIPSCs were recorded from two slices at a HP of -60 mV in the
presence of 10 µM CNQX, 100 µM APV and 0.5 µM TTX, respectively. Cells were first
treated with Gd³⁺ (100 mM, Left) or TPEN (40 mM, Right) for 10 – 15 min and then 20 µM
MeHg plus Gd³⁺ (100 mM) or TPEN (40 mM) until all events were blocked. Pretreatment of
Purkinje cells with Gd³⁺ (100 mM, Left) or TPEN (40 mM, Right) failed to block induction of
the giant slow inward currents. (Right panel inset shows the giant current can be reversed in
direction when the HP was switched from -60 mV to +60 mV). Each trace is a representative
depiction of 5 – 9 individual Purkinje cells which exhibited giant inward currents.

**Figure 3.** Appearance of the MeHg-induced giant slow inward current is voltage- and [Cl⁻]-
dependent. A, A continuous recording of time course of effects of 100 µM MeHg on
spontaneous events recorded from a representative Purkinje cell at HPs switching alternatively
between 0 mV and -70 mV with a low [Cl⁻]-containing pipette solution (140 Cs gluconate).
Note: the arrowhead indicates the starting point of MeHg exposure. The lower case letters (a – j)
indicate specific noteworthy changes in spontaneous events during MeHg exposure. The cell
was first held at 0 mV until several giant outward currents appeared at 28 (G₂₈) and 37 min
(G₃₇ₐ, G₃₇ₖ and G₃₇ₑ) during MeHg exposure. Then the cell was held at a potential of -70 mV for
5 min starting at 38 min (e). No giant inward current was seen during this period of time. At 43
min, the HP was switched back to 0 mV (a one min interval was given after HP switching to
allow time for baseline stabilization). At this HP, giant outward currents appeared again within
5 min (G₄₈ and G₄₉). These procedures were repeated once at 50 min (-70 mV) and 56 min (+20
mV), respectively. No giant inward current was seen at -70 mV, whereas giant outward current remained observable at 56 min (G56) until all events disappeared. Each trace is a representative depiction of 5 individual experiments. B, Portions of the traces from the same sampling points indicated in A are shown on an expanded time scale: a, control at a holding potential of 0 mV; b, MeHg-induced changes in amplitude and frequency of spontaneous outward currents at a HP of 0 mV; c, d, f and g, MeHg-induced giant slow outward currents at 28, 37, 48, 49 and 56 min, respectively, during MeHg exposure (HP = 0 mV); e and h, representative traces recorded at HPs of -70 mV; j, complete block of all spontaneous events.

Figure 4. No MeHg-induced giant current was seen at a membrane potential close to the equilibrium potential for Cl⁻ (ECl⁻). sIPSCs were recorded from a representative Purkinje cell first at a HP of -70 mV under approximately symmetric [Cl⁻] conditions (using the 145 mM [Cl⁻] standard pipette solution). The cell was then held at 0 mV, which is close to ECl⁻ under symmetric [Cl⁻] conditions. Data were collected consecutively before {Control (0 mV)} and during the course of exposure to 100 µM MeHg. At HP = 0 mV, only tiny upward sIPSCs could be seen, but no giant events were ever observed during exposure to MeHg. After switching the HP back to -70 mV at 51 and 64 min, respectively, the downward sIPSCs were still observable, although their frequency and amplitude were reduced significantly, suggesting that the cell was still viable at those specific time points. Each trace is a representative depiction of 4 individual experiments.
Figure 5. The MeHg-induced giant slow inward current is insensitive to the glycine receptor antagonist strychnine. sIPSCs were recorded from a representative Purkinje cell in a cerebellar slice at a holding potential of -60 mV in the presence of 10 mM CNQX and 100 mM APV. Pretreatment of this Purkinje cell with 20 µM strychnine completely blocked sIPSCs. However, application of 100 µM MeHg plus 20 µM strychnine still induced giant inward currents at 8 and 10 min, respectively. Each trace is a representative depiction of 7 individual experiments.

Figure 6. Sensitivity of MeHg-induced giant currents the to Cl⁻ channel blocker DIDS is voltage-dependent. A. sIPSCs in this cell were recorded using the standard pipette solution in the presence of 10 µM CNQX and 100 µM APV at a HP of -60 mV. After treatment of the cell with 300 µM DIDS for 5 min, the frequency of sIPSCs was reduced significantly. Subsequent exposure of this cell to 100 µM MeHg plus 300 µM DIDS still induced giant inward currents at 6, 17, 20 and 22 min (data not shown for 22 min), respectively. B. In another Purkinje cell, sIPSCs were recorded first at a HP of -70 mV and then at +60 mV. Pretreatment of this cell with 200 µM niflumic acid not only blocked sIPSCs completely, but also effectively blocked appearance of the MeHg-induced giant inward current. Each trace is a representative depiction of 8 - 9 individual experiments.

Figure 7. MeHg-induced giant currents are also sensitive to block by niflumic acid (NA),
another blocker of voltage-sensitive and Ca\(^{2+}\)-activated Cl\(^{-}\) channels. sIPSCs were recorded using the standard pipette solution from a representative Purkinje cell in the presence of 10 \(\mu\)M CNQX and 100 \(\mu\)M APV at a holding potential of -60 mV. In the presence of 200 \(\mu\)M NA, sIPSCs virtually disappeared completely. With subsequent exposure of this cell to 100 \(\mu\)M MeHg plus 200 \(\mu\)M NA for up to 20 min, no giant inward current was ever observed. Each trace is a representative depiction of 8 individual experiments.

**Figure 8.** Generation of MeHg-induced giant current appears to be Ca\(^{2+}\)-independent. A. mIPSCs were recorded from a Purkinje cell in a cerebellar slice in the presence of 10 \(\mu\)M CNQX, 100 \(\mu\)M APV, 0.5 \(\mu\)M TTX and absence of Ca\(^{2+}\) in the external solution. The slice was pre-incubated with Ca\(^{2+}\)-free ACSF for 10 min (Control), and then exposed to 20 \(\mu\)M MeHg in Ca\(^{2+}\)-free ACSF solution. Note that without Ca\(^{2+}\)\(_{e}\), the membrane became very “noisy”. MeHg exposure still induced a giant inward current at 35 min. B. After dialysis of this Purkinje cell in a slice with pipette solution containing 10 mM BAPTA, mIPSCs were recorded in the presence of 10 \(\mu\)M CNQX, 100 \(\mu\)M APV, and 0.5 \(\mu\)M TTX in the regular ACSF solution (Control). Exposure to 20 \(\mu\)M MeHg again induced a giant inward current at 43 min. C. sIPSCs were recorded from this cell after intracellular dialysis with 10 mM BAPTA-containing pipette solution and incubation with 50 \(\mu\)M BAPTA/AM in the regular ACSF for 30 min prior to exposure to 100 \(\mu\)M MeHg plus 50 \(\mu\)M BAPTA/AM (Control). At 22 min, MeHg exposure still induced a giant inward current. D. This Purkinje cell was first dialyzed with a 10 mM BAPTA-
containing pipette solution and incubated in Ca\(^{2+}\)-free ACSF for 10 min and then exposed to 20 \(\mu\)M MeHg in Ca\(^{2+}\)-free ACSF until all events disappeared. The combination of intracellular dialysis of cell with 10 mM BAPTA-containing pipette solution and extracellular Ca\(^{2+}\)-free ACSF still failed to block induction by MeHg of giant inward current at 44 min. Each trace is a representative depiction of 3 - 8 individual experiments.

**Figure 9.** Comparison of the incidence of cells which expressed MeHg-induced giant slow inward current after different pretreatments. MeHg-cells were exposed only to 10, 20 or 100 \(\mu\)M MeHg; Gd\(^{3+}\)-cells were pretreated with 100 \(\mu\)M Gd\(^{3+}\) for 10 - 15 min before exposure to MeHg plus Gd\(^{3+}\); TPEN-cells were first treated with 40 \(\mu\)M TPEN and then MeHg plus TPEN; STR-cells were pretreated with 1 - 100 \(\mu\)M strychnine and then MeHg plus strychnine; BAPTA-cells were dialyzed either with 10 mM BAPTA-containing internal solution alone or a combination with continuous extracellular application of BAPTA/AM before MeHg exposure. The two sets of data were pooled as BAPTA; DIDS-cells were pretreated with 200 - 300 \(\mu\)M DIDS at a holding potential of +60 mV before subsequent exposure to MeHg plus DIDS; NA, cells were first treated with 200 - 300 \(\mu\)M niflumic acid and then 20 or 100 \(\mu\)M MeHg plus niflumic acid. Control cells were treated with normal ACSF for 90 min. The numbers in parenthesis are the number of cells which expressed the MeHg-induced giant inward currents out of all cells tested.
Table 1. MeHg concentration-dependent onset of initial maximum increase in frequency and amplitude of mIPSCs, appearance of the giant current and block of mIPSCs in Purkinje cells in cerebellar slices.

<table>
<thead>
<tr>
<th>MeHg Concentration (µM)</th>
<th>Time to Peak Frequency of mIPSCs&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>Time to Peak Amplitude of mIPSCs&lt;sup&gt;a&lt;/sup&gt; (min)</th>
<th>Time to Onset of Giant Current&lt;sup&gt;b&lt;/sup&gt; (min)</th>
<th>Time to Complete Block of mIPSCs&lt;sup&gt;c&lt;/sup&gt; (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0- control</td>
<td>n/a (6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>n/a (6)</td>
<td>none (6)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>n/a (6)</td>
</tr>
<tr>
<td>10</td>
<td>46.7 ± 13.3 (4)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>28.3 ± 13.6 (4)</td>
<td>48.7 ± 14.9 (3)</td>
<td>87.5 ± 13.9 (4)</td>
</tr>
<tr>
<td>20</td>
<td>23.5 ± 2.4 (17)*</td>
<td>13.8 ± 2.9 (17)</td>
<td>29.5 ± 2.6 (14)</td>
<td>64.6 ± 2.8 (17)</td>
</tr>
<tr>
<td>100</td>
<td>4.3 ± 0.8 (17)**</td>
<td>4.4 ± 1.3 (17)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>10.0 ± 1.2 (9)**</td>
<td>18.0 ± 1.1 (17)**</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean time to MeHg-induced initial maximum increase in the frequency or amplitude of mIPSCs.

<sup>b</sup> Time to appearance of the first MeHg-induced giant current.

<sup>c</sup> Time to complete block of mIPSCs.

<sup>d</sup> n/a, not applicable.

<sup>e</sup> In six cells recorded for 60 - 90 min in the absence of MeHg, none showed a giant inward current.

<sup>f</sup> All values are mean ± SEM (n).

<sup>g</sup> Significant difference between 10 and 20 µM MeHg (p < 0.05, ANOVA and Tukey-Kramer post hoc comparison).
** Significant difference between 10 or 20 and 100 µM MeHg (p < 0.05, ANOVA and Tukey-Kramer post hoc comparison).

# Significant difference only between 10 and 100 µM MeHg (p < 0.05, ANOVA and Tukey-Kramer post hoc comparison).
**Figure 2**

100 µM Gd$^{3+}$

- Control
- Gd$^{3+}$
- MeHg–Gd$^{3+}$ 20 minutes
- MeHg–Gd$^{3+}$ 35 minutes
- MeHg–Gd$^{3+}$ 45 minutes
- MeHg–Gd$^{3+}$ 50 minutes
- MeHg+Gd$^{3+}$ 55 minutes
- MeHg+Gd$^{3+}$ 75 minutes

40 µM TPEN

- Control
- TPEN
- MeHg+TPEN 15 minutes
- MeHg+TPEN 23 minutes
- MeHg–TPEN 32 minutes
- MeHg–TPEN 55 minutes
- MeHg+TPEN 57 minutes
- MeHg+TPEN 70 minutes
- MeHg+TPEN 85 minutes

200 pA

1 s
Figure 4

Control (-70 mV)

MeHg 5 min (0 mV)

MeHg 10 min (0 mV)

MeHg 15 min (0 mV)

MeHg 20 min (0 mV)

MeHg 25 min (0 mV)

MeHg 30 min (0 mV)

MeHg 35 min (0 mV)

MeHg 40 min (0 mV)

MeHg 45 min (0 mV)

MeHg 50 min (0 mV)

MeHg 51 min (-70 mV)

MeHg 55 min (0 mV)

MeHg 60 min (0 mV)

MeHg 64 min (-70 mV)

MeHg 65 min (-70 mV)
Figure 5

Control

20 μM Strichnine 5 min

MeHg – Strychnine 4 min

MeHg + Strychnine 6 min

MeHg + Strychnine 8 min

MeHg + Strychnine 10 min

MeHg – Strychnine 12 min

MeHg + Strychnine 16 min

400 pA

1 s
Figure 6

A

Control

300 μM DIDS

MeHg–DIDS 2 min

MeHg+DIDS 5 min

MeHg+DIDS 6 min

MeHg–DIDS 17 min

MeHg–DIDS 20 min

B

Control (-70 mV)

Control (-60 mV)

DIDS 5 min (+60 mV)

MeHg 5 min (-60 mV)

MeHg 8 min (-60 mV)

MeHg 10 min (+60 mV)

MeHg 12 min (+60 mV)

MeHg 15 min (+60 mV)

MeHg 20 min (-60 mV)
Figure 7

Control

MeHg + NA 2 min

MeHg - NA 6 min

MeHg - NA 10 min

MeHg + NA 15 min

200 μM NA

MeHg + NA 4 min

MeHg + NA 8 min

MeHg - NA 12 min

MeHg + NA 20 min

200 pA

1 s
Figure 8

A

Control

MeHg 35 min

B

Control

MeHg 43 min

C

Control

MeHg 22 min

D

Control

MeHg 42 min
Figure 9

The diagram shows the incidence rate (%) for different treatments. The treatments include MeHg, Gd\(^{3+}\), TPEN, STR, BAPTA, DIDS, NA, and Control. The incidence rates are represented by bars, with the number of positive cases out of the total number indicated for each treatment. The treatments are ordered from left to right: MeHg, Gd\(^{3+}\), TPEN, STR, BAPTA, DIDS, NA, and Control. The incidence rates are: MeHg (26/38), Gd\(^{3+}\) (5/6), TPEN (9/11), STR (4/6), BAPTA (11/14), DIDS (0/8), NA (0/7), and Control (0/6).