**FULL PAPER** *Pharmacology*

# Heavy metal chelator TPEN attenuates fura-2 fluorescence changes induced by cadmium, mercury and methylmercury

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ABSTRACT. Stimulation with heavy metals is known to induce calcium ($Ca^{2+}$) mobilization in many cell types. Interference with the measurement of intracellular $Ca^{2+}$ concentration by the heavy metals in cells loaded with $Ca^{2+}$ indicator fura-2 is an ongoing problem. In this study, we analyzed the effect of heavy metals on the fura-2 fluorescence ratio in human SH-SY5Y neuroblastoma cells by using TPEN, a specific cell-permeable heavy metal chelator. Manganese chloride (30–300 $\mu$M) did not cause significant changes in the fura-2 fluorescence ratio. A high concentration (300 $\mu$M) of lead acetate induced a slight elevation in the fura-2 fluorescence ratio. In contrast, stimulation with cadmium chloride, mercury chloride or MeHg (3–30 $\mu$M) elicited an apparent elevation of the fura-2 fluorescence ratio in a dose-dependent manner. In cells stimulated with 10 or 30 $\mu$M cadmium chloride, the addition of TPEN decreased the elevated fura-2 fluorescence ratio to basal levels. In cells stimulated with mercury or MeHg, the addition of TPEN significantly decreased the elevation of the fura-2 fluorescence ratio induced by lower concentrations (10 $\mu$M) of mercury or MeHg, but not by higher concentrations (30 $\mu$M). Pretreatment with $Ca^{2+}$ channel blockers, such as verapamil, 2-APB or lanthanum chloride, resulted in different effects on the fura-2 fluorescence ratio. Our study provides a characterization of the effects of several heavy metals on the mobilization of divalent cations and the toxicity of heavy metals to neuronal cells.

KEY WORDS: Fura-2, heavy metal, SH-SY5Y, TPEN

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Heavy metals are distributed as environmental pollutants, and human and animal exposure to excessive levels of heavy metals is a global public health problem. Although some heavy metals, such as copper ($Cu^{2+}$), zinc ($Zn^{2+}$), manganese ($Mn^{2+}$) and iron ($Fe^{2+}$), are essential for maintaining normal physiological functions, they can lead to poisoning at higher concentrations. Other metals, including mercury ($Hg^{2+}$), cadmium ($Cd^{2+}$) and lead ($Pb^{2+}$), are not considered essential for biological functions. Exposure to both essential and non-essential heavy metals through inhalation or ingestion of contaminated food and water is known to induce abnormal alterations in the central nervous system, liver, kidneys and hematopoietic system, thus presenting a significant health hazard [18, 20].

Calcium ($Ca^{2+}$) is a highly versatile intracellular signaling molecule that regulates many cellular processes, such as gene transcription, cell motility, exocytosis, cell growth and cell death [4, 41]. Although the antagonizing effect of heavy metals on $Ca^{2+}$ channels has been demonstrated [24, 29], stimulation with heavy metals can induce an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) in neuronal cells. An increase in $[Ca^{2+}]_i$ has been reported as induced by cadmium in cerebral cortical neurons [40], cerebellar granule neurons [25] and a neuronal cell line [35], by methylmercury (MeHg) in rat cerebellar slices [39], a neuronal cell line [13–15] and cerebellar neurons [9, 23, 26, 30], by mercury in cortical neurons [36], by manganese in cerebellar neurons [37] and by lead in rat hippocampal neurons [10]. Therefore, the disturbance of $Ca^{2+}$ homeostasis is believed to be involved in the toxicity of heavy metals.

BAPTA-based fluorescent calcium indicators, such as fura-2 and fluo-3, are widely used for measuring $[Ca^{2+}]_i$. However, some heavy metals can bind to the fluorescent calcium indicators and change their fluorescence excitation spectra. For example, $Cd^{2+}$ can bind to fura-2 with an extremely high affinity, activating spectral responses similar to $Ca^{2+}$ [17, 21]. $Zn^{2+}$, $Pb^{2+}$, strontium ($Sr^{2+}$), barium ($Ba^{2+}$) and lanthanum ($La^{3+}$) are also known to elicit changes of spectra similar to the $Ca^{2+}$ complex [2, 33]. In contrast, $Cu^{2+}$, $Fe^{2+}$, nickel ($Ni^{2+}$), cobalt ($Co^{2+}$) and $Mn^{2+}$ are known to quench the fluorescent signal of fura-2 [12, 19, 21], while $Hg^{2+}$ is believed to have no effect on fura-2 spectra [21]. These reports suggest that endogenous and exogenous heavy metals may interfere with the measurement of $[Ca^{2+}]_i$ using fura-2, and this potential problem makes it difficult to analyze the precise effect of heavy metals on $[Ca^{2+}]_i$.

In this study, we analyzed the effect and contribution of several heavy metals on $[Ca^{2+}]_i$ measurement in human neuroblastoma SH-SY5Y cells loaded with fura-2 in the presence of tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), which is a specific cell-permeable heavy metal chelator. In addition, we examined the effects of $Ca^{2+}$ channel blockers, such as verapamil, 2-Aminoethoxydiphenyl borate (2-APB)

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and lanthanum chloride, on changes in the fura-2 fluorescence ratio induced by heavy metals.

## MATERIALS AND METHODS

*Cell culture*: Human neuroblastoma cell line SH-SY5Y (ATCC, Manassas, VA, U.S.A.), were grown in Dulbecco's Modified Eagle's Medium (DMEM) mixed 1:1 with Ham's F-12 nutrient mixture (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum, 100 unit/m*l* penicillin and 100 $\mu$g/m*l* streptomycin at 37°C in a humidified 5% $CO_2$ atmosphere. Two days before experimentation, cells were seeded at a density of $7 \times 10^4$ cells/cm$^2$ in a 96-well plate.

*Measurement of fura-2 fluorescence changes*: Cells in a 96-well plate were serum-starved for 4 hr; calcium indicator fura-2 was then loaded into the cells by using Calcium kit II fura-2 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, SH-SY5Y cells were incubated with 5 $\mu$M fura-2/AM in the presence of 0.04% Pluronic F-127, a dispersing agent to improve the efficiency of loading with fura-2, and 1.25 mM probenecid, a blocker of organic anion transport to prevent leakage of fura-2 from cells [8]. After 1 hr incubation at 37°C, fura-2 fluorescence was measured at 500 nm emission after excitation at 340 nm (F340) or 380 nm (F380) using an Infinite M200 plate reader (Tecan, Männedorf, Switzerland) at 37°C. The change in $[Ca^{2+}]_i$ was reflected by the ratio of F340 and F380. To determine the changes in fura-2 fluorescence ratio induced by heavy metal compounds, cells were treated with manganese chloride (Sigma-Aldrich), lead acetate (Wako, Osaka, Japan), cadmium chloride (Kanto Chemical, Tokyo, Japan), mercuric chloride (Wako) and MeHg chloride (Kanto Chemical) dissolved in distilled water. We confirmed that the cells adhered to the bottom of the plate after 6 hr exposure to heavy metal compounds. The cells were also treated with three $Ca^{2+}$ channel blockers, lanthanum chloride (Wako) dissolved in distilled water, verapamil (Sigma-Aldrich) and 2-APB dissolved in DMSO, 30 min before heavy metal exposure. The heavy metal chelator TPEN was dissolved in DMSO and added 3 hr after the stimulation with heavy metals to determine the contribution of endogenous and exogenous heavy metals on fura-2 fluorescence changes. We measured the effect of TPEN (20 $\mu$M) on the fura-2 fluorescence ratio after a 10 min treatment with TPEN, since our preliminary experiments showed that the effect of TPEN on fura-2 fluorescence reached maximum and stabilized within 10 min of the treatment.

*Data analysis*: Results are expressed as the mean ±SEM. Differences between means were evaluated by Student's *t*-test or Bonferroni's correction for multiple comparisons; *P* values<0.05 were considered significant.

## RESULTS

*Fura-2 fluorescence changes induced by heavy metals*: Figure 1 shows the changes in the fura-2 fluorescence ratio in SH-SY5Y cells by stimulation with manganese chloride, lead acetate, cadmium chloride, mercury chloride or MeHg. In cells without heavy metal stimulation (controls), we did not observe significant changes in the fura-2 fluorescence ratio within 6 hr under experimental conditions (data not shown). First, we examined the effect of manganese chloride and lead acetate on fura-2 fluorescence, since these metals are well known to be neurotoxic substances and have been shown to stimulate $[Ca^{2+}]_i$ [31, 37]. Manganese chloride (30–300 $\mu$M) did not cause a significant change in the fura-2 fluorescence ratio within 6 hr of exposure (Fig. 1A). Although manganese chloride had no effect on the fura-2 fluorescence ratio, we observed that it induced a quenching of fluorescent signals at both F340 and F380 in a dose-dependent manner (data not shown). Lead (30–100 $\mu$M) did not cause changes in the fura-2 fluorescence ratio (Fig. 1B). However, a higher concentration (300 $\mu$M) of lead induced a slight but significant elevation in the fura-2 fluorescence ratio. In contrast, stimulation with cadmium chloride, mercury chloride or MeHg elicited an apparent elevation of the fura-2 fluorescence ratio in a dose-dependent manner (Fig. 1C, 1D and 1E). The peak increase in the fura-2 fluorescence ratio from basal level (ΔRatio (340/380)) was induced by 30 $\mu$M cadmium chloride (2.9 ± 0.1 at 6 hr), 30 $\mu$M mercury chloride (2.3 ± 0.4 at 3 hr) and 30 $\mu$M MeHg (3.3 ± 0.1 at 4 hr). We confirmed that $Ca^{2+}$ ionophore ionomycin (3 $\mu$M) induced significant increase in the ΔRatio (340/380) (0.5 ± 0.1 at 3 min).

*Effect of TPEN on fura-2 fluorescence changes induced by cadmium chloride, mercury chloride or MeHg*: Since we observed an apparent elevation of the fura-2 fluorescence ratio induced by stimulation with cadmium chloride, mercury chloride and MeHg in SH-SY5Y cells, we examined the contribution of heavy metal cations to the increase in the fura-2 fluorescence ratio by using TPEN, a cell-permeable chelator for heavy metal cations with a low affinity for $Ca^{2+}$ [1]. In cells stimulated with 10 or 30 $\mu$M cadmium chloride, the addition of TPEN at 3 hr after exposure significantly decreased the elevated fura-2 fluorescence ratio to the basal levels within 10 min (119.6 ± 2.4% or 109.0 ± 1.5% decrease in ΔRatio (F340/F380) induced by 10 or 30 $\mu$M cadmium chloride, respectively), suggesting that a cadmium chloride-induced increase in the fura-2 fluorescence ratio was dependent on an increase in intracellular heavy metal cations but not intracellular $Ca^{2+}$ (Fig. 2A). We observed that the effect of TPEN on the elevated fura-2 fluorescence ratio induced by 30 $\mu$M cadmium chloride was dose-dependent (2.4 ± 3.4%, 69.3 ± 3.5% or 98.3 ± 0.5% decrease in ΔRatio (F340/F380) by 5, 10 or 20 $\mu$M TPEN, respectively, n=4). In mercury chloride or MeHg treated cells, the addition of TPEN significantly decreased the elevation of ΔRatio (F340/F380) induced by a lower concentration (10 $\mu$M) of mercury chloride (173.7 ± 19.4% decrease in ΔRatio (F340/F380)) or MeHg (110.7 ± 11.1% decrease in ΔRatio (F340/F380)) (Fig. 2B and 2C). However, although the addition of TPEN partially decreased the elevation of ΔRatio (F340/F380) induced by a higher concentration (30 $\mu$M) of mercury chloride (44.1 ± 18.0% decrease in ΔRatio (F340/F380)) or MeHg (34.4 ± 15.1% decrease in ΔRatio (F340/F380)), the effect was not significant.

*Effect of $Ca^{2+}$ channel blockers on fura-2 fluorescence*

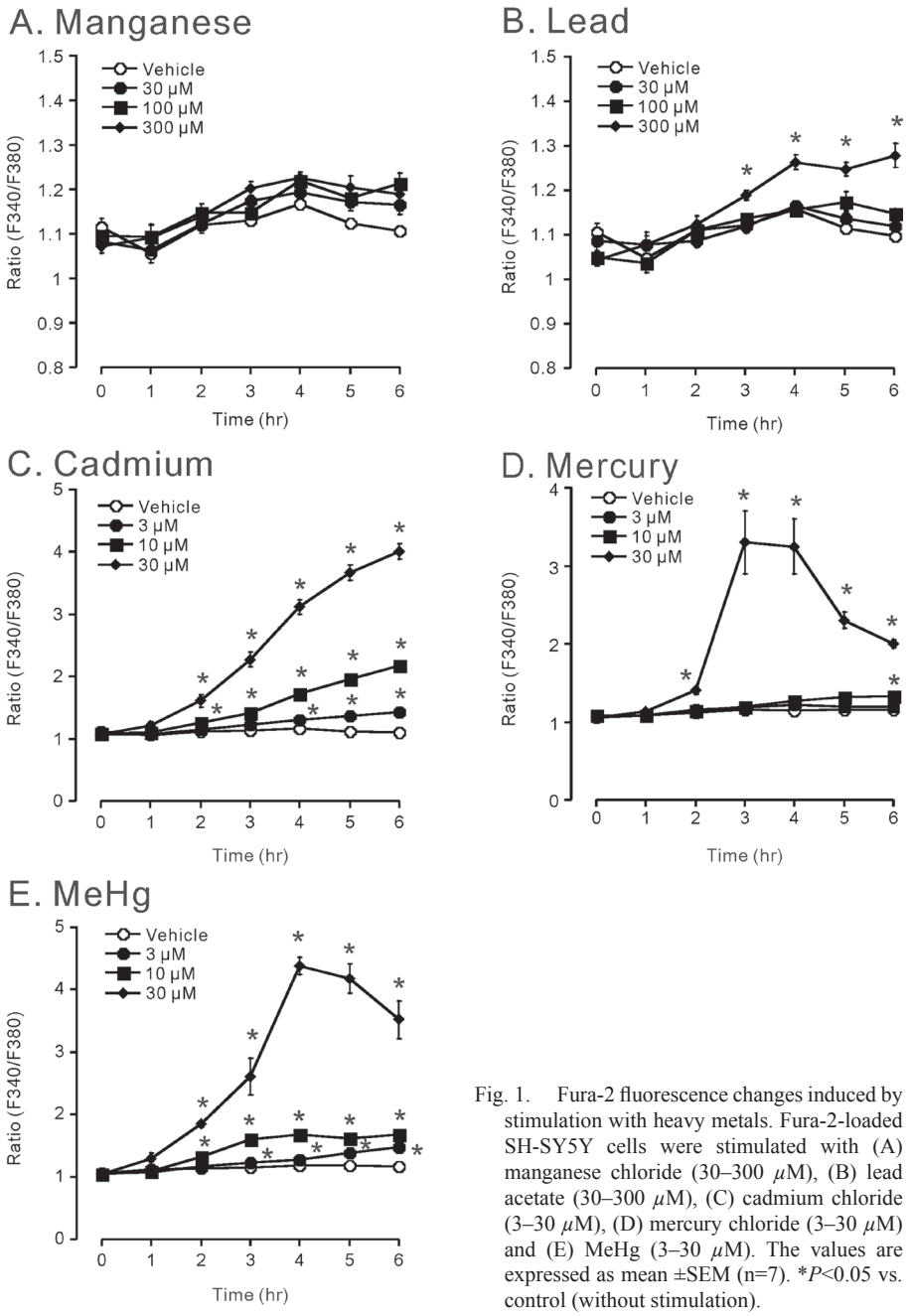


Fig. 1. Fura-2 fluorescence changes induced by stimulation with heavy metals. Fura-2-loaded SH-SY5Y cells were stimulated with (A) manganese chloride (30–300 $\mu$M), (B) lead acetate (30–300 $\mu$M), (C) cadmium chloride (3–30 $\mu$M), (D) mercury chloride (3–30 $\mu$M) and (E) MeHg (3–30 $\mu$M). The values are expressed as mean ±SEM (n=7). *$P$<0.05 vs. control (without stimulation).

*changes induced by cadmium chloride, mercury chloride or MeHg*: Finally, we analyzed the effect of pretreatment with $Ca^{2+}$ channel blockers on the increase in the fura-2 fluorescence ratio induced by cadmium chloride, mercury chloride or MeHg. Pretreatment with verapamil, an L-type $Ca^{2+}$ channel blocker [24], or lanthanum chloride, a non-specific cation channel blocker, significantly inhibited the fura-2 fluorescence ratio induced by cadmium chloride (Fig. 3). In contrast, 2-APB, an inositol 1,4,5-trisphosphate ($IP_3$) receptor and canonical transient receptor potential cation (TRPC) channel blocker [27], caused only a slight inhibition of the cadmium chloride-induced increase in the fura-2 fluorescence ratio. The fura-2 fluorescence ratio induced by mercury chloride was not affected by treatment with verapamil, 2-APB or lanthanum chloride (Fig. 4). Although verapamil and lanthanum chloride did not cause significant effects, 2-APB significantly suppressed the increase in the fura-2 fluorescence ratio induced by MeHg (Fig. 5).

## DISCUSSION

Fura-2 is a UV-excited ratiometric indicator dye for mea-

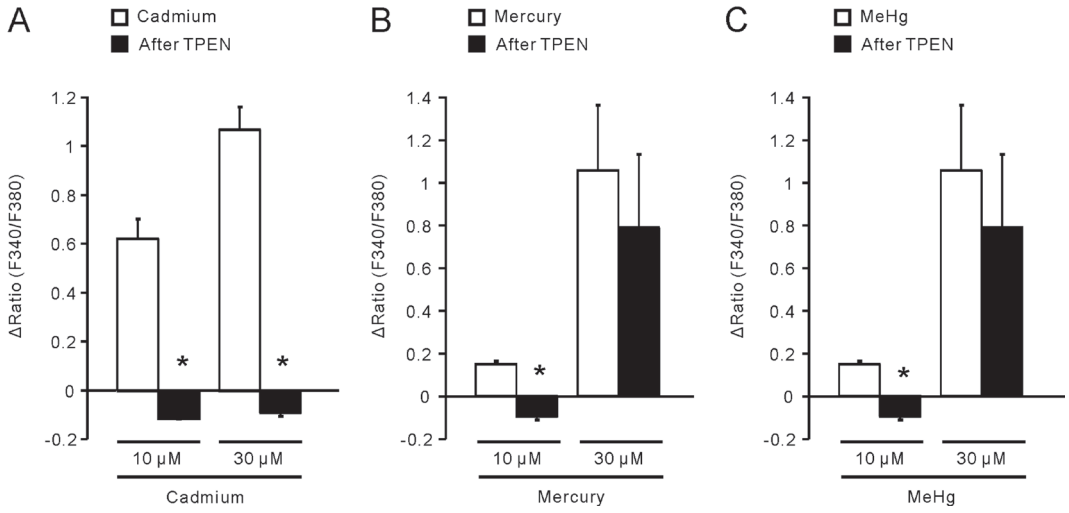


Fig. 2. Effect of TPEN on fura-2 fluorescence changes induced by stimulation with cadmium chloride, mercury chloride or MeHg. Contribution of heavy metal cations to the increase in the fura-2 fluorescence ratio was estimated by addition of TPEN (20 $\mu$M) 3 hr after stimulation with 10 or 30 $\mu$M cadmium chloride (A), mercury chloride (B) or MeHg (C) by measurement of ΔRatio (F340/F380) before and after the addition of TPEN. The values are expressed as mean ±SEM (n=8). *$P$<0.05 vs. ΔRatio (F340/F380) before the addition of TPEN.

suring $[Ca^{2+}]_i$ [12]. Upon binding of fura-2 to $Ca^{2+}$, $Cd^{2+}$, $Pb^{2+}$ and $Zn^{2+}$, the emission fluorescence intensity increases at 340 nm (F340) and decreases at 380 nm (F380) for the unbound form [2, 17, 33]. This change in fluorescence intensity results in elevation of the fura-2 fluorescence ratio (F340/F380). In contrast, the binding of fura-2 to some heavy metals, such as $Mn^{2+}$ and $Fe^{2+}$, quenches the fluorescence (both at F340 and F380) [12, 19, 21]. In this study, we found that manganese chloride did not cause significant changes in the fura-2 fluorescence ratio in SH-SY5Y cells. However, we observed a quenching of the fluorescent signal at both F340 and F380. Thus, it is likely that the $Mn^{2+}$ penetrates cells and quenches the fura-2 fluorescence.

Lead acetate caused a significant increase in the fura-2 fluorescence ratio only at a higher concentration (300 $\mu$M). Sukumar and Beech (2010) reported that stimulation with low lead acetate concentrations induced an increase in $[Ca^{2+}]_i$ through the TRPC5 channel in fura-2-loaded HEK 293 cells with TRPC5 overexpression [29]. Since expression of the TRPC5 channel in SH-SY5Y cells has been suggested [5], TRPC5 might mediated the increase in $[Ca^{2+}]_i$ by a higher concentration of lead acetate, while it may not have been enough to induce an apparent increase in the fura-2 fluorescence ratio at lower concentrations.

We found that cadmium chloride, mercury chloride and MeHg caused an apparent increase in the fura-2 fluorescence ratio in a dose-dependent manner. Therefore, we analyzed the contribution of endogenous or exogenous metal cations to the fura-2 fluorescence change using TPEN, a cell-permeable chelator for heavy metals. The increase in the fura-2 fluorescence ratio by stimulation with 10 or 30 $\mu$M cadmium chloride was inhibited to the basal level by the addition of TPEN, suggesting that the increase in the fura-2 fluorescence ratio was dependent on heavy metal cations but not intracellular $Ca^{2+}$. Given that $Ca^{2+}$ channels mediate $Cd^{2+}$ influx in many cell types [6], it is likely that the change in the fura-2 fluorescence ratio induced by cadmium chloride is caused by an influx of $Cd^{2+}$, which binds fura-2 with high affinity and activates its spectral responses, as $Ca^{2+}$ does [17]. In support of this explanation, we observed that verapamil and lanthanum chloride partially inhibited the increase in the fura-2 fluorescence ratio induced by cadmium chloride. Furthermore, Hinkle *et al.* (1987) reported that verapamil reduced cell death induced by cadmium chloride in a pituitary cell line [16]. On the other hand, cellular uptake of $Cd^{2+}$ by metal transporters, such as divalent metal transporter 1 (DMT1), Zrt/Irt-related protein (ZIP) 8 and ZIP14, had been reported [32]. Although DMT1 and ZIP14 are expressed in SH-SY5Y cells [11, 34], the contribution to the $Cd^{2+}$ uptake has not been known. The involvement of these metal transporters in the cadmium-induced increase in fura-2 fluorescence ratio needs to be clarified in future studies.

The addition of TPEN to cells exposed to a low concentration of mercury chloride or MeHg (10 $\mu$M) returned the increased fura-2 fluorescence ratio to basal level, while a higher concentration (30 $\mu$M) of mercury chloride or MeHg caused an increase in the fura-2 fluorescence ratio that was only partially sensitive to the TPEN treatment. These results suggest that higher concentrations of mercury chloride and MeHg induce an increase in the fura-2 fluorescence ratio through an increase in intracellular $Ca^{2+}$ in SH-SY5Y cells, in addition to the increase in intracellular metal cation levels. Since $Hg^{2+}$ and MeHg do not cause perturbation of fura-2 fluorescence [15, 21], the increase in the fura-2 fluorescence ratio which was sensitive to treatment with TPEN may be dependent on endogenous metal cation mobilization. A contribution by endogenous $Zn^{2+}$ mobilization in measurement of $[Ca^{2+}]_i$ by fura-2 has been proposed [22], and, in fact,

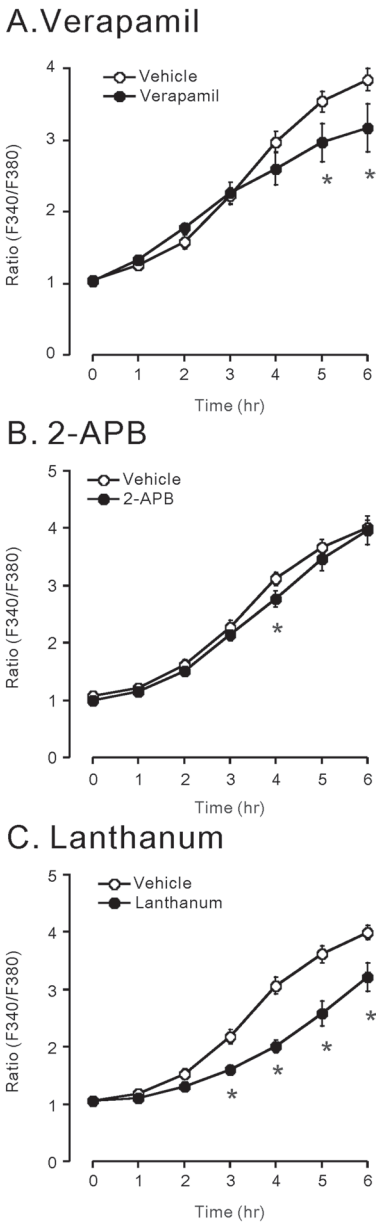


Fig. 3. Effect of $Ca^{2+}$ channel blockers on fura-2 fluorescence change induced by stimulation with cadmium chloride. Cells were treated with (A) verapamil (10 $\mu$M), (B) 2-APB (10 $\mu$M) or (C) lanthanum chloride (100 $\mu$M) 30 min before stimulation with cadmium chloride (30 $\mu$M). The values are expressed as mean ± SEM (n=6). *$P$<0.05 vs. vehicle-treated cells.

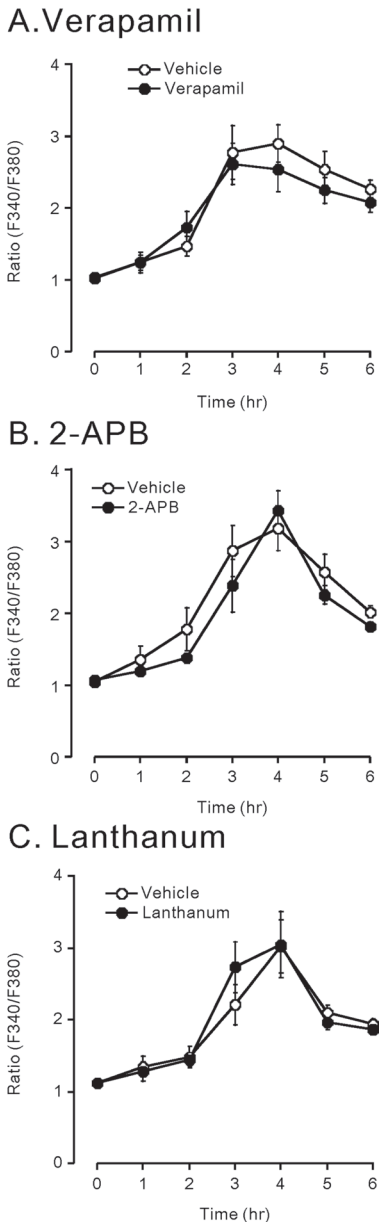


Fig. 4. Effect of $Ca^{2+}$ channel blockers on fura-2 fluorescence change induced by stimulation with mercury chloride. Cells were treated with (A) verapamil (10 $\mu$M), (B) 2-APB (10 $\mu$M) or (C) lanthanum chloride (100 $\mu$M) 30 min before stimulation with mercury chloride (30 $\mu$M). The values are expressed as mean ± SEM (n=9). *$P$<0.05 vs. vehicle-treated cells.

elevation of $[Zn^{2+}]_i$ by MeHg was reported in synaptosomes [7]. Taken together, it is likely that the increase in $[Zn^{2+}]_i$, at least in part, contributes to a TPEN-sensitive increase in the fura-2 fluorescence ratio induced by exogenous heavy metals. In contrast with cadmium chloride, a MeHg-induced increase in the fura-2 fluorescence ratio was suppressed by 2-APB but not by verapamil or lanthanum chloride, although alteration of MeHg-induced $Ca^{2+}$ mobilization by treatment with nifedipine, an L-type $Ca^{2+}$ channel blocker, has been reported in cerebellar granule cells [23], a neuronal cell line [13, 14, 23] and spinal motor neurons [28]. In agreement with previous reports that MeHg induced an increase in inositol phosphate levels and activated TRPC channels [14, 30, 38], 2-APB, an $IP_3$ receptor and TRPC channel blocker [27], inhibited an increase in the fura-2 fluorescence ratio induced by MeHg. In this study, significant inhibitory effects were not

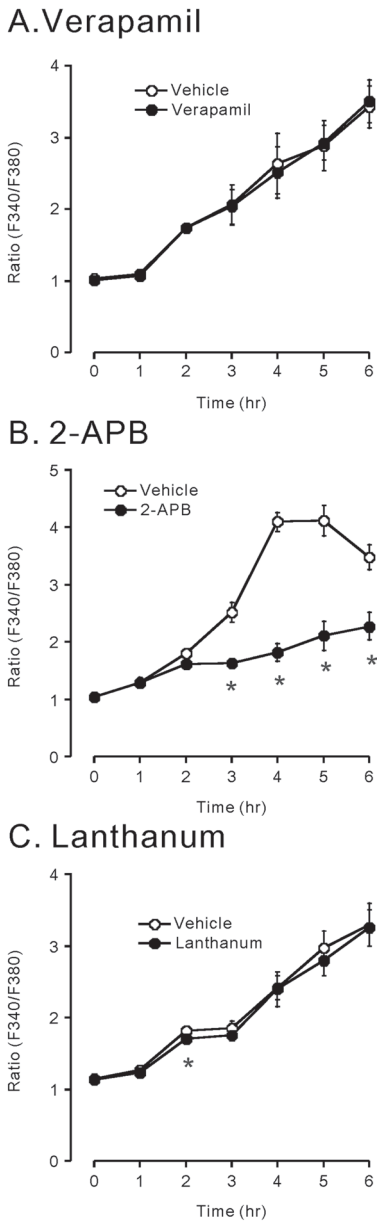


Fig. 5. Effect of $Ca^{2+}$ channel blockers on fura-2 fluorescence change induced by stimulation with MeHg. Cells were treated with (A) verapamil (10 $\mu$M), (B) 2-APB (10 $\mu$M) or (C) lanthanum chloride (100 $\mu$M) 30 min before stimulation with MeHg (30 $\mu$M). The values are expressed as mean ± SEM (n=9). *$P$<0.05 vs. vehicle-treated cells.

observed in mercury chloride-induced increases in the fura-2 fluorescence ratio after treatment with verapamil, 2-APB or lanthanum chloride. Recently, Xu *et al.* (2012) reported that a mercury-induced increase in $[Ca^{2+}]_i$ was inhibited by MK801, an antagonist of the NMDA receptor, in cultured cortical neurons [36]. Expression of the NMDA receptor in SH-SY5Y cells has also been reported [3]. The involvement of NMDA receptors or other types of $Ca^{2+}$ channels, with the mobilization of $[Ca^{2+}]_i$ by heavy metals in SH-SY5Y cells remains to be clarified.

In this study, we showed that the elevation of the fura-2 fluorescence ratio is dependent not only on $Ca^{2+}$ but also on heavy metal cations in SH-SY5Y cells. Furthermore, the contribution of heavy metal cations to the changes in the fura-2 fluorescence ratio induced by exogenous heavy metals was metal- and concentration-specific. Our study provides a characterization of the effects of several heavy metals on the mobilization of divalent cations and the toxicity of heavy metals to neuronal cells.

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