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Effect of thimerosal, a preservative in vaccines, on intracellular Ca^{2+} concentration of rat cerebellar neurons

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Abstract

The effect of thimerosal, an organomercurial preservative in vaccines, on cerebellar neurons dissociated from 2-week-old rats was compared with those of methylmercury using a flow cytometer with appropriate fluorescent dyes. Thimerosal and methylmercury at concentrations ranging from 0.3 to 10 μM increased the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) in a concentration-dependent manner. The potency of 10 μM thimerosal to increase the $[\text{Ca}^{2+}]_i$ was less than that of 10 μM methylmercury. Their effects on the $[\text{Ca}^{2+}]_i$ were greatly attenuated, but not completely suppressed, under external Ca^{2+} -free condition, suggesting a possibility that both agents increase membrane Ca^{2+} permeability and release Ca^{2+} from intracellular calcium stores. The effect of 10 μM thimerosal was not affected by simultaneous application of 30 μM L-cysteine whereas that of 10 μM methylmercury was significantly suppressed. The potency of thimerosal was similar to that of methylmercury in the presence of L-cysteine. Both agents at 1 μM or more similarly decreased the cellular content of glutathione in a concentration-dependent manner, suggesting an increase in oxidative stress. Results indicate that thimerosal exerts some cytotoxic actions on cerebellar granule neurons dissociated from 2-week-old rats and its potency is almost similar to that of methylmercury.

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Keywords: Thimerosal; Vaccine; Preservative; Cerebellar neurons; Calcium

1. Introduction

Thimerosal is an organomercurial preservative in vaccines to prevent contamination with harmful microbes and its derivative is ethylmercury. There is a concern on the part of public health community that adverse health consequences by thimerosal may occur among infants during immunization schedule although it is generally believed that the safety of thimerosal use for humans have been established (Mahaffey, 1999; Ball et al., 2001).

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In recent laboratory studies, thimerosal is used as a sensitizer of intracellular inositol-1,4,5-triphosphate (IP₃) receptor and it increases the intracellular Ca²⁺ concentration ([Ca²⁺]_i) of neurons (Carey and Matsumoto, 1999, 2000; Snider et al., 2000; Faure et al., 2001). The disturbance of intracellular Ca²⁺ homeostasis in neurons leads to cell injury and death (Choi, 1988; Siesjo and Bengtsson, 1989; Orrenius et al., 1992). Methylmercury also increases the [Ca²⁺]_i of cerebellar granule neurons that are severely damaged in the case of methylmercury poisoning (Sarafian, 1993; Oyama et al., 1994). The best-known epidemic of organomercurial poisoning is “Minamata” disease resulted from the consumption of methylmercury-contaminated fishes and the in utero exposure to methylmercury resulted in infants exhibiting severe neurologic injury (Harada, 1995). It is very important to compare the toxicity of thimerosal with that of methylmercury since the guidelines for mercury toxicity are based on laboratory and epidemiological studies on the methylmercury toxicity. Therefore, we have compared the effect of thimerosal on the [Ca²⁺]_i of cerebellar neurons dissociated from 2-week-old rats with that of methylmercury using a flow cytometer and appropriate fluorescent dyes.

2. Materials and methods

2.1. Preparation

Experiments were performed on the cerebellar neurons enzymatically obtained from 2-week-old Wistar rats. Technique for enzymatic dissociation of rat cerebellar neurons was previously described elsewhere (Oyama et al., 1992a,b, 1995). In brief, rat cerebellum was sliced at a thickness of 400–500 μm by a microslicer (DTK-1500, Dosaka EM, Kyoto, Japan). Slices were treated with dispase (1000 protease units per ml; Godo Shusei, Tokyo, Japan) for 60 min in the HEPES-buffered Tyrode’s solution (NaCl 150 mM; KCl 5 mM; CaCl₂ 2 mM; MgCl₂ 1 mM; glucose 5 mM; HEPES 5 mM; and an appropriate amount of NaOH to adjust to pH 7.4) which was oxygenated at a temperature of 36 °C. After enzymatic treatment, slices were gently triturated by pipetting in Tyrode’s solution to dissociate single cerebellar neurons. Tyrode’s solution containing dissociated neurons was

passed through a mesh (diameter of 53 μm) to remove residues. Since the neurons (more than 95%) in the filtered cell suspension were characterized by their small size (diameter of about 10 μm or less) and spherical shape under microscopic observation, the cells were likely cerebellar granule cells. Furthermore, neurons could be selected from the cytogram (forward-angle light scatter versus side light scatter; cell diameter versus cell density) for fluorescence measurement. Of all neurons freshly dissociated from mammalian brains, cerebellar granule cells are very suitable for flow-cytometric analysis at present (Oyama et al., 1992a,b, 1993a,b).

2.2. Fluorescence measurements and analysis

To estimate the [Ca²⁺]_i of living cells, a combination of two fluorescent probes, fluo-3-AM (Dojindo Laboratory, Kumamoto, Japan) and propidium iodide (Katayama Chemical Industries, Osaka, Japan) was used (Oyama et al., 1995). Respective dye was added into the cell suspension to achieve a final concentration of 500 nM for fluo-3-AM or 5 μM for propidium iodide. Before any measurement of fluo-3 fluorescence, the cerebellar neurons were incubated with fluo-3-AM for 60 min. Fluorescence obtained from the neurons incubated with propidium iodide was measured at an appropriate time (2 min at least) after the dye application. Fluo-3 is used for monitoring the [Ca²⁺]_i of intact living cells (Minta et al., 1989). Since propidium, a highly impermeant dye to intact plasma membrane, cannot stain living neurons, living neurons show only fluo-3 fluorescence (Fig. 1A). There is propidium fluorescence in dead and damaged neurons since propidium stains neurons that are dead or have compromised membranes (Fig. 1A). Therefore, the simultaneous measurements of fluo-3 and propidium fluorescence may provide a useful information concerning the [Ca²⁺]_i of living neurons, which retain fluo-3 in the presence of cytotoxic substances.

5-Chloromethylfluorescein diacetate (5CMF-DA, Molecular Probes Inc., Eugene, USA) was used to estimate cellular content of glutathione in thymocytes (Chikahisa et al., 1996). 5CMF-DA (1 mM in DMSO) was added to cell suspension to achieve a final concentration of 1 μM. The cells were incubated with 5CMF-DA for 30 min before measurement. The

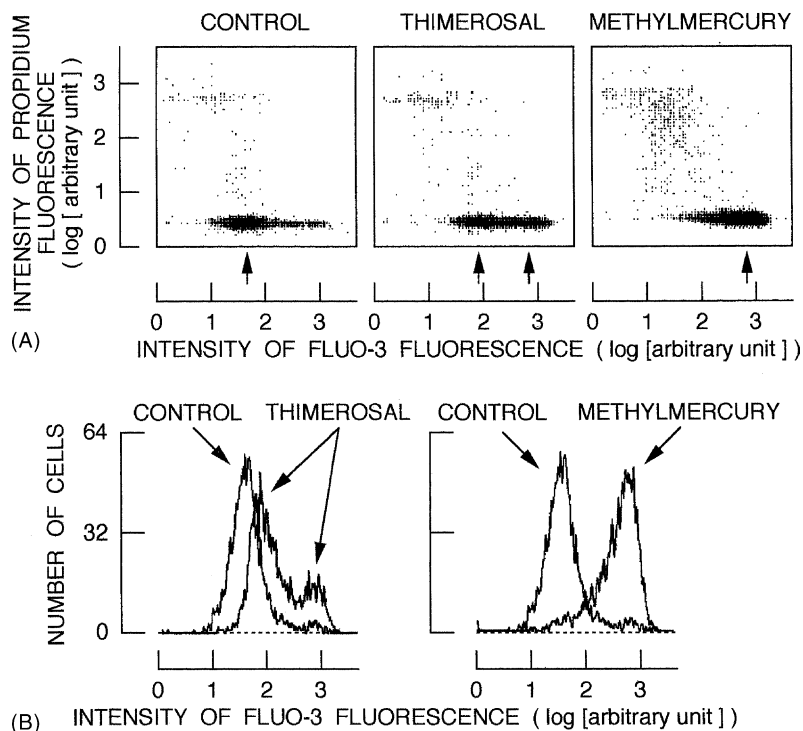


Fig. 1. Flow-cytometric measurements of fluo-3 and propidium fluorescence from dissociated cerebellar neurons (2500 cells). Upper three panels (A) show the fluorescence cytograms, fluo-3 fluorescence (abscissa) vs. propidium fluorescence (ordinate), before (left panel, CONTROL) and after application of $10\ \mu\text{M}$ thimerosal (center panel, THIMEROSAL) and $10\ \mu\text{M}$ methylmercury (right panel, METHYLMERCURY). The neurons under dotted line in each panel indicate the cells that were stained with fluo-3, but not with propidium. The neurons over the line were stained with propidium. Arrows at abscissa indicate the peak intensity of fluo-3 fluorescence of each cytogram. Lower two panels (B) show the histogram of fluo-3 fluorescence obtained from the neurons that were not stained with propidium (intact neurons) in the absence (CONTROL) and presence of $10\ \mu\text{M}$ thimerosal (middle panel, THIMEROSAL) and $10\ \mu\text{M}$ methylmercury (right panel, METHYLMERCURY). The histograms were constructed from 2500 cells.

5CMF fluorescence was also monitored from the neurons that were not stained with propidium iodide.

Measurements of fluorescence from brain neurons were made by a flow cytometer (Cyto ACE-150, JASCO, Tokyo, Japan). Excitation wavelength for fluo-3 and propidium was 488 nm produced by an argon laser. Emission was detected at wavelength of $530 \pm 20\ \text{nm}$ for fluo-3 and 5CMF and $600 \pm 20\ \text{nm}$ for propidium. Statistical analysis was performed with two sample *t* test with and without Welch's correction. A *P* value of <0.05 was considered to be significant.

2.3. Chemicals

Thimerosal was purchased from Sigma (St. Louis, USA). Methylmercury chloride was obtained from

Nacalai Chemicals (Osaka, Japan). Thimerosal and methylmercury were initially dissolved in dimethyl sulfoxide (Wako Pure Chemicals, Osaka, Japan). Dimethyl sulfoxide as a solvent at a final concentration (0.3% or less) did not affect the measurements of any fluorescence. The solutions of thimerosal and methylmercury were prepared just before use. Other chemical reagents were obtained from Katayama Chemical Industries (Osaka, Japan).

One may argue that ethylmercury chloride should be used as a reference agent since thimerosal contains ethylmercury. However, methylmercury chloride was employed since the guidelines for mercury toxicity are based on laboratory and epidemiological studies on the methylmercury toxicity.

2.4. Data presentation and statistics

Numerical values of experimental data are presented as mean \pm standard deviation (S.D.) of the mean. Statistical analysis was initially performed by two-sample *t*-test. *P* value of <0.05 was considered significant. Statistical significance was also confirmed by an overall test of significance using an *F*-ratio derived from analysis of variance.

3. Results

3.1. Effects of thimerosal and methylmercury on fluo-3 fluorescence of brain neurons under normal Ca^{2+} condition

As shown in the control fluorescence cytogram (left panel of Fig. 1A), the large population of neurons exerted fluo-3 fluorescence but not propidium fluorescence, indicating living neurons. Thimerosal at $10 \mu\text{M}$ slightly increased the population of neurons stained with propidium and increased the intensity of fluo-3 fluorescence in the living neurons (center panel of

Fig. 1A), indicating that thimerosal slightly increased the population of dead neurons, and increased the $[Ca^{2+}]_i$ of living neurons. Methylmercury at $10 \mu\text{M}$ increased the population of neurons stained with propidium and greatly augmented the fluo-3 fluorescence of living neurons (right panel of Fig. 1A). Thimerosal at $10 \mu\text{M}$ shifted the histogram of fluo-3 fluorescence toward a direction of higher intensity with double peaks (left panel of Fig. 1B) whereas methylmercury shifted it with single peak (right panel of Fig. 1B). Result suggests that the potency of $10 \mu\text{M}$ methylmercury to increase the $[Ca^{2+}]_i$ is greater than that of $10 \mu\text{M}$ thimerosal. The concentration-dependent changes in relative intensity of fluo-3 fluorescence by thimerosal and methylmercury are summarized in Fig. 2.

3.2. Effects of thimerosal and methylmercury on fluo-3 fluorescence of brain neurons under Ca^{2+} -free condition

To reveal the source for $[Ca^{2+}]_i$ increased by thimerosal, the effect of thimerosal on fluo-3

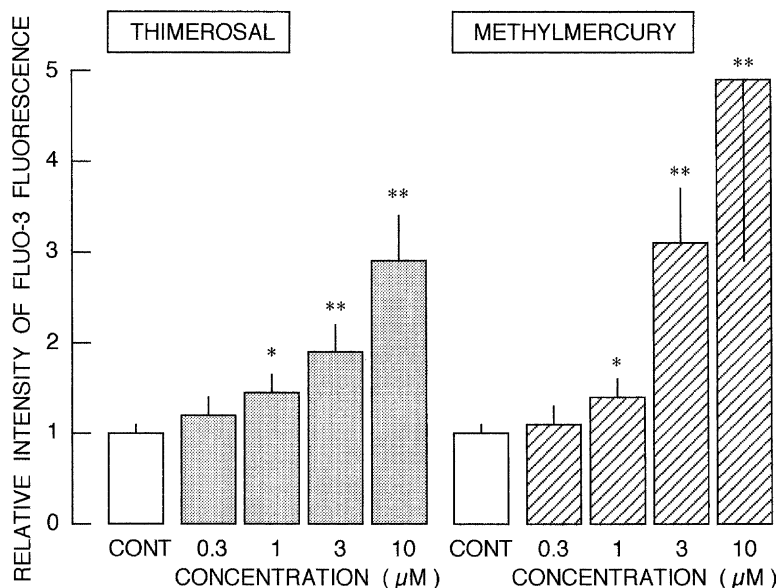


Fig. 2. Concentration-dependent effects of thimerosal (left panel, THIMEROSAL) and methylmercury (right panel, METHYLMERCURY) on the mean intensity of fluo-3 fluorescence monitored from 2500 living neurons. The effects were examined at 30min after the drug application. Each column and bar indicate the average and S.D. of 4–8 experiments. Asterisks (*) and (**) show the significant change ($P < 0.01$ and $P < 0.005$) between the control and test group, respectively.

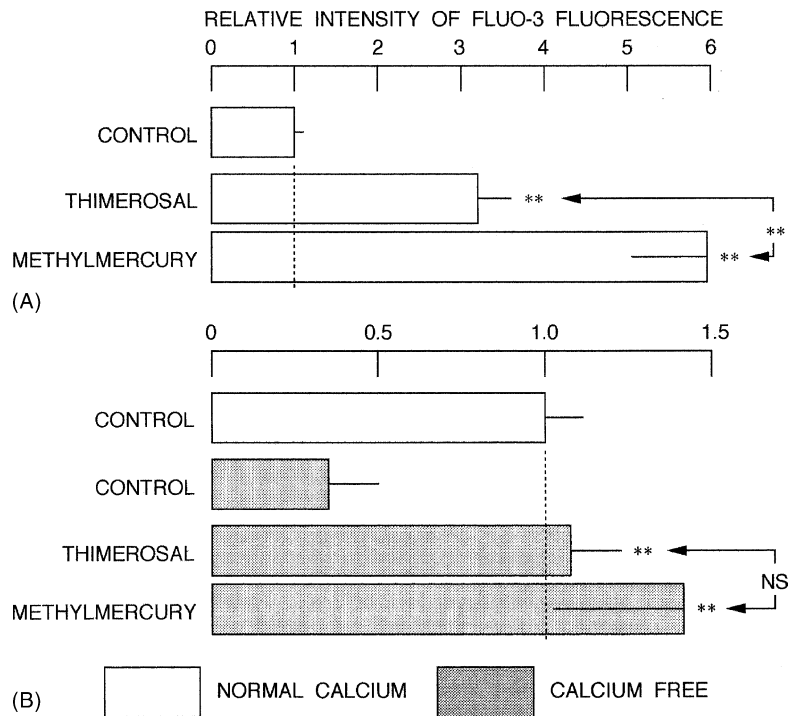


Fig. 3. Effects of thimerosal (THIMEROSAL) and methylmercury (METHYLMERCURY) on the mean fluo-3 fluorescence intensity of 2500 living neurons under Ca^{2+} -free condition. Upper panel (A) show the effects under normal Ca^{2+} condition (open columns). Lower panel (B) the effects under Ca^{2+} -free condition (filled columns). Each column and bar show the average and S.D. of three to five experiments, respectively. Dotted line indicates the control intensity of fluo-3 fluorescence under normal Ca^{2+} condition. Asterisks (**) show the significant change ($P < 0.005$) between the respective control and test group.

fluorescence was tested in Ca^{2+} -free Tyrode's solution where CaCl_2 was replaced with equimolar MgCl_2 and 2 mM EGTA was added. As shown in Fig. 3, the increase in mean intensity of fluo-3 fluorescence by $10 \mu\text{M}$ thimerosal and $10 \mu\text{M}$ methylmercury was greatly attenuated under the Ca^{2+} -free condition, suggesting that the increase in $[\text{Ca}^{2+}]_i$ by thimerosal and methylmercury was largely dependent on the external Ca^{2+} . Whereas, the increase in mean intensity of fluo-3 fluorescence by $10 \mu\text{M}$ methylmercury was significantly greater than that by $10 \mu\text{M}$ thimerosal under normal Ca^{2+} condition (Fig. 3A), there was no significant difference between them under the Ca^{2+} -free condition (Fig. 3B). Result may suggest that the potency of thimerosal to release Ca^{2+} from intracellular stores is similar to that of methylmercury.

3.3. Effect of L-cysteine on thimerosal- and methylmercury-induced increase in fluo-3 fluorescence intensity of brain neurons

Methylmercury is present in a form of methylmercury-S conjugate under in vivo condition (Yasutake et al., 1989) and the efficacy of methylmercury to increase the $[\text{Ca}^{2+}]_i$ of living neurons is greatly reduced by conjugating methylmercury with L-cysteine or trimethylbenzylmercaptan (Oyama et al., 1998). One may argue a possibility that the action of thimerosal on the $[\text{Ca}^{2+}]_i$ is also affected by L-cysteine. Therefore, the effects of $10 \mu\text{M}$ thimerosal and $10 \mu\text{M}$ methylmercury on the fluo-3 fluorescence of living neurons were compared in the presence and absence of $30 \mu\text{M}$ L-cysteine. As shown in Fig. 4, whereas the increase in mean intensity of fluo-3 fluorescence

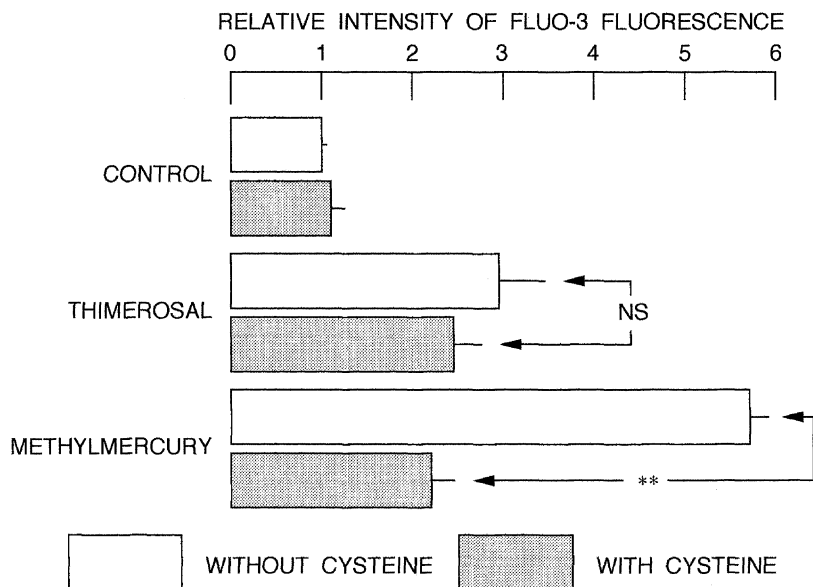


Fig. 4. Effects of L-cysteine on the thimerosal- and methylmercury-induced increases in the mean intensity of fluo-3 fluorescence of brain neurons. Each column and bar show the average and S.D. of three to four experiments, respectively. Asterisk (**) and NS respectively show the significant change ($P < 0.005$) and no significant change between the cells treated with (filled columns) and without (open columns) cysteine.

by methylmercury was significantly suppressed in the presence of L-cysteine as previously shown (Oyama et al., 1998, 2000), it was not the case for thimerosal. Therefore, it is likely that the potency of thimerosal to increase the $[Ca^{2+}]_i$ is similar to that of methylmercury in the presence of nonprotein thiols.

3.4. Effects of thimerosal and methylmercury on 5CMF fluorescence of brain neurons

An increase in $[Ca^{2+}]_i$ increases oxidative stress while oxidative stress induces an increase in $[Ca^{2+}]_i$ of brain neurons (Oyama et al., 1996) and methylmercury increases oxidative stress, resulting in reduction of cellular content of glutathione in rat cerebellar neurons (Oyama et al., 1994; Okazaki et al., 1997). Therefore, to see if thimerosal reduces the cellular content of glutathione in brain neurons, the effect of thimerosal on the intensity of 5CMF fluorescence was compared with that of methylmercury. As shown in Fig. 5, the mean intensity of 5CMF fluorescence was concentration-dependently decreased by the application of thimerosal at concentrations ranging from 1 to 10 μ M. Thimerosal at 10 μ M almost-completely

diminished the 5CMF fluorescence of brain neurons, indicating a depletion of cellular glutathione. The potency of thimerosal to reduce the content of cellular glutathione was almost similar to that of methylmercury at concentrations tested (Fig. 5).

4. Discussion

Thimerosal, one of organomercurials, is used as a preservative in vaccines and there is no evidence of neurologic dysfunction caused by concentrations of thimerosal in vaccine among the 2-week-olds (Ball et al., 2001). It is metabolized to ethylmercury and thiosalicylate under the in vivo condition. The estimated half-life of ethylmercury is 7 days and the concentrations of mercury in the blood of infants receiving vaccines containing thimerosal do not exceed the safe limits for mercury (Pichichero et al., 2002). Ethylmercury is less neurotoxic than methylmercury in adult male and female rats (Magos et al., 1985). However, thimerosal itself at concentrations ranging from 50 to 500 ng/ml exerts genotoxic action on primary human lymphocytes (Westphal et al., 2003). Therefore, the

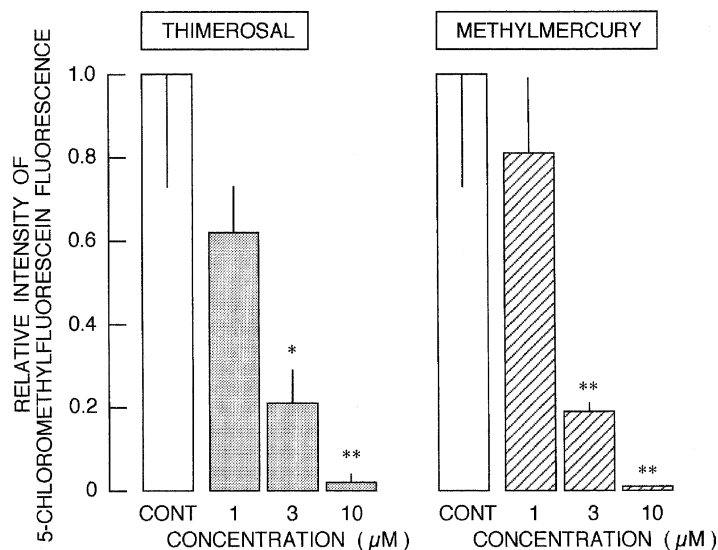


Fig. 5. Effects of thimerosal (left panel, THIMEROSAL) and methylmercury (right panel, METHYLMERCURY) on mean intensity of 5-chloromethylfluorescein (5CMF) fluorescence of brain neurons. Each column and bar show the average and S.D. of three to four experiments. Asterisks (*) and (**) show the significant change ($P < 0.01$ and $P < 0.005$) between the control and test group, respectively.

effect of thimerosal should be also compared with that of methylmercury.

In the present study, thimerosal at micromolar concentrations significantly increased the $[Ca^{2+}]_i$ (Figs. 2 and 3) and decreased the cellular content of glutathione (Fig. 5), suggesting an increase in oxidative stress, in cerebellar neurons dissociated from 2-week-old rats. Methylmercury at same concentrations also did so (Figs. 2, 3 and 5) (Sarafian and Ferity, 1991; Sarafian, 1993; Oyama et al., 1994). It is likely that the effects of thimerosal on rat brain neurons resemble those of methylmercury under the in vitro condition. Furthermore, the potency of thimerosal to increase the $[Ca^{2+}]_i$ of cerebellar neurons dissociated from 2-week-old rats was similar to that of methylmercury conjugated with L-cysteine (Fig. 4). The actions of thimerosal and methylmercury in the presence of L-cysteine may partly mimic those under the in vivo condition since the blood concentration of nonprotein thiols in the rats is about $30 \mu\text{M}$ (Adachi et al., 1994). Both thimerosal and methylmercury increased the $[Ca^{2+}]_i$ and oxidative stress in cerebellar granule cells (Figs. 2, 3 and 5). In rat cerebellar granule neurons, the increase in $[Ca^{2+}]_i$ induces an increase in oxidative stress while the

oxidative stress increases the $[Ca^{2+}]_i$ (Oyama et al., 1996). It is a possibility that uncontrolled and sustained elevation of $[Ca^{2+}]_i$ increases the formation of reactive oxygen species that induce a further increase in $[Ca^{2+}]_i$. If so, such insults induced by thimerosal and methylmercury would lead to cell injury or death in brain neurons (Choi, 1988; Siesjo and Bengtsson, 1989; Orrenius et al., 1992). It can be concluded that the potency of thimerosal to induce the cytotoxic action on brain neurons dissociated from 2-week-old rats under the in vitro condition is similar to that of methylmercury.

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