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METHYLMERCURY INDUCES AN INITIAL INCREASE IN GABA-EVOKED CURRENTS IN *XENOPUS* OOCYTES EXPRESSING α_1 AND α_6 SUBUNIT-CONTAINING GABA$_A$ RECEPTORS

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Abstract

Early onset effects of methylmercury (MeHg) on recombinant $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ subunit-containing GABA$_A$ receptors were examined. These are two of the most prevalent receptor types found in cerebellum–a consistent target of MeHg-induced neurotoxicity. Heterologously expressed receptors were used in order to: 1) isolate receptor-mediated events from extraneous effects of MeHg due to stimulation of the receptor secondary to increased release of GABA seen with MeHg in neurons *in situ* and 2) limit the phenotypes of GABA$_A$ receptors present at one time. Initial changes in I_{GABA} in *Xenopus laevis* oocytes expressing either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors were compared during continuous bath application of MeHg. A time-dependent increase in I_{GABA} mediated by both receptor subtypes occurred following the first 25–30 min of MeHg (5 μM) exposure. In $\alpha_6\beta_2\gamma_{2S}$ containing receptors, the MeHg-induced increase in I_{GABA} was less pronounced compared to that mediated by $\alpha_1\beta_2\gamma_{2S}$ containing receptors, although the pattern of effects was generally similar. Washing with MeHg-free solution reversed the increase in current amplitude. Application of bicuculline at the time of peak potentiation of I_{GABA} rapidly and completely reversed the MeHg-induced currents. Therefore these MeHg-increased inward currents are mediated specifically by the two subtypes of GABA$_A$ receptors and appear to entail direct actions of MeHg on the receptor. However bicuculline did not affect stimulation by MeHg of oocyte endogenous Cl^--mediated current, which presumably results from increased $[Ca^{2+}]_i$. Thus, MeHg initially potentiates I_{GABA} in oocytes expressing either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors prior to its more defined later effects, suggesting that MeHg may initially interact directly with GABA$_A$ receptors in a reversible manner to cause this potentiation.

Keywords

Methylmercury; GABA$_A$ receptor; α_1 and α_6 subunit; heterologous expression

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INTRODUCTION

Methylmercury (MeHg) is a potent environmental neurotoxicant that preferentially affects the somatosensory, visual and auditory cortices and the cerebellum (See review by Ekino *et al.*, 2007.) Each of these regions contains large numbers of small diameter granular cells, which are especially sensitive to MeHg. In the cerebellar cortex, for example, the granule cells are much more sensitive to MeHg exposure than are their neighboring Purkinje cells (Chang, 1988; Leyshon-Sorland and Morgan, 1991; Patel and Reynolds, 2013). This relative sensitivity can be recaptured *in vitro*- both in freshly isolated brain slices (Yuan and Atchison, 2003; 2007) and organotypic slice culture (Bradford *et al.*, 2016) as well as in single cells in primary culture (Edwards *et al.*, 2005).

Among the numerous differences between cerebellar granule and Purkinje cells are those of $GABA_A$ receptors. The two cell types express $GABA_A$ receptors with different subunit compositions (Fritschy *et al.*, 1992; Laurie *et al.*, 1992; Thompson *et al.*, 1992; Thompson and Stephenson, 1994; Gao and Fritschy, 1995; Wisden *et al.*, 1996; Mäkelä *et al.*, 1999). This is important because the pharmacological and electrophysiological properties of $GABA_A$ receptors vary markedly based on the composition of $GABA_A$ receptor subunits and subtypes (Smith, 2001; Trincavelli *et al.*, 2012; Nikas *et al.*, 2015). Thus subtype-specific effects of MeHg on $GABA_A$ receptors could contribute to specific cell cytotoxicity. Mature granule cells express α_1 or α_6 subunits alone or in combination, whereas Purkinje cells express only the α_1 subunit (Lüddens *et al.*, 1990; Varecka *et al.*, 1994; Nusser *et al.*, 1995; Wisden *et al.*, 1996; Siegel, 1998; Fritschy and Panzanelli, 2006). Furthermore, diversity is provided to granule cells by substitution in some receptors of a δ for a γ subunit. However this co-expression is strictly dependent on the presence of α_6 subunits (Quirk *et al.*, 1994; Jones *et al.*, 1997; Nusser *et al.*, 1999; Tretter *et al.*, 2001). Differential expression of α_1 or α_6 subunits confers unique pharmacological and biophysical properties on recombinant $GABA_A$ receptors (Whiting *et al.*, 1999, Olsen and Sieghart, 2008, 2009; Brickley and Mody, 2012). This difference could be important to granule cell vulnerability, because in granule cells a tonic GABA-mediated conductance regulates granule cell excitability (Brickley *et al.*, 1996; Mody 2001; Semyanov *et al.*, 2004; Brickley and Mody, 2012; Lee and Maguire, 2014). Thus, preferential block of the $GABA_A$ receptors responsible for this conductance could cause the granule cell to become more excitable, leading to membrane depolarization and subsequent increase of $[Ca^{2+}]_i$. Both of these latter effects occur in granule cells in response to MeHg (Marty and Atchison, 1997, 1998; Yuan and Atchison, 2003, 2007; Limke *et al.*, 2003; Yuan and Atchison, 2016)

Early studies suggested that $GABA_A$ receptors could be a sensitive target to MeHg. Following administration of MeHg to neonatal rats, morphological examination of the visual cortex indicated that aspinous or sparsely-spinous GABAergic interneurons in layer IV had degenerated selectively (O'Kusky, 1985; O'Kusky and McGeer, 1985, 1989; O'Kusky *et al.*, 1988). MeHg also affects $GABA_A$ receptors in several cell types in culture (Arakawa *et al.*, 1991; Komulainen *et al.*, 1995; Fonfría *et al.*, 2001; Herden *et al.*, 2007; Suñol *et al.*, 2008). GABAergic neurons are more sensitive to effects of MeHg than are glutamatergic neurons in hippocampal (Yuan and Atchison, 1995; 1997) and cerebellar slices (Yuan and Atchison, 2003). In the latter, granule cell $GABA_A$ receptor-mediated spontaneous inhibitory

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postsynaptic currents (sIPSCs) are additionally more sensitive to MeHg-induced block than are those of Purkinje cells (Yuan and Atchison, 2003). Furthermore, among a series of ion channels examined, $GABA_A$ receptor-mediated Cl^- channels were the most sensitive to MeHg (Yuan *et al.*, 2005; Yuan and Atchison, 2005). Thus, the relative sensitivity of GABAergic systems to MeHg could play a role in the MeHg-induced differential sensitivity of cerebellar granule and Purkinje cells.

The effects of MeHg on GABAergic function at intact CNS synapses are complex; they involve both a transient stimulation of inhibitory postsynaptic currents (IPSCs) amplitude, followed by reduction to their complete block (Yuan and Atchison, 2003, 2005, 2007). While MeHg exposure ultimately decreases I_{GABA} to complete block, an early effect appears to be transient increase in I_{GABA} amplitude. However, this effect is difficult to isolate at intact synapses due to the multiplicity of MeHg-induced effects that are time-dependent. These include a pronounced stimulation of spontaneous IPSC (sIPSC) frequency, so both pre- and postsynaptic effects contribute to actions of MeHg on cerebellar inhibitory circuits. Whereas in granule cells, GABAergic currents are blocked with a faster time course than those of Purkinje cells, the transient stimulation of IPSC amplitude was lesser in magnitude and frequency; it occurred in less than 50% of the granule cells examined compared to that which occurred in all Purkinje cells (Yuan and Atchison, 2003). This may reflect cell-specific subtype differences in combinations of $GABA_A$ receptors. However, studies of selective effects of MeHg on different $GABA_A$ receptor subtypes in culture cells or slices can be hindered by uncertainty about the receptor phenotype because granule cells contain a mixture of $GABA_A$ receptor subunits that is both developmentally and spatially regulated (Zheng *et al.*, 1993; Thompson and Stephenson, 1994; Varecka *et al.*, 1994; Carlson *et al.*, 1998; Takayama and Inoue, 2004).

Consequently, in this study, we focused specifically on the initial effect of MeHg on GABA-evoked currents in *Xenopus laevis* oocytes expressing either subtype of $GABA_A$ receptor in isolation. We sought to determine if MeHg has an initial stimulatory effect on the GABA-induced currents in *Xenopus* oocytes expressing heterologously α_1- or α_6- subunit-containing $GABA_A$ receptors as it does on native cerebellar neurons in slices, and if the two subtypes of $GABA_A$ receptors respond to MeHg differently. Consistent with results seen in native cerebellar neurons in slices, MeHg caused an initial and reversible potentiation of GABA-evoked currents in both subtypes of receptors expressed in oocytes. However unlike the situation in slices, in which intact synapses can contribute enhanced GABA release and thereby confound the source of the facilitated response, there was no endogenous source of GABA for the isolated oocyte. Thus a stimulated response caused by MeHg would reflect a direct action on the receptor/channel complex.

MATERIALS AND METHODS

Solutions and Chemicals

Methylmercuric chloride (MeHg) (ICN Biomedical Inc., Costa Mesa, CA, USA) was applied continuously by oocyte perfusion. A stock solution (10 mM) was prepared in deionized water. On the day of experiments, MeHg solutions (5 μM) were constituted in ND 96 extracellular solution consisting of (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5

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HEPES, (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, Sigma-Aldrich Chemicals, St. Louis, MO, USA) titrated to pH 7.4 with NaOH, supplemented with 2.5 mM Na pyruvate (Sigma) and 50 μg/ml gentamicin (Sigma). Consistent with our previous observations obtained from acutely isolated brain slices of rat (Yuan and Atchison, 1993, 2003), preliminary experiments revealed that MeHg at different concentrations (1 – 10 μM) produced a similar pattern of effects on GABA currents but varying with different time courses inversely related to the concentration employed (data not shown). Consequently, only a single concentration of MeHg (5 μM) was used for the data depicted. This is within the range of concentrations (~19.5 μM) found in the blood of patients poisoned with MeHg in Iraq in the 1970's (Bakir *et al.*, 1973). It is also at the low range of concentrations used in cerebellar slice and at which stimulatory effects of MeHg were observed previously (Yuan and Atchison, 2003, 2005, 2007).

GABA, bicuculline, niflumic acid, type IV collagenase, HEPES, deoxyribonuclease (DNase I), and ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′,-tetraacetic acid (EGTA), trypsin, poly-L-lysine were all purchased from Sigma Chemical Co. (St. Louis, MO). Qiagen kits, used for plasmid purification, were purchased from Qiagen Inc. (Valencia, CA) and Fugene 6 was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Preparation of cRNA

The plasmid cDNAs from rat for α_1, β_2 and γ_{2S} $GABA_A$ receptor subunits were generously provided by Dr. Cynthia Czjkowski, University of Wisconsin-Madison, while that for α_6 was generously provided by Dr. Bill Wisden (University of Heidelberg, Heidelberg, Germany). Plasmids containing cDNAs for the $GABA_A$ subunits were linearized after the poly (A) signal sequences. The linear plasmid DNA was 'agarose' gel purified and used for enzymatic cRNA synthesis using a mMessage mMachine T7 kit (Ambion, Austin, TX). Linearized plasmid DNA (1 mg) was mixed with reaction buffer, NTP/Cap and enzyme mix from the kit and incubated for 2 hr at 37°C. RNAase-free DNaseI was then added and incubation continued for another 15 min. The resulting capped, DNA-free cRNA was used to add poly-A tails with Poly (A) Tailing Kit (Ambion) by adding water, $MnCl_2$, ATP, E-PAP (*E. coli* Poly (A) polymerase) and E-PAP buffer and then incubating for 1 hr at 37°C. The products of the capped cRNA synthesis reaction and the poly (A) tailing reactions were compared using mobility shift and denaturing agarose gel electrophoresis to verify addition of the poly (A) tails. The final products were purified to remove unincorporated nucleotides and other reaction components by gel filtration using a MEGAclear kit also from Ambion. Purified cRNA was quantitated using UV spectrophotometery on a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE), and stored frozen at −80°C until use.

Xenopus oocyte preparation and electrophysiological recordings

All animal procedures complied with the National Institutes of Health of the USA guidelines on animal care and were approved by Michigan State University Institutional Animal Care and Use Committee. Clusters of *Xenopus laevis* oocytes were removed surgically from adult female frogs (Xenopus One, Ann Arbor, MI) under tricaine (Sigma-Aldrich) anesthesia (0.17% w/v) and were incubated in ND96 solution. Defolliculated oocytes were obtained by incubating oocytes for 30 – 120 min in Ca^{2+}-free ND96 medium containing 0.6 mg/ml type

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IV collagenase; any remaining follicular layers were manually removed using fine forceps. Only stage IV–V oocytes were collected for further use. The $GABA_A$ receptors expressed in oocytes in the present study were either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ subtype, because the former is the most common subtype of $GABA_A$ receptor in the brain including the cerebellum, whereas the latter is found specifically in cerebellar granule cells (Benke *et al.*, 1991, 1994; Laurie *et al.*, 1992). Each oocyte received a 50 nl injection of a mixture of α_1 or α_6, β_2 and γ_{2S} in the proportion of 1:1:10 (Boileau *et al.*, 2003). After cRNA injection, occytes were incubated in ND96 at 19°C for at least 2 days before electrophysiological experiments.

Electrophysiological recordings were made within 3 to 7 days after cRNA injection. For each experiment, oocytes from two or more frogs were used. Whole oocyte recordings of GABA-evoked currents were made using two-microelectrode voltage-clamp recording. Microelectrodes for voltage-sensing and current-passing were fabricated from thick-wall borosilicate glass (o.d. = 1.0 mm, i.d. = 0.5 mm) (WPI, Inc., Sarasota, FL) and had an impedance of 0.5 – 2.0 MΩ when filled with 3 M KCl. Signals from the current-passing electrode were amplified using an OC-725C amplifier (Warner Instruments Corp., Hamden, CT). Data were acquired using a Digidata 1200 interface and pClamp 9.0 software (Molecular Devices, Sunnyvale, CA). Currents were filtered at 20 – 100 Hz and digitized at 50 – 200 Hz, respectively, for off-line analysis. To do this, two computer recording systems were used: one was devoted specifically to episode recordings of GABA-evoked currents at a sampling rate of 200 Hz; the other was tasked for continuous recording of baseline currents at a sampling rate of 50 Hz. All recordings were made at a holding potential of −60 mV and at room temperature of ~22 °C. Oocytes were superfused at a constant rate ~4 ml/min. $GABA_A$ receptor-mediated currents were evoked by sequential 15-sec pulse applications of 0.2–1000 μM GABA in bath solution. A standard stimulus protocol was used in most experiments. It consisted of a 1.0 sec ramp protocol with voltage changing from −140 mV to +60 mV at a rate of 1 mV/5 ms, followed by a 15-sec pulse application of GABA to evoke I_{GABA}. In some cases, mostly $\alpha_1\beta_2\gamma_{2S}$ receptors, a second identical voltage ramp was applied at the end of the 15-sec GABA application (Figure 1). The ramp protocols were used to monitor changes in voltage-dependent responses, particularly those mediated by endogenous voltage-gated Cl^- channels before and after GABA application. A 5-min interval between two consecutive GABA applications was given to allow receptors to recover from deactivation/desensitization and currents to return to baseline. This interval was adequate to reverse desensitization associated with $GABA_A$ receptors (data not shown). When MeHg and other receptor antagonists or inhibitors were applied, they were perfused continuously with ND96 recording solution controlled by a programmable six channel valve perfusion system (VC-6, Warner Instrument, Hamden, CT). The composition of ND96 recording solution was similar to that used for incubation, but without Na pyruvate and gentamicin, and the concentration of HEPES was increased from 5 mM to 10 mM.

Data analysis

Data were collected prior to and during application of MeHg and analyzed statistically using one-way analysis of variance (ANOVA) or Student's paired *t* test. Dunnett's procedure was used for *post hoc* comparison. Values were considered statistically significant at $P < 0.05$.

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Each experiment were repeated at least three times in oocytes from different frogs. Values are expressed as mean ± SEM of individual experiments.

RESULTS

Differential sensitivity of $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors to GABA and niflumic acid

Among $GABA_A$ receptors the α subunit subfamily, especially the α_1 or α_6 subunits have the most divergent properties (Pritchett *et al.*, 1989; Draguhn *et al.*, 1990; Korpi *et al.*, 1995; Tia *et al.*, 1996; Saxena and Macdonald, 1996; Fisher *et al.*, 1997; Fisher and Macdonald, 1998; Zhu *et al.*, 1998; Sigel and Baur, 2000; Smith, 2001; Fisher, 2004). In both native and recombinant $GABA_A$ receptors, presence of the α_6 subunit confers distinct pharmacological properties including higher sensitivity to receptor agonists and insensitivity to desensitization. To ensure that the $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ subtype receptors expressed in our oocyte expression system retain these properties, we compared the sensitivity of the two receptors subtypes to GABA. As shown in Figure 2, sequential pulse application of 0.2 – 1000 μM GABA for 15 sec to oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ subtypes induced a concentration-dependent increase in GABA-evoked inward current (I_{GABA}) in both receptor subtypes at a holding potential of –60 mV. However, I_{GABA} evoked by the same agonist concentrations in $\alpha_6\beta_2\gamma_{2S}$-containing receptors was much larger than that in $\alpha_1\beta_2\gamma_{2S}$ subtype. The averaged EC_{50} values for $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptor were 9.2 μM (n = 5) and 2.0 μM (n = 6), respectively ($P<0.05$). Thus, these data are consistent with the general concept that α_6 subunit-containing $GABA_A$ receptors have a relatively higher affinity for GABA than do α_1 subunit-containing receptors (Saxena and Macdonald, 1996). For this reason, in the subsequent experiments, the whole cell I_{GABA} in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors was evoked by 5 μM GABA, whereas that in oocytes expressing $\alpha_6\beta_2\gamma_{2S}$ receptors was evoked by 1 μM GABA, about half of the EC50 value for either subtype, respectively. In addition, data shown in Figure 2 suggest that the 5 min interval between two GABA pulse applications was sufficient to allow I_{GABA} to return to baseline level.

α_1 or α_6 subunit-containing receptors expressed in oocytes also respond differentially to niflumic acid (NA), a nonsteroidal anti-inflammatory drug and anion channel blocker (Sinkkonen *et al.*, 2003). We compared sensitivity of α_1 and α_6- containing receptors to NA (500 μM). I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ subtype, was potentiated whereas that mediated by $\alpha_6\beta_2\gamma_{2S}$ subtype was inhibited by niflumic acid (Figure 3A). Both the potentiation and inhibition of I_{GABA} are statistically significant (Figure 3B, $p < 0.05$, n = 3 – 4). Thus, these results again suggest that the recombinant $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptor subtypes expressed in our oocyte expression system retain, at least in part, those pharmacological properties that are typical of α_1 or α_6 subunit-containing native $GABA_A$ receptors. Therefore, no further pharmacological characterization of the two receptor subtypes was carried out.

MeHg potentiates I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors in a similar pattern

Initial preliminary studies utilized 1, 5, or 10 μM MeHg to examine its early onset effects on I_{GABA}. Consistent with our previous observations in brain slices (Yuan and Atchison, 1993; 1999; 1997; 2003, 2007), 1 – 10 μM MeHg produced a similar pattern but with very

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different time-courses of effects on I_{GABA} expressed in oocytes. At 1 μM MeHg, it took much longer time to produce an effect similar to that induced by 5 μM MeHg, whereas 10 μM MeHg produced an effect that was too rapid to allow for other subsequent modulations. Considering the potential current run-down of $GABA_A$ receptors expressed in oocytes and the lack of a clearly defined concentration-dependent effect of MeHg on I_{GABA} (data not shown), all data presented in this and subsequent figures were collected from oocytes treated with 5 μM MeHg. As the primary purpose of the present study was to determine if MeHg initially stimulated GABA-evoked currents mediated by the two recombinant $GABA_A$ receptors, we limited the MeHg exposure duration to 30 min, or the point at which a MeHg-induced peak stimulation was achieved. Also, after ~40–50 min, continuous oocyte recordings in the presence of MeHg became unstable. Figure 4A shows two representative recordings of effects of MeHg on I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$, respectively. In both cases, exposure of oocytes to 5 μM MeHg for 25 min caused an increase in GABA-evoked currents. I_{GABA} recorded from both receptor subtypes appears to decay faster in the presence of MeHg, suggesting that MeHg may affect the deactivation or/and desensitization process of the two subtypes of receptors. However, detailed kinetic analysis using ultrafast-step application of $GABA_A$ receptor agonist is needed to substantiate further this effect and was beyond the scope of the study. Figure 4B summarizes the peak increases in I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors, respectively, during the first 30 min exposure to 5 μM MeHg. Clearly, MeHg caused a significant increase in I_{GABA} mediated by both $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors compared with their own controls. The peak increases in I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors are 215 ± 16% (n = 7) and 115± 5% (n = 7) of their own control, respectively (P<0.05). MeHg appears to potentiate the I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ more strongly than it does that mediated by the $\alpha_6\beta_2\gamma_{2S}$ receptor subtype ($p < 0.05$), although the pattern of effects of MeHg on both receptor subtypes is generally similar.

The time course of 5 μM MeHg-induced potentiation of I_{GABA} in $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors is shown in Figures 5 and 6, respectively. In Figure 5, the top three current traces from a representative experiment depict I_{GABA} mediated by $\alpha_1\beta_2\gamma_{2S}$ receptors that were collected before, at peak increase during MeHg exposure, and after 10 min of MeHg-washout. The bottom curve is the averaged time course of effects of MeHg on I_{GABA} in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors (n = 7). Application of MeHg began (0 min) after the baseline had remained stable for at least 10 min, and continued for 30 min. MeHg was then washed out with MeHg-free ND96 solution for another 10 min. There was a time-dependent increase of I_{GABA} in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors. It reached a maximum at 30 min. The increase might not be the real peak effect of MeHg because of the limited MeHg exposure duration. This increase could possibly continue if longer exposure time is allowed, which is actually demonstrated later in an experiment shown in the inset. However, because recordings under our experimental conditions usually became unstable after 40 – 50 min, most experiments were terminated after washing for 10 min. Therefore, only a partial recovery is shown in this figure. In two individual recordings in which longer duration of MeHg exposure and washing were made successfully, complete recovery of MeHg-induced increase in I_{GABA} was attained (The inset shows one of them.). A similar pattern of time-course of effects of MeHg on I_{GABA} was seen in oocytes expressing $\alpha_6\beta_2\gamma_{2S}$ receptors

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(Figure 6). In these experiments, oocytes were exposed to MeHg for 25 min because enhancement of I_{GABA} usually reached the peak after 20 min of exposure; thus wash out began at 25 min and lasted for 10 min. Again, the continuous recordings showed that the effect of MeHg on I_{GABA} mediated by $\alpha_6\beta_2\gamma_{2S}$ receptors was less pronounced as compared to that mediated by $\alpha_1\beta_2\gamma_{2S}$ receptors. In fact, two of the seven α_6-expressing oocytes examined had no detectable increase in I_{GABA}. However, when it occurred, the MeHg-induced peak increase in I_{GABA} mediated by $\alpha_6\beta_2\gamma_{2S}$ receptors had a more rapid onset than in those cells with α_1-containing receptors. Thus although the general pattern of effect of MeHg on the two recombinant receptors is similar, the time course of MeHg effects differed substantially. The inset in Figure 6 demonstrates a representative recording that was sufficiently stable to permit one to observe a complete recovery of MeHg-induced effect on I_{GABA} mediated by α_6-containing receptors. Overall, these data suggest that potentiation by MeHg of I_{GABA} occurred with both subtypes of recombinant $GABA_A$ receptors is MeHg exposure time-dependent, and at least partially reversible.

MeHg-increased I_{GABA} was sensitive to block by $GABA_A$ receptor antagonist

We next sought to ascertain whether the action of MeHg on these receptors could be reversed by a $GABA_A$ receptor antagonist, that is, if the MeHg-induced stimulatory effect was mediated directly by access to the receptor. Bicuculline (20 μM) was applied at the time when MeHg caused peak potentiation of I_{GABA} in oocytes expressing either α_1 or α_6-containing receptors (Figure 7). Bicuculline rapidly and completely (100%) blocked GABA-evoked inward currents in oocytes expressing either subunit-containing receptors. As expected, the bicuculline effect was completely reversible when washing with bicuculline-free, but MeHg-containing solution. Thus the inward currents were indeed mediated specifically by these two subtypes of $GABA_A$ receptors and appear to be due to a direct and reversible action of MeHg at the receptor.

As shown in Figures 1, 5 and 7 for oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors, the second voltage ramp immediately following GABA application induced a much larger outward current than that induced by one immediately prior to GABA application. MeHg also potentiated both sets of outward currents. Bicuculline did not affect the outward currents evoked by the first voltage ramp, but reduced those evoked by second voltage ramp to a level equal to that of the first voltage ramp. This suggests that GABA-evoked currents may contribute to or facilitate an increase in the outward currents evoked by the second voltage ramp, whereas the MeHg-induced increase in the residual outward currents evoked by the first and second voltage ramps in the presence of bicuculline is not mediated by $GABA_A$ receptors.

To test whether the MeHg-increased, bicuculline-insensitive outward currents were due to MeHg-induced stimulation of the endogenous Ca^{2+}-activated Cl^- channels in oocytes (Philips *et al.*, 2003), we next examined the effect of MeHg on whole-cell currents in oocytes injected with water only. Figure 8 representatively shows exposure of water-injected oocytes to MeHg induced a significant increase in voltage-dependent outward currents in a time-dependent manner (Figure 8A–D). In this case, the outward current was increased from 0.22 μA to 2.56 μA at a membrane potential of +60 mV after 25 min MeHg exposure

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(Figure 8D and 8F). As expected, the MeHg-increased voltage-dependent outward currents were blocked completely by 300 μM niflumic acid (NA), a Ca^{2+}-activated Cl^- channel blocker (Figure 8E and 8F). These results suggest that the MeHg-increased, bicuculline-insensitive outward currents are mediated, most likely, by the endogenous Ca^{2+}-activated Cl^- channels.

DISCUSSION

The primary objective of the present study was to examine the early onset effects of MeHg on two types of $GABA_A$ receptors expressed abundantly in the cerebellum. In native cerebellar neurons in cerebellar slices, MeHg causes an initial stimulatory effect on presumptive $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ $GABA_A$ receptors. This effect precedes the more commonly-described, and apparently irreversible, block of I_{GABA}, and appears to result from a transient increased sensitivity of the receptor. However, in slices, MeHg also dramatically increases the frequency of occurrence of GABA-mediated IPSCs (Yuan and Atchison, 2003, 2007), suggesting an involvement of presynaptic action. This presynaptic effect certainly complicated attempts to resolve postsynaptic effects in isolation. Thus a recombinant system was used in this study to examine this effect in isolation. In HEK293 cells, expressing either phenotype, the stimulatory effect could not be reproduced reliably for unknown reasons (Herden *et al.*, 2007). Thus we re-examined the effect in the well described oocyte expression system. MeHg potentiated I_{GABA} mediated by both subtypes of recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes. This potentiation is reversible and is sensitive to block by the specific $GABA_A$ receptor antagonist bicuculline.

α_1 Subunit-containing $GABA_A$ receptors display lower affinity to GABA than do α_6 subunit-containing receptors (Saxena and Macdonald, 1996; Fisher *et al.*, 1997; Sinkkonen *et al.*, 2003; Fisher, 2004). Differential sensitivity of recombinant $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors expressed in our oocyte expression system to GABA also occurred, with EC_{50} values similar to those reported for the α_1 or α_6 subunit-containing receptors expressed in L929 cells (Saxena and Macdonald, 1996; Fisher *et al.*, 1997), oocytes (Sinkkonen *et al.*, 2003) and HEK293 cells (Fisher, 2004). We also obtained a similar pattern of responses of $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors to NA as that reported by Sinkkonen *et al.* (2003) for positive and negative modulation of α_1 and α_6 subunit-containing $GABA_A$ receptors, respectively. These data clearly demonstrate that $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors expressed in our oocyte expression system were indeed two different subtypes of $GABA_A$ receptors. Thus, any difference between the two subtypes of receptors displayed in our experiments should be related to the unique pharmacological and electrophysiological properties of α_1 and α_6 subunits.

In acutely isolated cerebellar slices, we have consistently shown that acute bath application of MeHg causes an increase prior to subsequent suppression of both frequency and amplitude of $GABA_A$ receptor-mediated sIPSCs in both Purkinje and granule cells (Yuan and Atchison, 2003). The initial increases in sIPSCs in Purkinje cells appear to be more prominent than those in granule cells (Yuan and Atchison, 2003). In fact, almost half of the granule cells examined in cerebellar slices had no initial increase in either sIPSC frequency or amplitude. Consistently, MeHg-induced potentiation of I_{GABA} also appears to be more

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prominent in oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ receptors than those $\alpha_6\beta_2\gamma_{2S}$ receptors, and two of seven recordings from oocyte expressing $\alpha_6\beta_2\gamma_{2S}$ receptors exhibited no initial increase in I_{GABA}. If the response is subunit dependent, then cells expressing the sensitive subunit would be expected to express the response whereas those with fewer of the responsive receptors would not, or would only do at a reduced frequency. This makes sense because native Purkinje cells only express α_1 subunit-containing $GABA_A$ receptors, whereas granule cells express $GABA_A$ receptors containing α_1, α_6 or both α_1 and α_6 subunits. In addition, the relatively rapid time course to peak stimulation of I_{GABA} mediated by $\alpha_6\beta_2\gamma_{2S}$ receptors compared to that mediated by $\alpha_1\beta_2\gamma_{2S}$ receptors also appears to be consistent with the different time courses of effects of MeHg on sIPSCs in granule and Purkinje cells (Yuan and Atchison, 2003). Interestingly, the reversibility of MeHg-induced initial potentiation of I_{GABA} in both subtypes of receptors also appeared to be consistent with our observations in native cerebellar neurons (Yuan and Atchison, 2003). In cerebellar slices, MeHg-induced block of sIPSCs in granule cells occurred much more rapidly than it did those in Purkinje cells. Because of the relatively short time course of onset of block, sIPSCs in most granule cells could be recovered completely to the pretreatment control level by washing cells with D-penicillamine, a MeHg chelator. In contrast, it took about 3-fold longer for the same concentrations of MeHg to block sIPSCs in Purkinje cells than it did in granule cells. Once blocked, sIPSCs in Purkinje cells could not be restored by washing the cells with D-penicillamine. This irreversibility of sIPSCs in Purkinje cells by washing appeared to be due to MeHg-induced nonspecific effects such as irreversible membrane damage resulting from the longer time of MeHg exposure that is typically needed to cause complete block of sIPSCs in Purkinje cells. So, one should not be surprised that MeHg-induced potentiation of I_{GABA} in oocytes expressing either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors was reversible in the present study, because the oocytes were only exposed to MeHg for relatively brief durations and washed before the reduction of I_{GABA} or nonspecific MeHg effects occurred. Thus, these data suggest that $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors do respond to MeHg somewhat differently in terms of the peak stimulation and time course of effects, though the pattern is similar. This is generally consistent with the observations obtained in native cerebellar granule and Purkinje cells in slices (Yuan and Atchison, 2003).

Whether or not these differential responses of $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors to MeHg play any role in differential sensitivity of granule and Purkinje cells remains to be determined. Increases in sIPSC frequency are usually related to increased transmitter release from presynaptic terminals, whereas increased sIPSC amplitude could be due to either pre- or postsynaptic mechanisms. In the present study, GABA was applied directly to $GABA_A$ receptors (equivalent to postsynaptic receptors), thereby bypassing any presynaptic actions. Therefore, potentiation by MeHg of I_{GABA} in oocytes expressing the two subtypes of $GABA_A$ receptors clearly indicates that it results from a direct postsynaptic action of MeHg on $GABA_A$ receptors. Then the question is how MeHg causes this initial increase or potentiation of I_{GABA}. One possibility is that MeHg may directly interact with the GABA receptor complex and modulate the benzodiazepine or barbiturate modulation sites to cause this initial potentiation since MeHg increases the total number of benzodiazepine binding sites of $GABA_A$ receptors in the retina and other brain areas including the cerebellum (Corda *et al.*, 1981; Concas *et al.*, 1983; Komulainen *et al.*, 1995; Fonfría *et al.*, 2001). If this

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is the case, α_6-containing receptors would be less responsive because the α_1 subunit confers greater benzodiazepine sensitivity (Smith, 2001; Trincavelli *et al.*, 2012). Alternatively, MeHg may interact with protein kinases such as the cyclic AMP-dependent protein kinase (PKA) or Ca^{2+}-phospholipid-dependent protein kinase C (PKC) to affect phosphorylation of $GABA_A$ receptors and their functions. $HgCl_2$-induced potentiation of $GABA_A$ receptor-mediated current in rat dorsal root ganglion cells involves changes in phosphorylation (Huang and Narahashi, 1997). However, whether or not these effects indeed underlie mechanisms by which MeHg causes this potentiation remain to be determined.

Although MeHg affects function of multiple ion channels, the sensitivity of MeHg-induced potentiation of I_{GABA} to bicuculline confirms that these effects of MeHg are mediated specifically by these two subtypes of $GABA_A$ receptors. In contrast, those bicuculline-insensitive, voltage ramp-activated outward currents also affected by MeHg are not mediated by $GABA_A$ receptors. Our recordings from oocytes injected with water only suggest that the MeHg-increased, bicuculline-insensitive outward currents are probably mediated by the endogenous Ca^{2+-}activated Cl^- channels since the Ca^{2+}-activated Cl^- channel blocker NA could block the MeHg-induced increase of these outward currents. In addition, 4′, 4′-diisothiocyanostibene-2,2′-disulfonic acid (DIDS) or pre-injection of Ca^{2+} chelator BAPTA could also block MeHg-induced increase of these outward currents (Unpublished observations).

The question is why the second voltage ramp caused a larger outward current compared with the first voltage ramp. One possible explanation is that the GABA-evoked, $GABA_A$ receptor-mediated inward currents (efflux of Cl^-) increases the driving force for influx of Cl^- (outward currents) mediated by voltage-gated Cl^- channels such as Ca^{2+}-activated Cl^- channels following GABA applications. When MeHg increases the $GABA_A$ receptor-mediated inward current, it will further enhance the driving force for influx of Cl^-, leading to a even bigger outward current evoked by the second voltage ramp.

In conclusion, consistent with our previous observations from native cerebellar neurons in slices, MeHg initially potentiates I_{GABA} in oocytes expressing either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors. This effect is reversible and is directed specifically at the receptor level, whereas the subsequent inhibition of receptor function involves irreversible effects of MeHg. The pattern of effects of MeHg on the two subtypes of $GABA_A$ receptor is generally similar, but slightly different in terms of time-courses and potency of MeHg effect. Whether or not these differences contribute to differential effects of MeHg on cerebellar granule and Purkinje cells remains to be determined.

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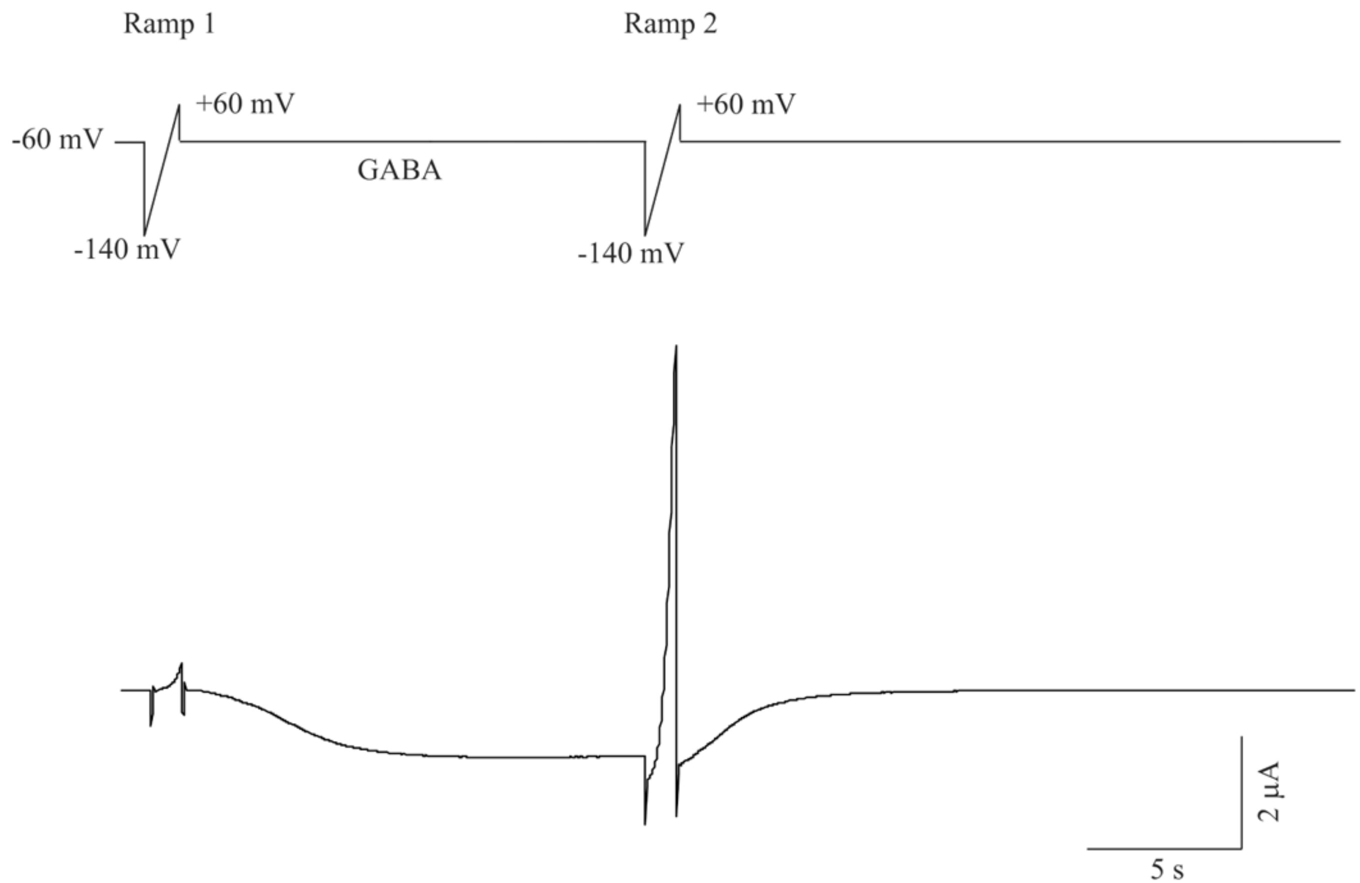

Figure 1.

Current recording protocols used for two-electrode voltage clamp recordings in *Xenopus* oocytes. **Top:** A given oocyte is voltage clamped at a holding potential of −60 mV and is activated first by a voltage ramp (Ramp 1, 1 sec duration, voltage changes at a rate of 1mV/5 ms from −140 mV to 60 mV), followed by a 15 sec pulse application of 1 μM GABA. Immediately following GABA application, a second ramp (Ramp 2) with identical features as the first one is applied. **Bottom:** A representative current trace evoked by this protocol in an oocyte expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors.

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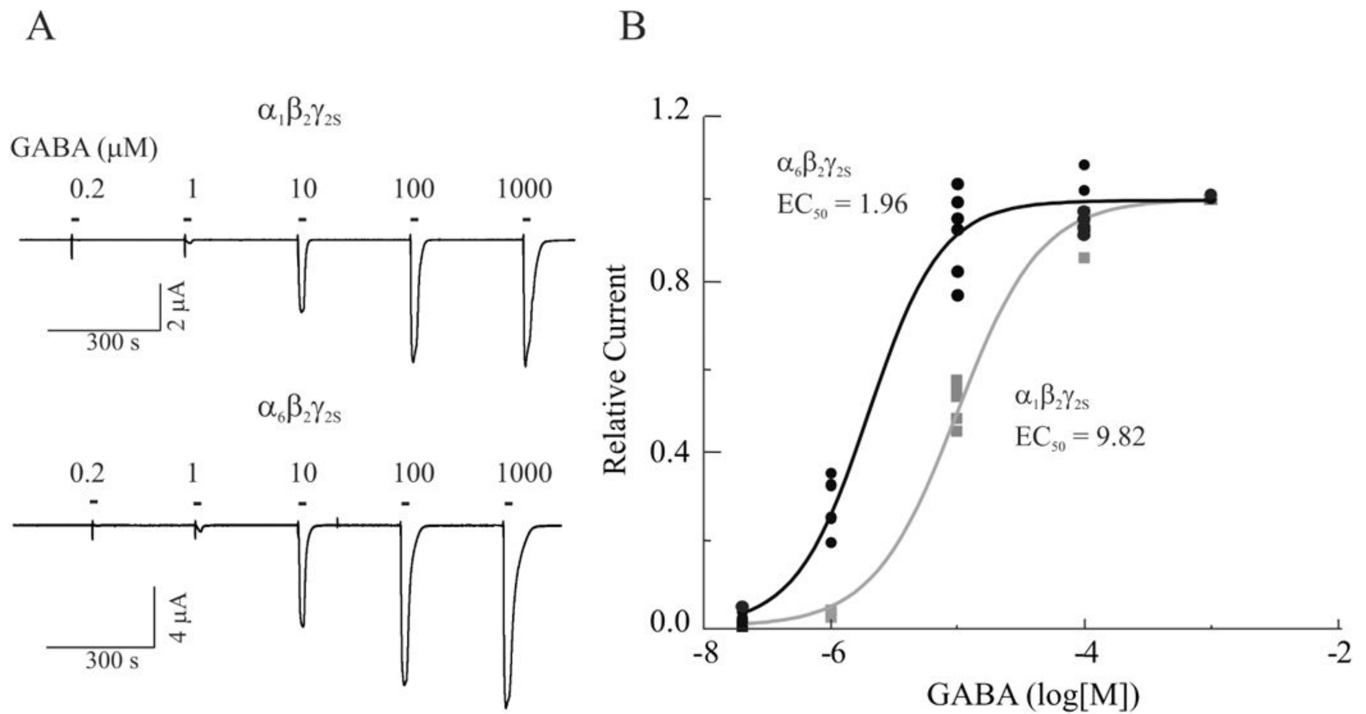

Figure 2.

Differential sensitivity of recombinant $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors to GABA. A, Whole cell currents recorded from representative oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ **(Top)** and $\alpha_6\beta_2\gamma_{2S}$ **(Bottom)** in response to 0.2–1000 μM GABA. B, Concentration-dependent response curves were generated by nonlinear regression fit of normalized GABA-evoked currents recorded from oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ (Black) and $\alpha_6\beta_2\gamma_{2S}$ (Grey) receptors. The peak current evoked by each concentration of GABA is normalized to that evoked by 0.2 μM GABA. Each value is a representative example of 5 – 6 individual experiments.

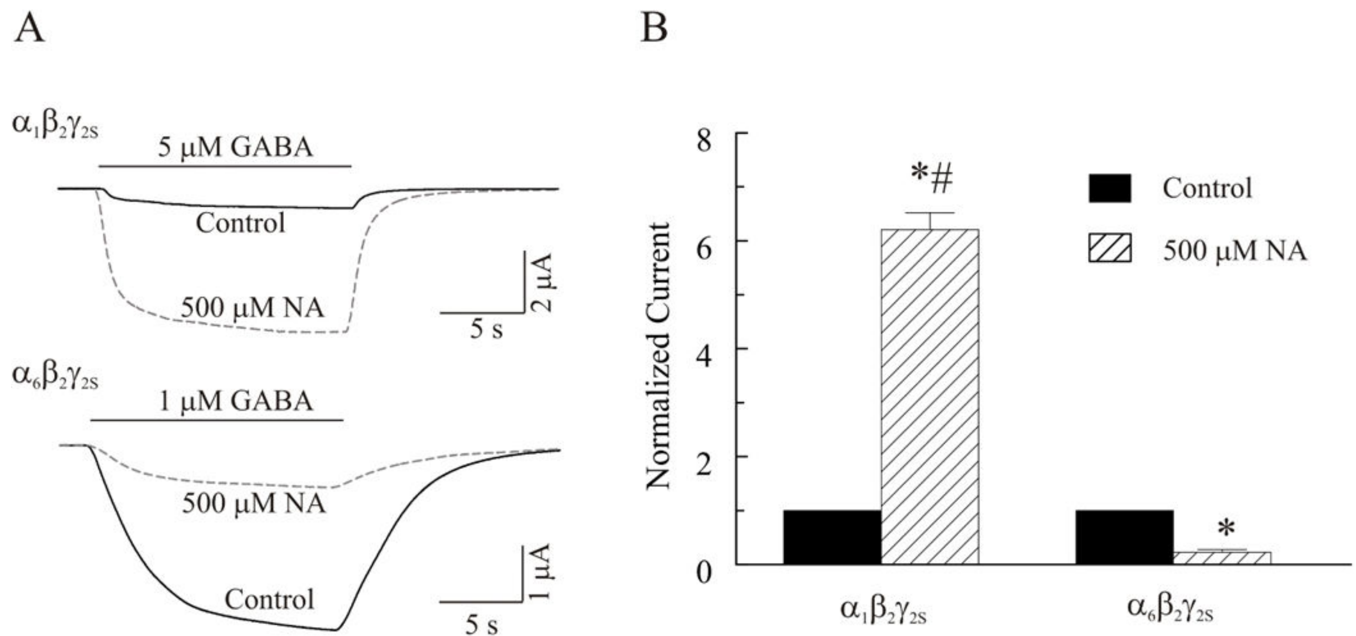

Figure 3.

Differential sensitivity of recombinant $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ $GABA_A$ receptors to niflumic acid (NA). A, Whole cell currents were recorded from oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ **(Top)** or $\alpha_6\beta_2\gamma_{2S}$ (Bottom) in response to 5 or 1 μM GABA, respectively, at a holding potential of −60 mV in the absence and presence of 500 μM NA. B, Comparison of effects of NA on I_{GABA} on the two subtype receptors (n = 3 – 4). The asterisk (*) indicates a significant difference between control and NA treatment (p 0.05). The number sign (#) indicates a significant difference between $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ containing receptors (p 0.05).

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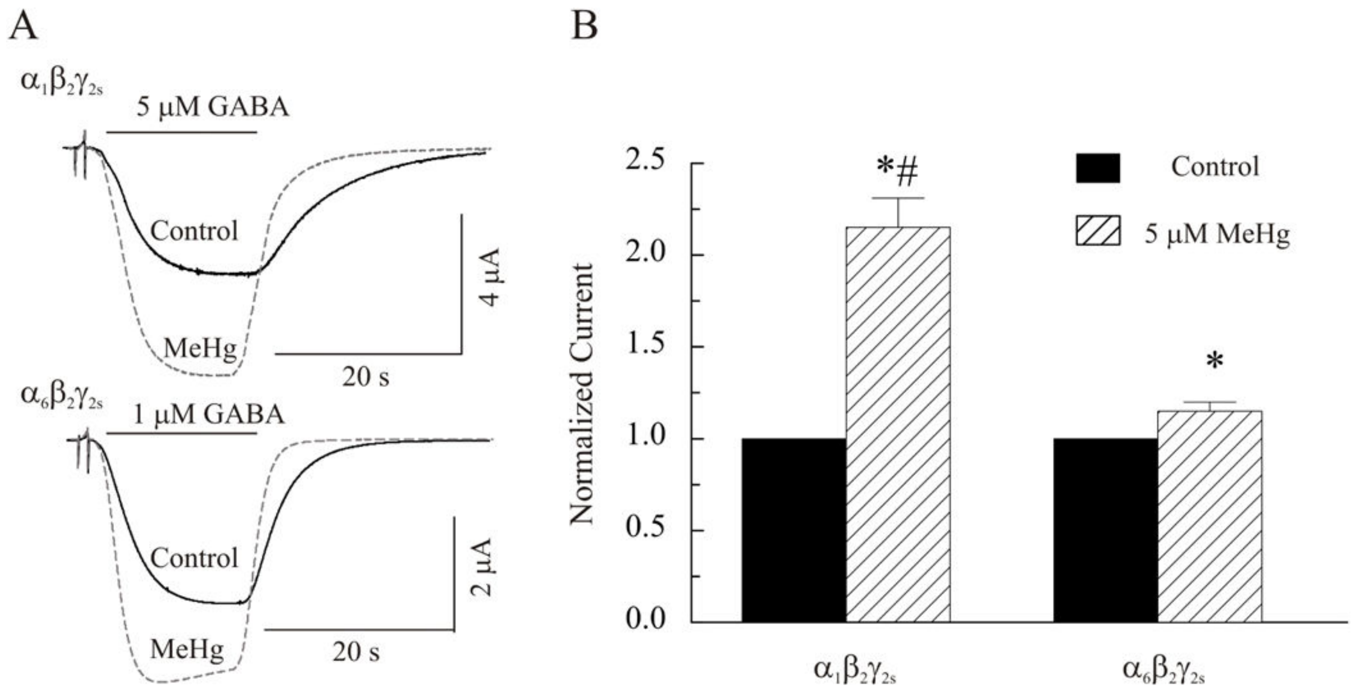

Figure 4.

MeHg potentiates GABA-evoked currents mediated by either $\alpha_1\beta_2\gamma_{2S}$ or $\alpha_6\beta_2\gamma_{2S}$ receptors. A, Whole cell currents were recorded from oocytes expressing $\alpha_1\beta_2\gamma_{2S}$ **(Top)** or $\alpha_6\beta_2\gamma_{2S}$ **(Bottom)** in response to 5 or 1 μM GABA, respectively, at a holding potential of −60 mV in the absence and presence of 5 μM MeHg. B, Comparison of effects of MeHg on I_{GABA} amplitude of the two receptor subtypes (n = 7). The asterisk (*) indicates a significant difference between control and MeHg treatment (p 0.05). The number sign (#) indicates a significant difference between $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_6\beta_2\gamma_{2S}$ receptors (p 0.05).

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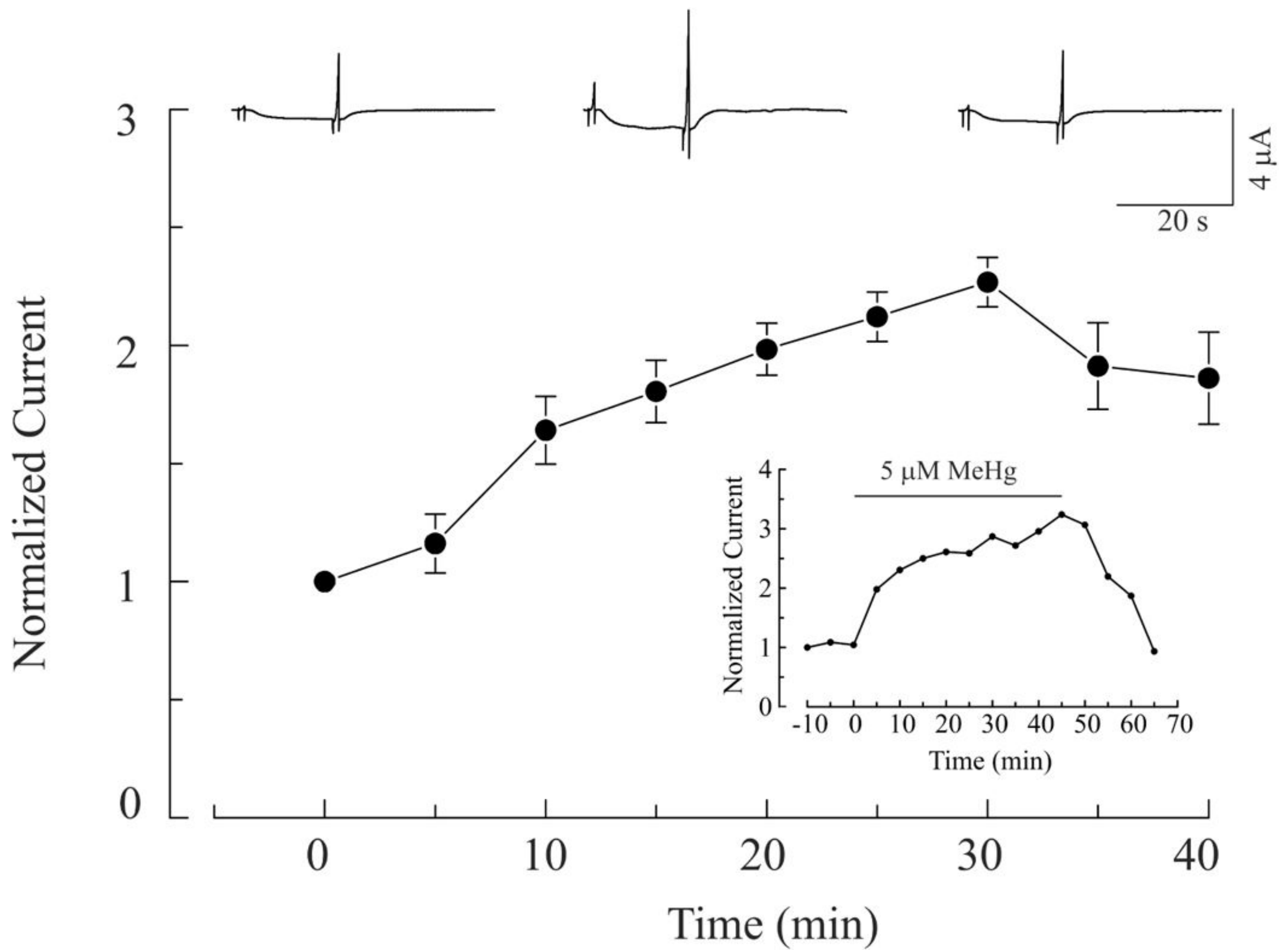

Figure 5.

MeHg-induced potentiation of I_{GABA} in oocytes expression $\alpha_1\beta_2\gamma_{2S}$ receptors is time-dependent and reversible. **Top:** whole cell currents were recorded before, at 30 min after MeHg exposure, and following a 10 minute wash respectively. **Bottom:** Time-course of MeHg-induced potentiation of GABA currents mediated by $\alpha_1\beta_2\gamma_{2S}$ receptors. Application of 5 μM MeHg began at 0 min, continued for 30 min, was subsequently stopped, and the oocyte washed with MeHg-free solution. Values are mean ± SEM (n = 7).

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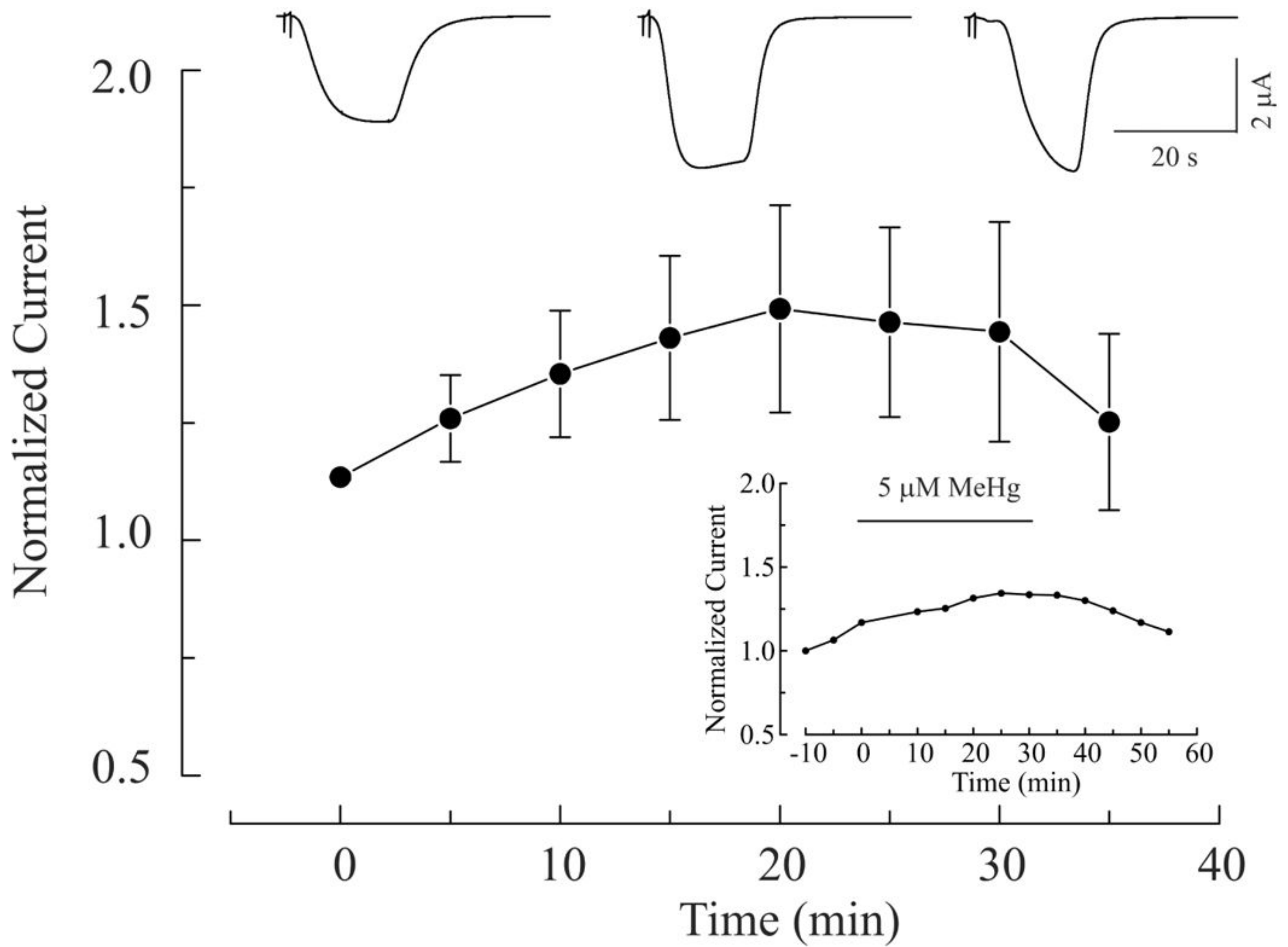

Figure 6.

MeHg-induced potentiation of I_{GABA} in oocytes expressing $\alpha_6\beta_2\gamma_{2S}$ receptors is time-dependent and reversible. **Top:** whole cell currents recorded before, at 20 min after MeHg exposure and following a 10 min wash with MeHg-free solution respectively. **Bottom:** Time-course of MeHg-induced potentiation of GABA currents mediated by $\alpha_6\beta_2\gamma_{2S}$ receptors. Application of 5 μM MeHg began at 0 min, continued for 30 min, was subsequently stopped, and the oocyte washed with MeHg-free solution. Values are mean ± SEM (n = 7).

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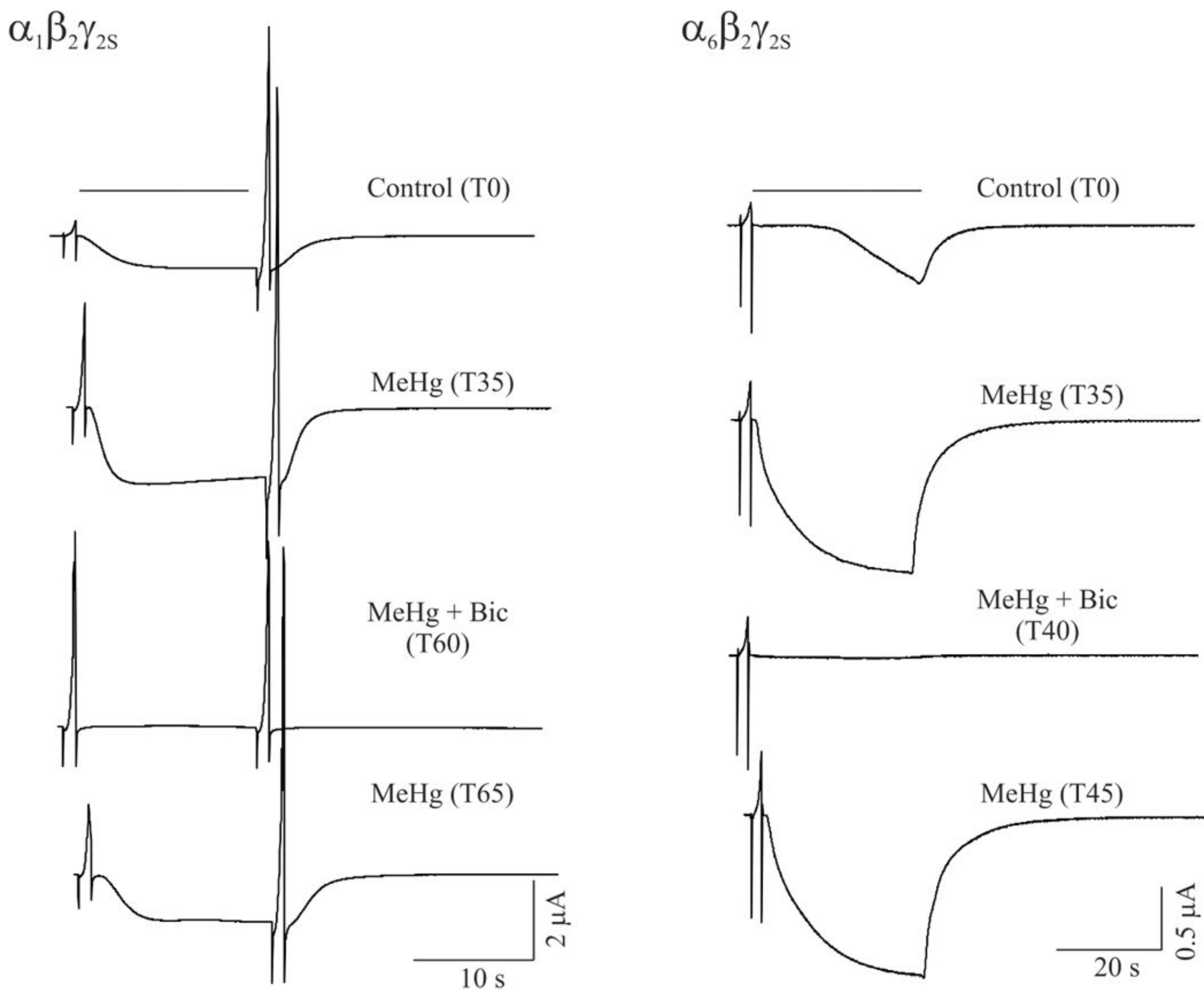

Figure 7.

MeHg-induced potentiation of I_{GABA} mediated by both subtype receptors is sensitive to block by the $GABA_A$ receptor antagonist bicuculline. **Left**, whole cell currents were recorded from a representative oocyte expressing recombinant $\alpha_1\beta_2\gamma_{2S}$ containing receptors ar a holding potential of −60 mV. Currents were recorded before (control), at 35 min (T35) in the presence of 5 μM MeHg, at 60 min (T60) in the presence of both 5 μM MeHg and 20 μM bicuculline and 65 min again in the presence of MeHg alone. **Right**, whole cell currents were recorded from a representative oocyte expressing recombinant $\alpha_6\beta_2\gamma_{2S}$ receptors under similar conditions as for $\alpha_1\beta_2\gamma_{2S}$ receptors, but with different time points.

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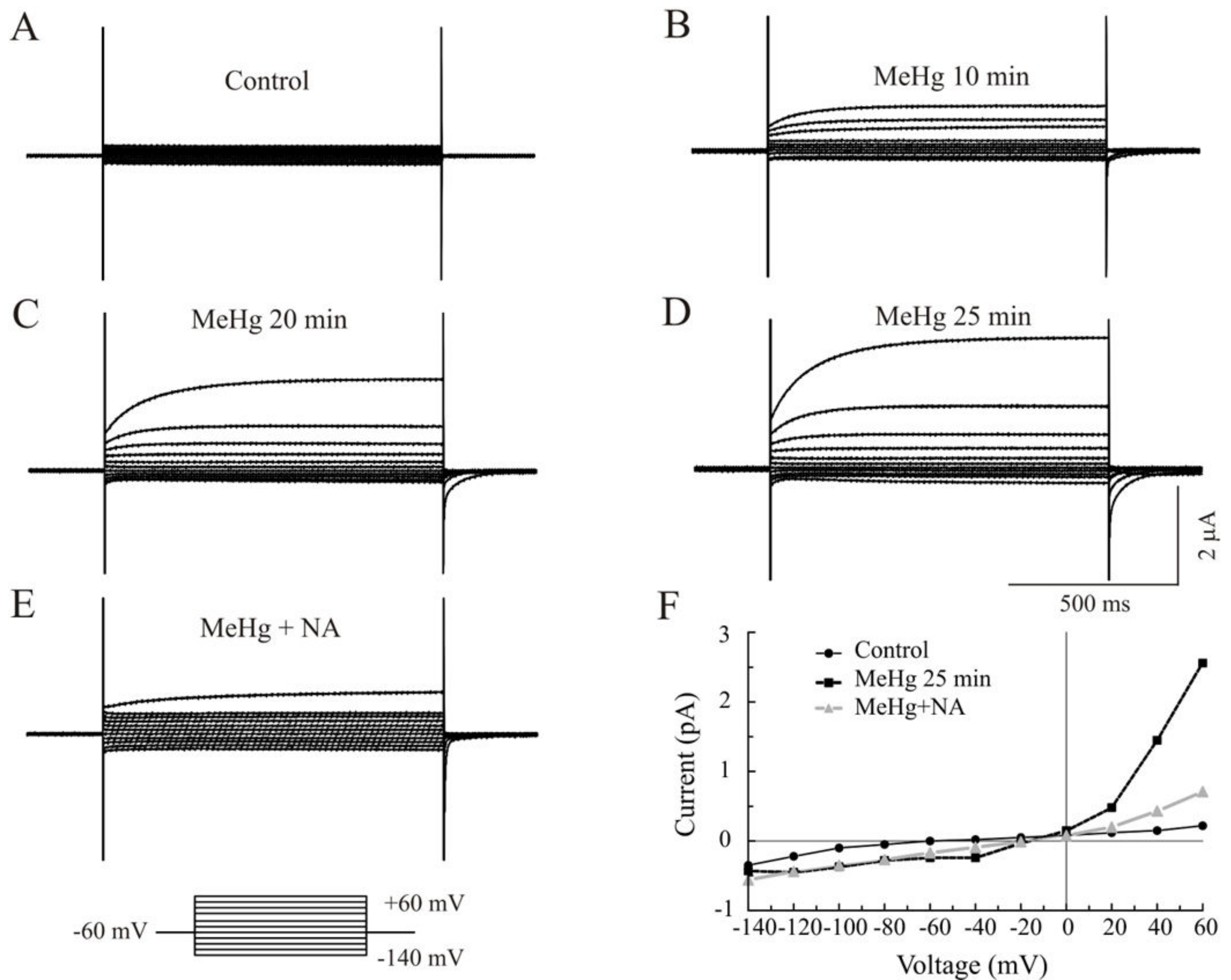

Figure 8.

MeHg caused an increase in outward currents in the water-injected oocytes. A family of whole cell currents was recorded from a water-injected oocyte at a holding potential of −60 mV before (A) and after exposure to MeHg for 10 (B), 20 (C) and 25 min (D). Treatment with niflumic acid (NA, 300 μM) (B) for 5 min, a Ca^{2+}-activated chloride channel blocker, caused a significant decrease in MeHg-induced outward currents (E). Effect of MeHg and NA on whole cell current-voltage relationships are shown in F. Clearly, MeHg increased both inward and outward currents, but primarily so outward currents. Each trace is a representative depiction of 5 individual experiments.