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Blockade of 5-HT_3 receptor with MDL72222 and Y25130 reduces β-amyloid protein (25–35)-induced neurotoxicity in cultured rat cortical neurons

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Abstract

The present study was performed to examine neuroprotective effects of 5-hydroxytryptamine (5-HT)$_3$ receptor antagonists against β-amyloid protein (25–35)-, a synthetic 25–35 amyloid peptide, induced neurotoxicity using cultured rat cortical neurons. β-Amyloid protein (25–35) produced a concentration-dependent reduction of cell viability, which was significantly reduced by (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cyclohepten-5,10-imine (MK-801), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, verapamil, an L-type Ca^{2+} channel blocker, and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor. The 5-HT_3 receptor antagonists, tropanyl-3,5-dichlorobenzoate (MDL72222, 0.1–10 μM) and *N*-(1-azabicyclo[2.2.2.]oct-3-yl)-6-chloro-4-ethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide hydrochloride (Y25130, 0.05–5 μM), decreased the β-amyloid protein (25–35) (10 μM)-induced neuronal cell death as assessed by a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and the number of apoptotic nuclei, evidenced by Hoechst 33342 staining. MDL72222 and Y25130 inhibited the β-amyloid protein (25–35) (10 μM)-induced elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) and glutamate release, generation of reactive oxygen species, and caspase-3 activity. These neuroprotective effects of MDL72222 (10 μM) and Y25130 (5 μM) were completely blocked by the simultaneous treatment with 100 μM 1-phenylbiguanide, a 5-HT_3 receptor agonist, indicating that the protective effects of these compounds were due to 5-HT_3 receptor blockade. These results suggest that the activation of the 5-HT_3 receptor may be partially involved in β-amyloid protein-induced neurotoxicity, by membrane depolarization for Ca^{2+} influx. Therefore, the blockade of 5-HT_3 receptor with MDL72222 and Y25130, may ameliorate the β-amyloid protein-induced neurotoxicity by interfering with the increase of $[Ca^{2+}]_c$, and then by inhibiting glutamate release, generation of reactive oxygen species and caspase-3 activity.

Keywords: 5-HT_3 receptor antagonist; MDL72222; Y25130; 5-HT_3 receptor; β-Amyloid protein; Neurotoxicity; Cortical neuron; Neuroprotection

1. Introduction

Alzheimer's disease is characterized by neuronal loss and extracellular senile plaque, whose major constituent is β-amyloid protein, a 39–43 amino acid peptide derived from amyloid precursor protein (Ivins et al., 1999). Both in vitro (Iversen et al., 1995) and in vivo (Chen et al., 1994) studies have reported the toxic effects of β-amyloid protein or β-amyloid peptide fragments suggesting an important role for β-amyloid protein in the pathogenesis of Alzheimer's disease. In cultures, β-amyloid protein can directly induce neuronal cell death (Ueda et al., 1994) and can render neurons vulnerable to excitotoxicity (Koh et al., 1990) and oxidative insults (Goodman and Mattson, 1994). The mechanisms underlying β-amyloid protein-neurotoxicity are complex but may involve *N*-methyl-D-

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aspartate (NMDA) receptor, a glutamate receptor subtype, modulation induced by glutamate release, sustained elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and oxidative stresses (Forloni, 1993; Gray and Patel, 1995; Ueda et al., 1997; Ekinci et al., 2000). NMDA receptor acts either as a selective substrate of β-amyloid protein binding or as a mediator of β-amyloid protein-triggered glutamate excitotocixity (Harkany et al., 1999). NMDA receptor is a ligand-gated/voltage-sensitive cation channel, especially highly permeable to Ca^{2+}. Extensive elevation of the $[Ca^{2+}]_i$ may lead directly to cellular dysfunction, overexcitation or death (Horn et al., 1999). Therefore, Ca^{2+} influx through NMDA receptor activation by β-amyloid protein may be a critical role in β-amyloid protein-induced neurotoxicity. Formation of reactive oxygen species is also believed to be involved in the pathogenesis of neurodegenerative disorders (Olanow, 1993). Several lines of evidence support the involvement of oxidative stress as an active factor in β-amyloid protein-mediated neuropathology, by triggering or facilitating neurodegeneration through a wide range of molecular events that disturb neuronal homeostasis (Ekinci et al., 2000).

The 5-hydroxytryptamine $(5\text{-HT})_3$ receptor is the only 5-HT-activated ligand-gated ion channel that increases intracellular cation ions, such as Ca^{2+} as well as Na^+ and K^+, by its activation. The stimulation of the receptor induces neuronal depolarization and excitation (Derkach et al., 1989; Maricq et al., 1991). 5-HT_3 receptors are concentrated in limbic and cortical areas thought to be involved with learning and memory (Kilpatrick et al., 1987; Waeber et al., 1989). Antagonists at the 5-HT_3 receptor have been considered as potential cognitive enhancers for the treatment of dementia (Altman and Normile, 1988; Gower, 1992). Studies in rodents have demonstrated that 5-HT_3 receptor antagonists such as ondansetron and SEC-579 can enhance cognitive function (Barnes et al., 1990; Costal and Naylor, 1994; Hodges and Fletcher, 1995) and central acetylcholine release (Robinson, 1983; Barnes et al., 1989). Moreover, ondansetron was reported to attenuate the deficit in various learned behaviors caused by systemically administered scopolamine (Carey et al., 1992; Carli et al., 1997). In addition, it was also demonstrated that the blockade of 5-HT_3 receptors plays a neuroprotective role in ischemia-induced damage (Kagami-ishi et al., 1992). Therefore, it seems reasonable to explore neuroprotective effects of 5-HT_3 receptor antagonists against β-amyloid protein-induced apoptotic death and to study associated potential underlying mechanisms. The current study aims at determining whether blockade of 5-HT_3 receptor with tropanyl-3,5-dichlorobenzoate (MDL72222) and *N*-(1-azabicyclo[2.2.2.]oct-3-yl)-6-chloro-4-ethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide hydrochloride (Y25130) is able to protect the neuronal cells against β-amyloid protein (25–35)-induced neurotoxicity in cultured rat cortical neurons.

2. Materials and methods

2.1. Chemicals (reagents) and physiological solution

β-Amyloid protein (25–35) was purchased from Bachem (Bubendorf, Switzerland). MDL72222 and Y25130 and 1-phenylbiguanide hydrochloride were purchased from Tocris Cookson Inc. (St. Ballwin, MO, USA). (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*] cyclohepten-5,10-imine (MK-801) and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from RBI (Natick, MA, USA). Verapamil, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *o*-phthaldialdehyde, 2-mercaptoethanol, Dulbecco's modified Eagle's medium (DMEM), Joklik-modified MEM, poly-L-lysine and amino acids for HPLC standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hoechst 33342 dye, fluo-4 AM and 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum was purchased from Gibco (Logan, UT, USA). PRO-PREP protein extraction solution was purchased from iNtRON Biothechnology Inc. (Seoul, Korea). Western Lightening™ chemiluminescence reagent was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, USA). Anti-caspase 3 (rabbit polyclonal IgG) and horse-radish peroxidase conjugated anti-rabbit IgG were purchased from Upstate Biotechnology (Lake Placid, NY, USA). All other chemicals used were of the highest grade available.

β-Amyloid protein (25–35) stock solution of 2 mM was prepared in sterile distilled water, stored at −20 °C, and incubated for more than 2 days at 37 °C to aggregate before use. MDL72222 was dissolved in dimethylsulfoxide (DMSO) with the concentration of 10 mM and further diluted with experimental buffers. The final concentration of DMSO was 0.1%, which did not affect cell viability (99.8 ±2.1%). Y25130, 1-phenylbiguanide, MK-801, verapamil and L-NAME were solubilized in experimental buffers. For every experiment, MDL72222 (0.1–10 μM), Y25130 (0.05–5 μM), 1-phenylbiguanide (100 μM), MK-801 (10 μM), verapamil (10 μM) and L-NAME (1 mM) or their vehicle were applied 15 min prior to the treatment with β-amyloid protein (25–35) and were present in the medium during the incubation period with β-amyloid protein (25–35). For some experiments, a HEPES-buffered solution (incubation buffer) containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl and 2.3 mM $CaCl_2$ at pH 7.4 was used.

2.2. Animals

Specific pathogen-free pregnant Sprague–Dawley (SD) rats (Daehan Biolink Co. Ltd., Chungbuk, Korea) were housed in an environmentally controlled room with temperature of 23±2 °C, relative humidity of 55±5%, and a 12-h light/dark cycle, and food and water were available ad libitum. The procedures involving experimental animals adhered to the 'Guide Principles in the Use of Animals in

Toxicology' which had been adopted by the Society of Toxicology in 1989.

2.3. Primary culture of cerebral cortical neurons

Primary cortical neuronal cultures were prepared using SD rat fetuses on embryonic days 15 to 16. Fetuses were isolated from a dam anaesthetized with ether. Cortical hemispheres were dissected under sterile conditions and placed into Joklik-modified Eagle's medium containing trypsin (0.25 mg/ml). After slight trituration through a 5-ml pipette five to six times, the cells were incubated for 10 min at 37 °C. Dissociated cells were collected by centrifugation (1500 rpm, 5 min) and resuspended in DMEM supplemented with sodium pyruvate (0.9 mM), L-glutamine (3.64 mM), sodium bicarbonate (44 mM), glucose (22.73 mM), penicillin (40 U/ml), gentamicin (50 μg/ml), KCl (5 mM) and 10% fetal bovine serum at a density of about 2×10^6 cells/ml. Cells were plated onto poly-L-lysine coated 12-well plates (Corning 3512, NY, USA) for the measurements of cell death and glutamate release, coverslips (Fisher Scientific 12CIR, Pittsburgh, PA, USA) for the measurements of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), reactive oxygen species and apoptosis, and 30 mm culture dishes for the measurement of caspase-3 activation. After 2 days' incubation, the medium was replaced with a new growth medium in which the concentrations of fetal bovine serum and KCl were changed to 5% and 15 mM, respectively. Cultures were kept at 37 °C in a 5% CO_2 atmosphere, changing the medium twice a week. Neurotoxicity experiments were performed on neurons grown for 5–7 days in vitro. Immunochemical staining with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided cell cultures containing about 90% neurons.

2.4. Analysis of neuronal viability

2.4.1. MTT colorimetric assay

This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase (Berridge and Tan, 1993). Therefore, the amount of formazan produced is proportional to the number of viable cells. The culture medium was removed and replaced with serum-free growth medium. Neurons were then incubated for 20 min in the medium, and incubated for a further 24 h in the presence of β-amyloid protein (25–35) at 37 °C. After completion of incubation with β-amyloid protein (25–35), the culture medium was replaced by a solution of MTT (0.5 mg/ml) in serum-free growth medium. After a 4 h incubation at 37 °C, this solution was removed, and the resulting blue formazan was solubilized in 0.4 ml of acid-isopropanol (0.04 N HCl in isopropanol), and the optical density was read at 570 nm using microplate reader (Bio-Tek EL_X808, Vermont, USA). Serum-free growth medium was used as a blank solution.

2.4.2. Masurement of apoptotic neuronal death

The bis-benzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells without permeabilization (Ishikawa et al., 1999). In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy. Exposed to 10 μM β-amyloid protein (25–35) in serum-free growth medium for 24 h as described in MTT assay, neurons on coverslips were fixed in 4% paraformaldehyde at room temperature for 20 min, and then stained with Hoechst 33342 dye at the concentration of 1 μg/ml in the incubation buffer for 15 min. The morphological change was examined under UV illumination using a fluorescence microscope (Olympus IX70-FL, Tokyo, Japan). The dye was excited at 340 nm, and emission was filtered with a 510 nm barrier filter. To quantify the apoptotic process, neurons with fragmented or condensed DNA and normal DNA were counted. Data were shown as apoptotic neurons as a percentage of total neurons.

2.5. Measurement of $[Ca^{2+}]_c$

Neurons grown on coverslips were loaded with 3 μM fluo-4 AM (dissolved in DMSO) in serum-free growth medium for 45 min at 37 °C in the CO_2 incubator, and washed with the incubation buffer. The coverslips containing fluo-4 AM labeled neurons were mounted on a perfusion chamber containing incubation buffer, subjected to a laser scanning confocal microscope (Carl Zeiss LSM 510, Oberkochen, Germany), and then scanned every 2 s with a 488 nm excitation argon laser and a 515 nm longpass emission filter. After the baseline of $[Ca^{2+}]_c$ was observed for 30 s, 10 μM β-amyloid protein (25–35) was added to the perfusion chamber for the measurement of $[Ca^{2+}]_c$ change. In order to test effects of various compounds on β-amyloid protein (25–35)-induced $[Ca^{2+}]_c$ change, neurons were pretreated with the compounds 15 min before the treatment with β-amyloid protein (25–35) after being loaded with fluo-4 AM and washed. The compounds were also present in the perfusion chamber during the $[Ca^{2+}]_c$ measurement period. All images, about 100 images from the scanning, were processed to analyze changes of $[Ca^{2+}]_c$ in a single cell level. The results were expressed as the relative fluorescence intensity (Lee et al., 1998).

2.6. Measurement of glutamate concentration

After being washed and equilibrated for 20 min with the incubation buffer, neurons were incubated with the buffer containing 10 μM β-amyloid protein (25–35) for 6 h at 37 °C. At the end of the incubation, glutamate secreted into the

medium from the treated neurons was quantified by high performance liquid chromatography (HPLC) with an electrochemical detector (BAS MF series, IN, USA) (Ellison et al., 1987). Briefly, after a small aliquot was collected from the culture wells, glutamate was separated on an analytical column (ODS2; particle size, 5 μm; 4.6×100 mm) after pre-derivatization with *o*-phthaldialdehyde/2-mercaptoethanol. Derivatives were detected by electrochemistry at 0.1 μA/V, and the reference electrode was set at 0.7 V. The column was eluted with mobile phase (pH 5.20) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a flow rate of 0.5 ml/min.

2.7. Measurement of reactive oxygen species generation

The microfluorescence assay of 2′,7′-dichlorofluorescin (DCF), the fluorescent product of H_2DCF-DA, was used to monitor the generation of reactive oxygen species. Neurons, grown on coverslips, were washed with phenol red-free DMEM 3 times and incubated with the buffer in the presence of 10 μM β-amyloid protein (25–35) at 37 °C for 24 h (unless otherwise indicated). The uptake of H_2DCF-DA (final concentration, 5 μM) dissolved in DMSO was carried out for the last 10 min of the incubation with β-amyloid protein (25–35). After being washed, coverslips containing cortical neurons loaded with H_2DCF-DA were mounted on the confocal microscope stage, and the neurons were observed by a laser scanning confocal microscope (Bio-Rad, MRC1024ES, Maylands, UK) using 488 nm excitation and 510 nm emission filters. The average pixel intensity of fluorescence was measured within each cell in the field and expressed in the relative units of DCF fluorescence. Values for the average staining intensity per cell were obtained using the image analyzing software supplied by the manufacturer. Challenge of H_2DCF-DA and measurement of fluorescence intensity were performed in the dark.

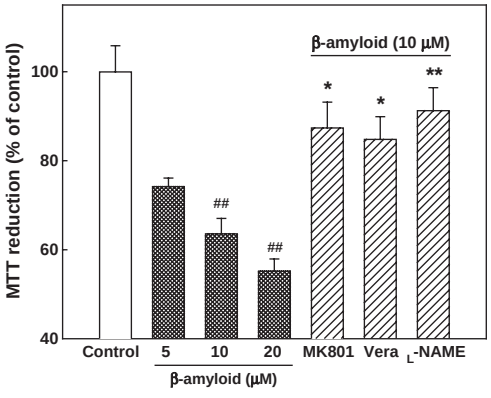

Fig. 1. Concentration-response of β-amyloid protein (25–35) on cell viability of cultured cortical neurons. Cell viability was determined by MTT assay. The absorbance of non-treated cells was regarded as 100%. Results are expressed as mean±S.E.M. values of the data obtained from three independent experiments performed in duplicate. $^{\#\#}p<0.01$ vs. control,. $^{*}p<0.05$, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35).

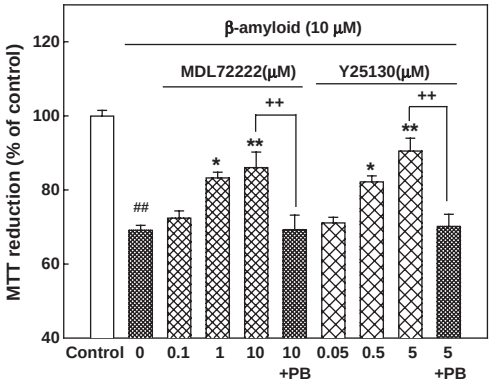

Fig. 2. Concentration-response of 5-HT_3 receptor antagonists on β-amyloid protein (25–35)-induced decrease of MTT reduction in cultured cortical neurons. Results are expressed as mean±S.E.M. values of the data obtained from four independent experiments performed in duplicate. $^{\#\#}p<0.01$ vs. control, $^{*}p<0.05$, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35), $^{++}p<0.01$ vs. the presence of agonist, 1-phenylbiguanide (PB).

2.8. Western blot analysis of caspase-3

Incubated as described in MTT assay, neurons on dishes were washed with PBS and lysed with Proprep buffer. The amount of protein was measured by Lowry's (1951) method. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membrane. Transferred to membrane, caspase-3 was detected with a rabbit anti-caspase-3 antiserum. The antigen antibody reaction was visualized by using a secondary antibody conjugated with horse-radish peroxidase and enhanced chemiluminescence detection reagents.

2.9. Statistical analysis

Data were expressed as mean±S.E.M. and statistical significance was assessed by one-way analysis of variance (ANOVA) with subsequent Turkey's tests. *P* values of <0.05 were considered to be significant.

3. Results

3.1. 5-HT_3 receptor antagonists protect neurons against cell death induced by β-amyloid protein (25–35)

To assess β-amyloid protein (25–35)-induced neuronal cell death, the MTT assay was performed. We now show that when cortical neurons were exposed for 24 h to β-amyloid protein (25–35), 25.8±1.9%, 36.4±3.5% and 44.8±2.7% of neuronal cell death were observed at 5, 10 and 20 μM, respectively (Fig. 1). Therefore, the concentration of 10 μM was used for the determination of β-amyloid protein (25–35)-induced neuronal cell damage in the following experiments. MK-801 (10 μM), an NMDA

receptor antagonist, verapamil (10 μM), an L-type Ca^{2+} channel blocker, and L-NAME (1 mM), a nitric oxide synthase inhibitor, significantly inhibited the decrease of MTT reduction rate caused by 10 μM β-amyloid protein (25–35). Fig. 2 shows the effect of 5-HT_3 receptor blockade on a 10 μM β-amyloid protein (25–35)-induced decrease of MTT reduction. MTT reduction rate decreased to 69.1±1.4% when using 10 μM β-amyloid protein (25–35). MDL72222 (0.1, 1 and 10 μM) and Y25130 (0.05, 0.5 and 5 μM) concentration-dependently reduced the β-amyloid protein (25–35)-induced decrease of MTT reduction showing 86.0±4.2% and 90.5±3.4% with 10 and 5 μM, respectively. However, MDL72222 (10 μM) and Y25130 (5 μM) failed to reduce the β-amyloid protein (25–35)-induced neuronal cell death in the presence of the receptor agonist, 1-phenylbiguanide (100 μM), indicating that the neuroprotective effect of 5-HT_3 receptor antagonists was mediated via 5-HT_3 receptor blockade. 1-

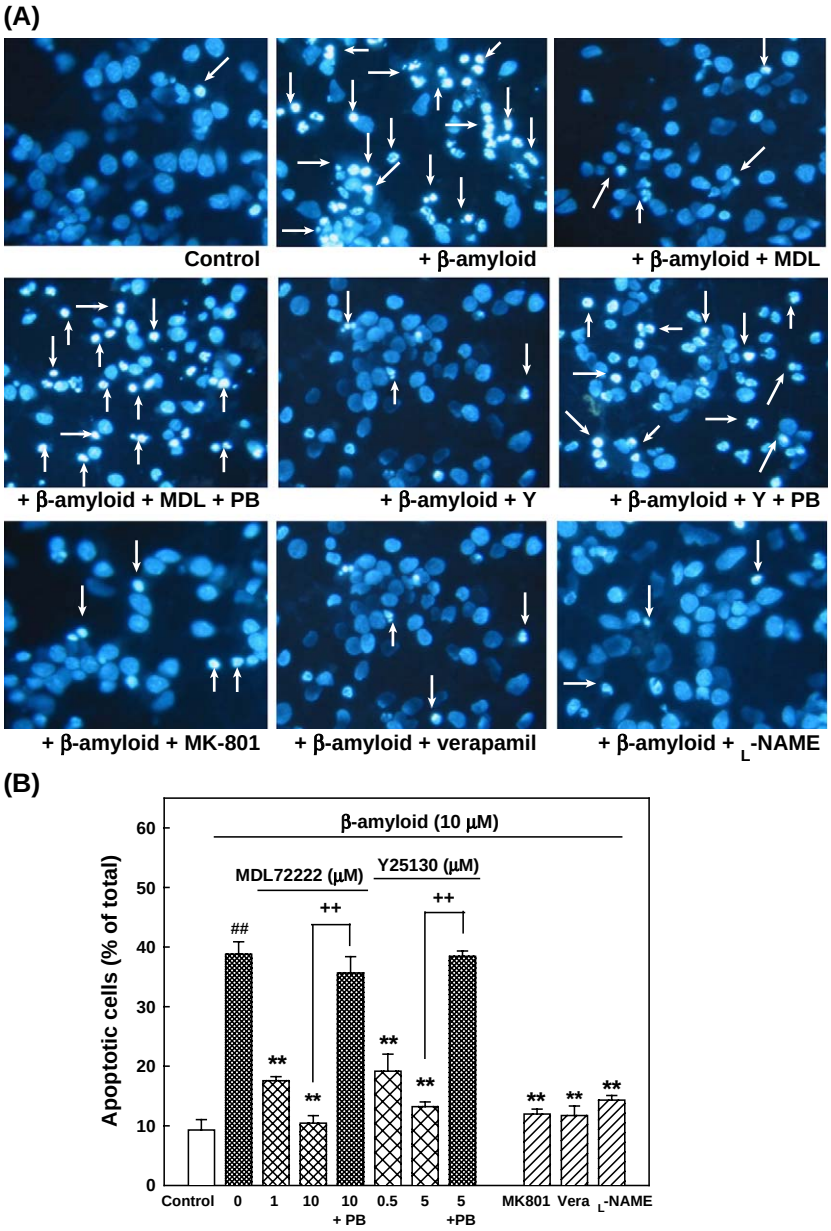

Fig. 3. Inhibitory effects of 5-HT_3 receptor antagonists on β-amyloid protein (25–35)-induced apoptosis of cultured cortical neurons as measured by Hoechst 33342 staining. Apoptotic cells were counted from 5 to 6 fields per well. (A) Representative photomicrographs of cultured neurons showing β-amyloid protein (25–35)-induced apoptosis. The arrows indicate fluorescence typical for apoptotic nuclei. MDL72222 (MDL) and Y25130 (Y) were treated with the concentration of 10 and 5 μM, respectively. (B) Results are shown as apoptotic cells as a percentage of total number of cells and expressed as mean±S.E.M. values of the data obtained from three independent experiments performed in two or three wells. $^{\#\#}p<0.01$ vs. control, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35), $^{++}p<0.01$ vs. the presence of agonist, 1-phenylbiguanide (PB).

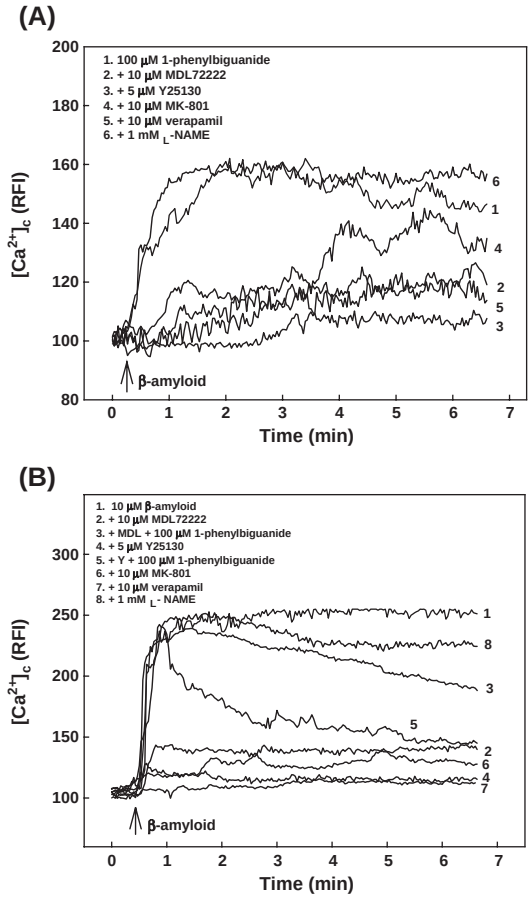

Fig. 4. Change of $[Ca^{2+}]_c$ in response to 5-HT_3 receptor agonist (A) and β-amyloid protein (25–35) (B) in the presence or absence of various compounds in cultured cerebral cortical neurons. $[Ca^{2+}]_c$ was monitored using a laser scanning confocal microscope. All images from the scanning were processed to analyze changes of $[Ca^{2+}]_c$ in a single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace is a single cell representative from at least three independent experiments.

Phenylbiguanie (100 μM) alone neither affected β-amyloid protein (25–35)-induced neuronal cell death nor produced neuronal cell death (data not shown). In addition, the toxicity of β-amyloid protein (1–42) in cultured cortical neurons was examined. When cortical neurons were exposed to 5 μM β-amyloid protein (1–42) for 24 h, the neuronal viability decreased to 79.5±1.7%. However, the β-amyloid protein (1–42)-induced decrease of neuronal viability was completely recovered to 100.5±0.9% with the treatment with 10 μM MDL72222.

An additional experiment was performed with Hoechst 33342 staining to assess the neurotoxicity of β-amyloid protein (25–35). Cell nuclei stained by Hoechst 33342 enable the occurrence of DNA condensate to be detected, a feature of apoptosis. In neurons treated with 10 μM β-amyloid protein (25–35), chromatin condensation and nuclear fragmentation were observed, whereas the control culture had round blue nuclei of viable neurons (Fig. 3A). As shown in Fig. 3B, the proportion of apoptotic neurons was calculated. The treatment of neurons with 10 μM β-amyloid protein (25–35) produced apoptosis of 38.8 ±2.1% of the total population of cultured cortical neurons, as compared with 9.3±1.7% of apoptotic neurons in control cultures. On the other hand, the addition of MDL72222 and Y25130 significantly decreased the β-amyloid protein (25–35)-induced apoptotic cell death, showing 10.5±1.2% and 13.2±0.8%, at the concentration of 10 and 5 μM, respectively. However, co-treatment of MDL72222 (10 μM) and Y25130 (5 μM) with 1-phenylbiguanide (100 μM) to neurons did not decrease the β-amyloid protein (25–35)-induced apoptotic cell death. MK-801 (10 μM), verapamil (10 μM) and L-NAME (1 mM) also inhibited the β-amyloid protein (25–35)-induced apoptotic cell death. MDL72222, Y25130, MK-801, verapamil and L-NAME did not affect neuronal viability (data not shown).

3.2. 5-HT_3 receptor antagonists inhibit β-amyloid protein (25–35)-induced elevation of $[Ca^{2+}]_c$

The increase of $[Ca^{2+}]_c$ has been postulated to be associated with β-amyloid protein-induced cell death in many studies. Firstly, we examined the effects of 5-HT_3 antagonists on 5-HT_3 agonist-induced $[Ca^{2+}]_c$ increase. As shown in Fig. 4A, $[Ca^{2+}]_c$ was rapidly increased by the treatment with 100 μM 1-phenylbiguanide, showing some fluctuation during the measurement period. Elevation of $[Ca^{2+}]_c$ by 1-phenylbiguanide was completely inhibited by 5-HT_3 receptor antagonists, MDL72222 (10 μM) and Y25130 (5 μM), and verapamil. MK-801 also showed a significant, but not complete, inhibition on the 1-phenylbiguanide-induced $[Ca^{2+}]_c$ elevation. However, L-NAME did not inhibit the 5-HT_3 receptor agonist-induced increase of $[Ca^{2+}]_c$. These results suggest that the

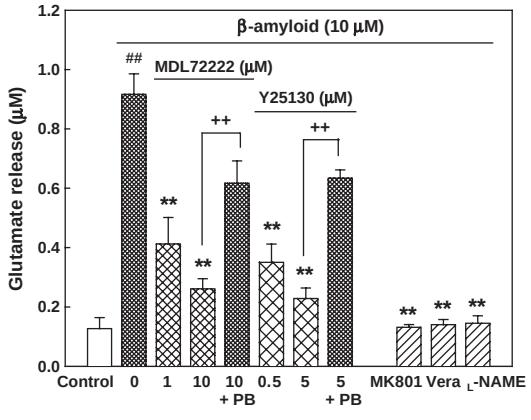

Fig. 5. Inhibitory effects of 5-HT_3 receptor antagonists on β-amyloid protein (25–35)-induced glutamate release in cultured cortical neurons. The amount of released glutamate was measured by HPLC with an electrochemical detector. Results are expressed as mean±S.E.M. values of the data obtained from three independent experiments performed in two or three wells. $^{\#\#}p<0.01$ vs. control, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35), $^{++}p<0.01$ vs. the presence of agonist, 1-phenylbiguanide (PB).

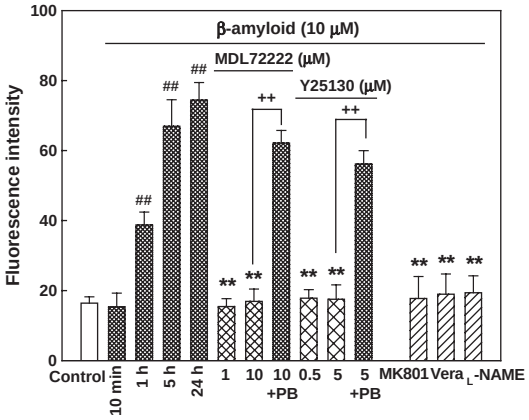

Fig. 6. Inhibitory effects of 5-HT_3 receptor antagonists on β-amyloid protein (25–35)-induced reactive oxygen species generation in cultured cortical neurons. Values represent mean±S.E.M. of relative fluorescence intensity obtained from three independent experiments performed in two or three wells. $^{\#\#}p<0.01$ vs. control, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35), $^{++}p<0.01$ vs. the presence of agonist, 1-phenylbiguanide (PB).

stimulation of 5-HT_3 receptors may cause the activation of voltage-dependent calcium channels which are coupled to 5-HT_3 receptor as well as NMDA type glutamate receptor. As shown in Fig. 4B, $[Ca^{2+}]_c$ rapidly increased with the treatment of 10 μM β-amyloid protein (25–35), and maintained plateau throughout the recording period of over 6 min. In contrast, 10 μM β-amyloid protein (25–35) application in the presence of MDL72222 (10 μM) and Y25130 (5 μM) failed to produce an increase of $[Ca^{2+}]_c$ throughout the measurement period. However, co-treatment of 1-phenylbiguanide (100 μM) with MDL72222 (10 μM) or Y25130 (5 μM), recovered the initial rapid increase of $[Ca^{2+}]_c$ induced by 10 μM β-amyloid protein (25–35). Verapamil (10 μM) and MK-801 (10 μM) completely inhibited the β-amyloid protein (25–35)-induced elevation of $[Ca^{2+}]_c$, whereas L-NAME did not block at all. MDL72222, Y25130, MK-801, verapamil and L-NAME did not affect basal $[Ca^{2+}]_c$ (data not shown).

3.3. 5-HT_3 receptor antagonists inhibit β-amyloid protein (25–35)-induced elevation of glutamate release

Glutamate released into the extracellular medium by the treatment with 10 μM β-amyloid protein (25–35) for 6 h was quantified. As shown in Fig. 5, 10 μM β-amyloid protein (25–35) markedly elevated the basal glutamate level from 0.13±0.04 of control neurons to 0.92±0.07 μM. MDL72222 (1 and 10 μM) and Y25130 (0.5 and 5 μM) strongly blocked the β-amyloid protein (25–35)-induced elevation of glutamate release showing 0.26±0.03 and 0.23±0.04 μM, at the concentration of 10 and 5 μM, respectively. However, these inhibitory effects of 5-HT_3 receptor antagonists were significantly recovered by a simultaneous treatment with 5-HT_3 receptor agonist, 1-phenylbiguanide (100 μM). In addition, MK-801 (10 μM), verapamil (10 μM) and L-NAME (1 mM) markedly inhibited β-amyloid protein (25–35)-induced elevation of glutamate.

3.4. 5-HT_3 receptor antagonists inhibit β-amyloid protein (25–35)-induced reactive oxygen species generation

β-Amyloid protein (25–35) increased the concentration of $[Ca^{2+}]_c$ and the glutamate release. Furthermore, the pathological condition induced by β-amyloid protein (25–35) is associated with accelerated formation of reactive oxygen species. After exposure of neurons to 10 μM β-amyloid protein (25–35) for 10 min, 1, 5 or 24 h, the time-response of reactive oxygen species generation was assessed in H_2DCF-DA-loaded cortical neurons. When neurons were incubated with β-amyloid protein (25–35) for more than 1 h, generation of reactive oxygen species significantly increased showing a maximum level after 5 h (Fig. 6). In β-amyloid protein (25–35) (10 μM)-treated neurons for 24 h, the fluorescence intensity increased about 4.5 folds to 74.4±5.0 compared to control neurons of 16.4±1.8. The β-amyloid protein (25–35)-induced increase of reactive oxygen species was completely inhibited by MDL72222 (1 and 10 μM) and Y25130 (0.5 and 5 μM), and these protective effects were completely disappeared by 1-phenylbiguanide (100 μM) (Fig. 6). MK-801 (10 μM), verapamil (10 μM) and L-NAME (1 mM) significantly blocked the β-amyloid protein (25–35)-induced increase of fluorescence intensity. MDL72222, Y25130, 1-phenylbiguanide, MK-801, verapamil and L-NAME did not show direct reaction with H_2DCF-DA to generate fluorescence (data not shown).

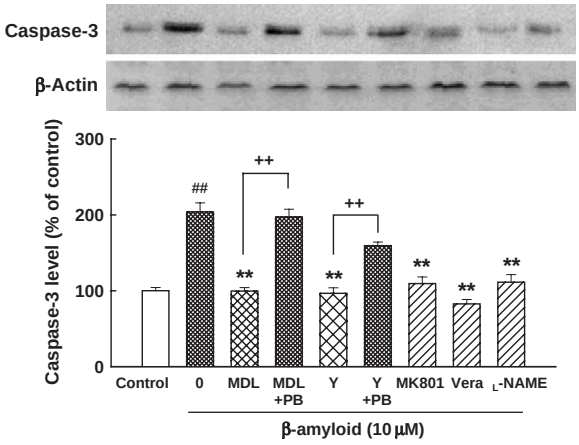

Fig. 7. Inhibitory effects of 5-HT_3 receptor antagonists on β-amyloid protein (25–35)-induced caspase-3 expression in cultured cortical neurons. Caspase-3 levels were determined by Western blotting. MDL72222 (MDL) and Y25130 (Y) were treated with the concentration of 10 and 5 μM, respectively. A representative band obtained from three similar results is shown. Quantified results of density are the mean±S.E.M. obtained from three independent experiments. $^{\#\#}p<0.01$ vs. control, $^{**}p<0.01$ vs. 10 μM β-amyloid protein (25–35), $^{++}p<0.01$ vs. the presence of agonist, 1-phenylbiguanide (PB).

3.5. 5-HT$_3$ receptor antagonists inhibit β-amyloid protein (25–35)-induced caspase-3 protein activation

Caspase-3, the 32 kDa protease constitutively expressed by many cell types and tissues, is implicated in apoptosis promoted by different death stimuli (Allen et al., 2001). Caspase-3 protein expression was evaluated by Western blotting. In 10 μM β-amyloid protein (25–35) treated neurons, caspase-3 activity markedly increased compared to control cultures. MDL72222 (10 μM) and Y25130 (5 μM) significantly blocked the β-amyloid protein (25–35)-induced increase of caspase-3 immunoreactivity. However, in the presence of 1-phenylbiguanide (100 μM), MDL72222 and Y25130 failed to block the β-amyloid protein (25–35)-induced increase of caspase-3 activity. MK-801 (10 μM), verapamil (10 μM) and L-NAME (1 mM) also markedly reduced the β-amyloid protein (25–35)-induced increase of caspase-3 activity (Fig. 7).

4. Discussion

The present study provides evidence that β-amyloid protein (25–35)-induced injury to rat cortical neurons can be prevented by 5-HT$_3$ receptor antagonists. 5-HT$_3$ receptor antagonists, MDL72222 (1 and 10 μM) and Y25130 (0.5 and 5 μM) were able to reduce the β-amyloid protein (25–35)-induced neuronal apoptotic death, $[Ca^{2+}]_c$ increase, glutamate release, reactive oxygen species generation and caspase-3 activation. The nearly complete reversal of the anti-neurotoxic effects of the 5-HT$_3$ receptor antagonists by the receptor agonist, 1-phenylbiguanide (Ireland and Tyers, 1987; Schmidt and Black, 1989), strongly suggests that this process is mediated by blockade of 5-HT$_3$ receptors.

β-Amyloid protein is a major contributor to the pathogenesis of Alzheimer's disease. β-Amyloid protein-induced neurotoxicity has been attributed in various studies to Ca^{2+} influx, generation of reactive oxygen species, and activation of caspase-3 (Behl et al., 1994; Arias et al., 1995; Harada and Sugimoto, 1999). The present study confirmed that β-amyloid protein (25–35) caused neuronal cell death, which was blocked by treatment with MK-801, verapamil and L-NAME. This result implies the involvement of NMDA-glutamate receptor activation, an increase of Ca^{2+} influx and generation of reactive oxygen species in the β-amyloid protein (25–35)-induced neurotoxicity in cultured cortical neurons, as previously evidenced in other studies (Gray and Patel, 1995; Ueda et al., 1997; Ekinci et al., 2000). Regardless of the relative contribution of these events to β-amyloid protein (25–35)-induced neurotoxicity, the primary event following β-amyloid protein (25–35) treatment of cultured neurons has been suggested to be Ca^{2+} influx, apparently via L-type voltage-dependent calcium channels, since blockage of this channel and/or Ca^{2+} chelation prevents all other consequences (Ekinci et al., 1999; Ueda et al., 1997), as demonstrated with verapamil in the present study. Furthermore, β-amyloid protein (25–35)-induced elevation of $[Ca^{2+}]_c$ and neurotoxicity were inhibited by MK-801, suggesting Ca^{2+} influx through NMDA receptor-coupled voltage-dependent calcium channels plays a critical role in the neurotoxicity. It has been reported that vitamin-E, an antioxidant, blocked the β-amyloid protein-induced generation of reactive oxygen species, but not Ca^{2+} influx, and reduction of extracellular Ca^{2+} inhibited the β-amyloid protein-induced increase in intracellular Ca^{2+} as well as generation of reactive oxygen species, indicating that the generation of reactive oxygen species is a consequence of Ca^{2+} accumulation (Ekinci et al., 2000). Confirming these reports, the present study showed that β-amyloid protein (25–35)-induced elevation of $[Ca^{2+}]_c$ occurred within seconds, but increase of reactive oxygen species generation, 1 h after the treatment with 10 μM β-amyloid protein (25–35). Furthermore, L-NAME, a nitric oxide synthase inhibitor, failed to inhibit the β-amyloid protein (25–35)-induced increase in $[Ca^{2+}]_c$ in the short period of measurement in contrast to the complete inhibition of verapamil on the β-amyloid protein (25–35)-induced reactive oxygen species generation. However, in many experiments, free radicals are responsible for the increase of $[Ca^{2+}]_i$. The reactive oxygen species-induced membrane damage causes further Ca^{2+} influx and resultant accentuated Ca^{2+} influx in turn will induce the generation of further reactive oxygen species (Cotman et al., 1992). Many researchers have demonstrated that β-amyloid protein triggered apoptotic degeneration in in vitro neuronal experiment (Harkany et al., 1999; Yan et al., 1999). Cultured cortical neurons exposed to β-amyloid protein (25–35) for more than 24 h showed increased chromatin condensation, a typical feature of apoptotic cell death in the present work. Caspases are aspartate-specific cysteine proteases, which have been proposed to play a pivotal role in apoptosis (Nicholson, 1997). In the caspase family, which consists of more than 10 homologues, caspase-3 has been suggested to play an important role in β-amyloid protein-induced apoptosis (Allen et al., 2001; Cardoso et al., 2002). β-Amyloid protein (25–35)-induced apoptotic neuronal death and increase of caspase-3 activity was blocked by MK-801, verapamil and L-NAME in the present study.

The 5-HT$_3$ receptor is the ligand-gated ion channel that increases intracellular cations such as Ca^{2+}, Na^+ and K^+, proved with 1-phenylbiguanide in the present study. Stimulation of the receptor induces rapid and transient depolarization and excitation of neurons. 5-HT$_3$ receptor antagonists directly decrease depolarization and elevation of $[Ca^{2+}]_i$ by blocking 5-HT$_3$ receptor. MDL72222 and Y25130 completely blocked β-amyloid protein (25–35)- as well as 1-phenylbiguanide-induced $[Ca^{2+}]_c$ elevation, and the blockade of β-amyloid protein (25–35)-induced $[Ca^{2+}]_c$ elevation, at least the initial rapid increase, was reversed by the presence of the receptor agonist, 1-phenylbiguanide. Therefore, it is strongly suggested that the neuroprotective

effect of 5-HT_3 receptor antagonists is mainly due to the inhibition on the β-amyloid protein (25–35)-induced increase of $[Ca^{2+}]_c$ through voltage-dependent calcium channels which are coupled to 5-HT_3 receptor. It might be concluded that β-amyloid protein (25–35) and 5-HT_3 receptor agonist share a characteristic in terms of neuronal excitation involved in L-type voltage-dependent calcium channels opening, since verapamil completely blocked β-amyloid protein (25–35)- as well as 1-phenylbiguanide-induced $[Ca^{2+}]_c$ elevation in the present study. It is also hypothesized that 5-HT_3 receptor and NMDA receptor activated by β-amyloid protein (25–35) share the L-type voltage-dependent calcium channels, since MK-801 inhibited both β-amyloid protein (25–35)- and 1-phenylbiguanide-induced increase of $[Ca^{2+}]_c$. The antagonists also significantly inhibited the β-amyloid protein (25–35)-induced glutamate elevation. This result indicates that the sustained inhibition of $[Ca^{2+}]_c$ elevation by the compounds resulted in the decrease of the β-amyloid protein (25–35)-induced glutamate release. It was also demonstrated that 5-HT_3 agonist 1-phenylbiguanide released glutamate from the rat medulla, and this effect was blocked by the receptor antagonist ondansetron (Ashworth-Preece et al., 1995). However, it might be suggested that β-amyloid protein (25–35)-induced increase of glutamate is attributable to the neuronal glutamate uptake inhibition by β-amyloid protein, as described by Fernández-Tomé et al. (2004). It should be further studied.

The elucidation of the variety of events occurring downstream of neuronal Ca^{2+} overloading is still a matter for further research. The generation of reactive oxygen species undoubtedly takes place in glutamate neurotoxicity and is likely due to Ca^{2+} influx in the cytosol (Pereira and Oliveira, 2000). Many reports demonstrated the involvement of reactive oxygen species formation in β-amyloid protein-induced neurotoxicity (Miranda et al., 2000; Cardoso et al., 2002). MDL72222 and Y25130 decreased the β-amyloid protein (25–35)-induced increase of reactive oxygen species generation. We demonstrated that L-NAME failed to show an inhibition on the β-amyloid protein (25–35)-induced $[Ca^{2+}]_c$ increase occurred in seconds to minutes, while verapamil, a Ca^{2+} channel antagonist, completely blocked reactive oxygen species generation. L-NAME also failed to inhibit the 1-phenylbiguanide-induced rapid increase of $[Ca^{2+}]_c$. Therefore, it is suggested that the antagonists inhibited the β-amyloid protein (25–35)-induced reactive oxygen species generation via the blockade of $[Ca^{2+}]_c$ increase. In many cell types, the β-amyloid protein-induced increase of caspase-3 activity has been demonstrated (Allen et al., 2001; Cardoso et al., 2002). An increase in expression of activated caspase-3 has been detected in the brains of Alzheimer's disease (Su et al., 2001). The activation of caspase-3 may be a downstream event following Ca^{2+} influx, glutamate release and reactive oxygen species generation in cortical neurons exposed to β-amyloid protein (25–35), since it was blocked by 5-HT_3 receptor antagonists as well as MK-801, verapamil, and L-NAME in the present study.

In conclusion, we demonstrated a novel pharmacological action of 5-HT_3 receptor antagonists and its mechanism in the present study. These results suggest that 5-HT_3 receptor blockade with antagonists such as MDL72222 and Y25130 could serve as a promising therapeutic approach to control the progression of neurodegeneration in the brain of Alzheimer's disease. Forthcoming studies will attempt to clarify the in vivo effect of 5-HT_3 receptor antagonists.

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