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Donepezil attenuates excitotoxic damage induced by membrane depolarization of cortical neurons exposed to veratridine

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ABSTRACT

Long-lasting membrane depolarization in cerebral ischemia causes neurotoxicity via increases of intracellular sodium concentration ($[Na^+]_i$) and calcium concentration ($[Ca^{2+}]_i$). Donepezil has been shown to exert neuroprotective effects in an oxygen–glucose deprivation model. In the present study, we examined the effect of donepezil on depolarization-induced neuronal cell injury resulting from prolonged opening of Na^+ channels with veratridine in rat primary-cultured cortical neurons. Veratridine (10 μM)-induced neuronal cell damage was completely prevented by 0.1 μM tetrodotoxin. Pretreatment with donepezil (0.1–10 μM) for 1 day significantly decreased cell death in a concentration-dependent manner, and a potent NMDA receptor antagonist, dizocilpine (MK801), showed a neuroprotective effect at the concentration of 10 μM. The neuroprotective effect of donepezil was not affected by nicotinic or muscarinic acetylcholine receptor antagonists. We further characterized the neuroprotective properties of donepezil by measuring the effect on $[Na^+]_i$ and $[Ca^{2+}]_i$ in cells stimulated with veratridine. At 0.1–10 μM, donepezil significantly and concentration-dependently reduced the veratridine-induced increase of $[Ca^{2+}]_i$, whereas MK801 had no effect. At 10 μM, donepezil significantly decreased the veratridine-induced increase of $[Na^+]_i$. We also measured the effect on veratridine-induced release of the excitatory amino acids, glutamate and glycine. While donepezil decreased the release of glutamate and glycine, MK801 did not. In conclusion, our results indicate that donepezil has neuroprotective activity against depolarization-induced toxicity in rat cortical neurons via inhibition of the rapid influx of sodium and calcium ions, and via decrease of glutamate and glycine release, and also that this depolarization-induced toxicity is mediated by glutamate receptor activation.

1. Introduction

The pathogenesis of ischemic brain is considered to involve excessive release of glutamate and subsequent elevation of intracellular Ca^{2+} levels. It is generally thought that cytosolic Ca^{2+} plays a critical role in neuronal death. The depolarization of membrane potential can trigger a massive Ca^{2+} increase in neuronal cells, mainly due to activation of NMDA-glutamate receptors, Na^+/Ca^{2+} exchangers and Ca^{2+} channels, thereby causing neuronal cell damage (Choi, 1988a,b; 1995). It has also been widely accepted that Na^+ influx through voltage-sensitive Na^+ channels upon depolarization exacerbates neuronal cell damage (Stys, 1998), and Na^+ channel blockers have been shown to protect against neuronal damage induced by hypoxia or head injury (Lynch et al., 1995; Taylor and Meldrum, 1995; Martínez-Sánchez et al., 2004). Activation of Na^+ channels by veratridine has been used as a depolarization-induced neuronal injury model eliciting Ca^{2+}-dependent neuronal damage (Pauwels et al., 1989, Takahashi et al., 1999, Takano et al., 2003).

Donepezil is a potent acetylcholinesterase inhibitor (Sugimoto et al., 1995). It is the most widely used drug for the treatment of Alzheimer's disease, and its clinical efficacy is based on acetylcholinesterase inhibition, leading to an increase of acetylcholine level at synapses, with amelioration of memory and cognitive impairments (Nordberg and Svensson, 1998; Giacobini, 2000). In addition, a neuroprotective effect of donepezil has been supported by the results of both clinical studies (Krishnan et al., 2003; Hashimoto et al., 2005) and preclinical studies in oxygen–glucose deprivation (Akasofu et al., 2003), glutamate insult (Takada et al., 2003), *N*-methyl-D-aspartate (NMDA)- and alpha-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA)/kainite-induced insult (Akasofu et al., 2006), and amyloid beta (Aβ) toxicity (Kimura et al., 2005) models using primary-cultured neurons. The precise mechanisms of neuroprotection by donepezil remain to be clarified. However, interference with the Ca^{2+}-dependent cell damage pathway may be one of them, as donepezil has marked effects in oxygen–glucose deprivation (Akasofu et al., 2003) and excitotoxicity models (Takada et al., 2003; Akasofu et al., 2006). It is of interest to examine

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whether donepezil has a neuroprotective effect against depolarization-induced neuronal cell injury. Therefore, we used veratridine as a tool for inducing depolarization in neuronal cells, and examined the effect of donepezil on neuronal cell injury and on the changes in intracellular sodium concentration ($[Na^+]_i$) and calcium concentration ($[Ca^{2+}]_i$) induced by veratridine. Further, a microdialysis study has shown that glutamate and glycine increase in rat cortex after cortical contusion injury, and depolarization has been proposed as the mechanism of release of excitatory amino acids (Rose et al., 2002). Therefore, release of the excitatory amino acid glutamate and the co-agonist glycine was also measured to elucidate whether these transmitters contribute to membrane depolarization injury.

2. Materials and methods

2.1. Drugs

Veratridine, dizocilpine (MK801), nifedipine, mecamylamine hydrochloride (mecamylamine) and carbamoylcholine chloride (carbachol) were obtained from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX), and scopolamine hydrobromide *n*-hydrate (scopolamine) and 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate (KB-R7943) were obtained from Seikagaku Kougyo Co., Ltd. (Tokyo, Japan), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Tocris (Ellisville, MO, USA), respectively. Caspase-3 inhibitor I was purchased from Calbiochem (La Jolla, CA, USA). ω-Conotoxin MVIIA and ω-agatoxin IVA were purchased from Peptide Institude Inc. (Osaka, Japan). Donepezil was synthesized in Eisai Co., Ltd. (Ibaraki, Japan). Before use, these drugs were dissolved in distilled water and then further diluted with culture medium or buffer for measuring $[Na^+]_i$ or $[Ca^{2+}]_i$ to yield the desired final concentrations. We used dimethyl sulfoxide to prepare stock solutions for water-insoluble compounds. "Vehicle" means the vehicle used to prepare the drugs.

2.2. Cortical cell cultures

Cortical cell cultures were prepared from fetal rats of the Wistar strain (gestational age of 17 to 19 days, Charles River Japan, Kanagawa, Japan) according to the procedures described previously (Ohgoh et al., 1998). Briefly, the cortex was dissected and kept in Neurobasal medium (Invitrogen Corp., Carlsbad, CA, USA) containing 2% B27 supplement (Invitrogen), 25 μM 2-mercaptoethanol (Wako), 0.5 mM L-glutamine (Invitrogen) and ×1/100 volume of antibiotic–antimycotic (Invitrogen), then incubated at 37 °C for 30 min in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS;10 mM HEPES, pH 7.3) containing 0.25% trypsin (Invitrogen) and 0.2 mg/ml deoxyribonuclease (DNase) (Sigma). The cortical tissues were dissociated to single cells by gentle trituration. The cell suspension was centrifuged at 310 ×g for 3 min and the resulting pellets were resuspended in the medium described above. All experiments were approved by the Animal Care and Use Committee of Eisai.

2.3. Veratridine-induced toxicity

Cells were plated at an initial cell density of 2.6×10^5 cells/cm^2 in poly-L-lysine (Sigma)-coated 96-well plates. The cells were cultured in a CO_2 incubator (5% (v/v), at 37 °C) in the Neurobasal medium described above for 5 days, and cultured following 5 days in culture medium containing without antioxidant B27 supplement and without 2-mercaptoethanol. The cultures were exposed to 10 μM veratridine for 7 h at 37 °C. The cells were treated with donepezil for 1 day or TTX for 15 min before and during the exposure to veratridine.

2.4. MTT assay

Viability of cells grown in 96-well plates was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay using a modification of the original methods of Mosmann (1983) and Morten et al. (1989). Ten microliters of 8 mg/ml MTT (Sigma) solution in D-PBS(−) (Dulbecco's phosphate-buffered saline, Sigma) was added to each well. During 10 min incubation at 37 °C, the active dehydrogenase in viable mitochondria reduces the tetrazolium ring of MTT, forming a blue-colored precipitate. The cells were completely lysed and the precipitate was taken up in 100 μl of MTT solubilizing buffer containing 20% sodium dodecyl sulfate (SDS: Wako) in 50% *N*,*N*-dimethyl formamide at pH 4.7, and quantified spectrophotometrically at 550–690 nm.

Wells without cells, containing only the medium and MTT solution, were measured to obtain the background value, which was subtracted from measured values at 550–690 nm, as follows: % of control = (sample − background)/(control − background) × 100.

2.5. Measurement of intracellular sodium concentration

$[Na^+]_i$ was measured by means of SBFI-based fluorometry with a Fluorescence Drug Screening System 6000 (FDSS6000 (Hamamatsu Photonics, Shizuoka, Japan)). Rat cortical cultures were loaded with 10 μM SBFI-AM (Sigma) and 1 mg/ml Pluronic F127 (Molecular Probes, Eugene, OR, USA) for 5 h in a CO_2 incubator (5% (v/v), 37 °C), then rinsed twice with buffer with the composition 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 1 mM $CaCl_2{\cdot}2H_2O$, 24 mM D-glucose, and 10 mM HEPES (pH 7.4) for 30 min using a modification of the procedure of Imanishi et al. (2006). Donepezil was contained in the buffer during the 1-day pretreatment and 5-h SBFI-AM loading. Buffer (90 μl) with or without test compounds was added to each well, then the culture plates were transferred to the FDSS6000, and measurement was started at room temperature. After 23 s for measurement of basal fluorescence intensity, 10 μl of veratridine solution (100 μM) was added to each well (final concentration: 10 μM). The change in $[Na^+]_i$ from the basal level for 163 s was measured as the change in the ratio of fluorescence emission (505 nm) intensities at the excitation wavelengths of 340 nm and 380 nm.

2.6. Measurement of intracellular calcium concentration

$[Ca^{2+}]_i$ was measured by means of Fura2-based fluorometry with a FDSS6000. Rat cortical cultures were loaded with 5 μM Fura2-AM (Dojindo, Kumamoto, Japan) for 5 h in a CO_2 incubator (5% (v/v), 37 °C), then rinsed twice with Krebs–Ringer bicarbonate buffer (KR buffer) with the composition 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 137 mM NaCl, 4.17 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 0.01% BSA, 10 mM glucose, and 10 mM HEPES (pH 7.4) according to the procedures described previously (Akasofu et al., 2006). Donepezil was contained in the buffer during the 1-day pretreatment and the 5-h Fura2-AM loading, which was conducted using a slight modification of the reported method (Gilbert et al., 1991). KR buffer (90 μl) with or without test compounds was added to each well, then the culture plates were transferred to the FDSS6000, and measurement was started at room temperature. After 23 s for measurement of basal fluorescence intensity, veratridine was added to each well (final concentration: 10 μM). The change in $[Ca^{2+}]_i$ from the basal level for 163 s was measured as the change in the ratio of fluorescence emission (505 nm) intensities at the excitation wavelengths of 340 nm and 380 nm.

2.7. Glutamate and glycine release by veratridine

Treatment with veratridine was performed on cultured rat cortical cells plated at an initial cell density of 5.2×10^5 cells/cm^2

in poly-L-lysine-coated 96-well plates. The cells were cultured in a CO_2 incubator (5% (v/v), at 37 °C in the Neurobasal medium described above for 10 days. The cells were treated with or without donepezil for 1 day and then rinsed twice with KR buffer. KR buffer (90 µl) with or without test compounds, TTX, MK801 and calcium channel blockers was added to each well. The plate was incubated in the presence or absence of 20 µM veratridine at 37 °C for 30 min. The supernatants were collected, and glutamate and glycine release into the KR buffer was measured using high-performance liquid chromatography (HPLC) with a fluorometric detector (excitation 340 nm, emission 450 nm) after derivatization with *o*-phthaldialdehyde (OPA) as described previously (Piepponen and Skujins, 2001), with some modifications. Briefly, the OPA reagent was prepared as follows: OPA (27 mg) was dissolved in 0.5 ml of methanol, and 2-mercaptoethanol (20 µl) was added. This solution was mixed with 4.5 ml of 0.4 M borate buffer (pH 9.3). Automated sample derivatization was carried out using a Waters 717plus autosampler at 6 °C. The autosampler was charged with 10 µl of the KR buffer together with 5 µl of internal standard solution (40 nM homoserine), and was programmed to add 15 µl of the OPA reagent to 15 µl of the sample mixture, to mix it once, and then to inject a 15 µl aliquot onto the analytical column (YMC-Pack ODS-AM, 75×4.6 mm ID, 3 µm particle size; YMC, Kyoto, Japan) after a reaction time of 1 min. The column temperature was maintained at 35 °C. Mobile phase A was 0.05 M phosphate buffer (pH 6.1) containing 13% (v/v) acetonitrile and 1% (v/v) tetrahydrofuran. Mobile phase B was acetonitrile–tetrahydrofuran–water (70:1:29, v/v). Mobile phase A was pumped at a flow-rate of 0.85 ml/min, and was switched to mobile phase B at 6.5 min after the beginning of the run in order to wash out superfluous amino acids. Then, the mobile phase was switched back to mobile phase A after 1 min. The whole step-gradient run took 10 min in total. In each experiment, 50 pmol of amino acids standard was injected into the HPLC for calibration, followed by sample measurements. Peaks of OPA-derivatized amino acids (isoindoles) were recorded on a Powerchrom integrator (Eicom, Kyoto, Japan), and identified on the basis of retention time (glutamate: 2.06 min, glycine: 6.5 min, homoserine: 5.60 min). The concentrations of glutamate and glycine were quantified from the peak areas, using homoserine as an internal standard.

2.8. Protein measurement

Cortical cell cultures were lysed with 1% sodium dodecyl sulfate and 0.5 N NaOH and the protein of the lysate was measured using a Micro BCA protein assay kit (Pierce, Rockford, IL, USA).

2.9. Statistical analysis

The data were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the software package SAS ver. 6.12 (SAS Institute Japan Ltd., Tokyo, Japan). $P<0.05$ was considered significant.

3. Results

3.1. Neuroprotective effect of donepezil against veratridine-induced toxicity

The neuroprotective profile of donepezil against 10 µM veratridine-induced toxicity to rat cultured cerebral cortical neurons was investigated. Fig. 1 shows the MTT values, an index of viability, of the cells after exposure to veratridine. In the control (without veratridine), the absolute value was 0.195±0.003. In the absence of veratridine, donepezil (0.1, 1 and 10 µM) had no significant effect on the cell viability under these conditions (MTT value % of control: donepezil 0.1 µM, 97%; 1 µM, 97%; 10 µM, 99%). The vehicle contained veratridine, and veratridine per se caused a reduction of 43.6% of MTT at 7 h after exposure, as compared with the veratridine-free control. Pretreatment with donepezil (0.1, 1, and 10 µM) for 1 day provided significant neuroprotection against veratridine-induced toxicity, and increased the cell viability by 18.8%, 25.9% and 64.7%, respectively, compared with the vehicle. Pretreatment with TTX (0.1 µM) for 15 min showed a strong protective effect against veratridine-induced toxicity, and significantly increased the cell viability by 76.5% compared with the vehicle ($P<0.05$).

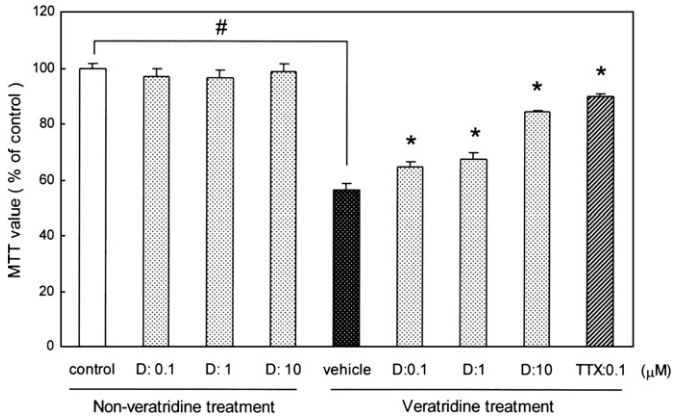

Fig. 1. Protective effect of donepezil against veratridine-induced toxicity in rat cultured cerebral cortical neurons. The cells were treated with veratridine (10 µM) for 7 h. Donepezil (D; 0.1, 1 and 10 µM) was applied from 1 day before and during the veratridine treatment. TTX (0.1 µM), used as a positive control, was applied from 15 min before and during veratridine treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means±S.E. $N=5$. $^{*}P<0.05$, $^{\#}P<0.05$ compared with the vehicle group (Dunnett's multiple comparison).

3.2. Influence of acetylcholine receptor antagonists on the neuroprotective effect of donepezil against veratridine insult

In addition, an enhanced neuroprotective effect could be observed in the donepezil plus TTX treatment group compared with the TTX treatment group (Fig. 2A). Donepezil is a potent acetylcholinesterase inhibitor, and this fact prompted us to investigate the role of muscarinic and nicotinic acetylcholine receptors in its neuroprotective action. As shown in Fig. 2B and C, neither mecamylamine (10 µM; a non-selective antagonist of nicotinic acetylcholine receptors) alone nor scopolamine (10 µM; a muscarinic receptor antagonist) alone had any effect on cell viability when applied 1 day before veratridine treatment. Moreover, these antagonists did not influence the neuroprotective effect of donepezil (Fig. 2B and C). Pretreatment with carbachol (1 nM–1 µM), a cholinomimetic agent, for 1 day had no effect on the viability of cells exposed to 10 µM veratridine (Fig. 2D).

3.3. Influence of caspase-3 inhibitor and NMDA receptor antagonist

As shown in Fig. 3, caspase-3 inhibitor I partially protected neuronal cells from veratridine toxicity with statistical significance (versus the vehicle). Its neuroprotective effect was weaker than that of donepezil, but this difference was not significant. A slight additive effect could be seen in cells treated with both donepezil and caspase-3 inhibitor I compared with caspase-3 inhibitor I alone. The NMDA-glutamate receptor antagonist, MK801, showed a marked, statistically significant neuroprotective effect at the concentration of 10 µM compared with the vehicle.

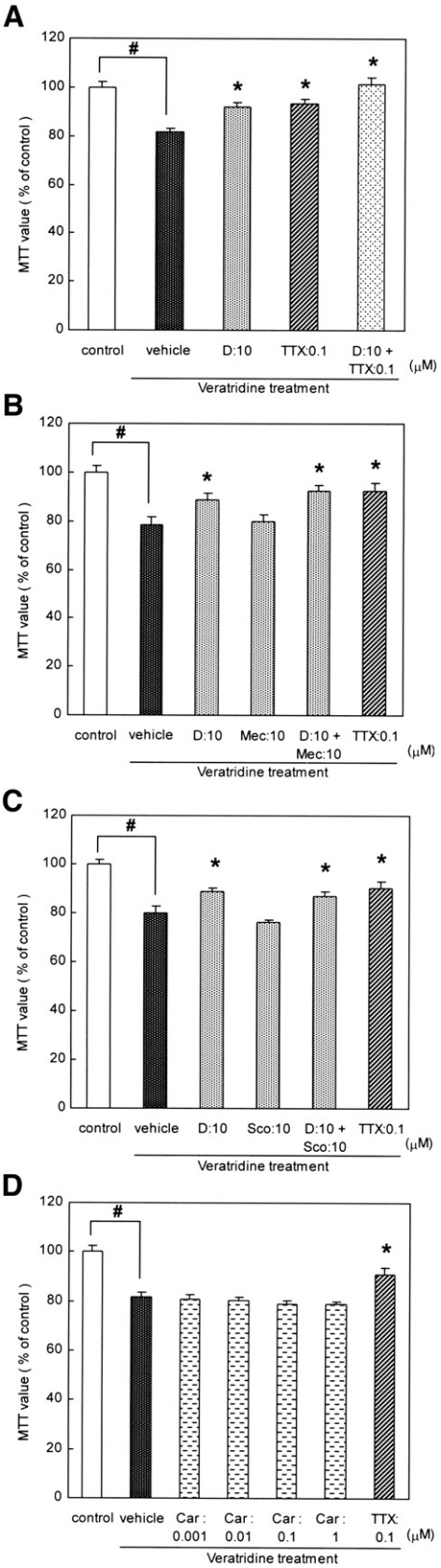

Fig. 2. Effects of acetylcholine receptor antagonists and a cholinomimetic agent against veratridine-induced toxicity in rat cultured cerebral cortical neurons. Donepezil (D; 10 μM), mecamylamine (Mec; 10 μM), scopolamine (Sco; 10 μM) and carbachol (Car; 0.001, 0.01, 0.1, 1, 10 μM) were given on the pre-1-day treatment schedule. TTX (0.1 μM) was given as a pre-15 min treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means ± S.E. $N=5$ or 10. $^{*}P<0.05$, $^{\#}P<0.05$ compared with the vehicle group (Dunnett's multiple comparison).

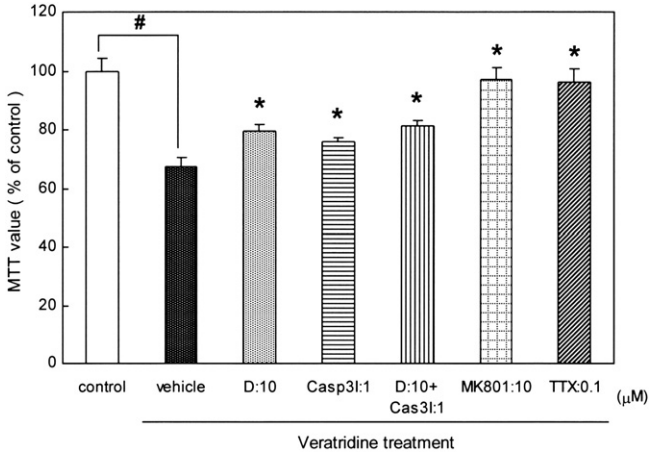

Fig. 3. Effects of caspase-3 inhibitor and MK801 on veratridine-induced toxicity in rat cultured cerebral cortical neurons. Caspase-3 inhibitor I (Casp3I; 1 μM) and donepezil (D; 10 μM) were given on the pre-1-day treatment schedule. MK801 (10 μM) and TTX (0.1 μM) were given as a pre-15 min treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means ± S.E. $N=5$. $^{*}P<0.05$, $^{\#}P<0.05$ compared with the vehicle group (Dunnett's multiple comparison).

3.4. Effect of donepezil on $[Na^+]_i$ increase induced by veratridine

Veratridine is known to activate sodium channels and increase $[Na^+]_i$. In order to examine whether donepezil affects the rise of $[Na^+]_i$ induced by veratridine, the change of $[Na^+]_i$ after application of veratridine was measured ratiometrically using the sodium indicator SBFI. The change in fluorescence ratio was plotted against time in the presence or absence of donepezil (left panel of Fig. 4). In the vehicle control group, $[Na^+]_i$ increased gradually with time and reached near-steady levels within 100 s after exposure to 10 μM veratridine. TTX at 0.1 μM markedly inhibited the rise of $[Na^+]_i$. Pretreatment with donepezil for 1 day also decreased the rise of $[Na^+]_i$. The right panel of Fig. 4 shows the area under the ratio value during exposure to veratridine for 163 s after subtraction of the basal level (before veratridine treatment). Donepezil at 10 μM and TTX at 0.1 μM decreased the rise of $[Na^+]_i$ with statistical significance.

3.5. Effect of donepezil on $[Ca^{2+}]_i$ increase induced by veratridine

Veratridine increases $[Na^+]_i$ and the rise of $[Na^+]_i$ indirectly elicits $[Ca^{2+}]_i$ increase via the Na^+/Ca^{2+} exchange system. Depolarization by veratridine induces the activation of voltage-dependent calcium channels (VDCCs), and this also contributes to the increase of $[Ca^{2+}]_i$. In order to examine whether donepezil affects the rise of $[Ca^{2+}]_i$ induced by veratridine, the change of $[Ca^{2+}]_i$ after application of veratridine was measured ratiometrically using the calcium indicator Fura2. The change in fluorescence ratio was plotted against time in the presence or absence of donepezil (left panel of Fig. 5). In the vehicle control group, $[Ca^{2+}]_i$ increased rapidly within 15 s after exposure to veratridine, then slowly further increased to a plateau during 163 s of observation. TTX and donepezil (10 μM), markedly decreased the rise of $[Ca^{2+}]_i$ compared with the vehicle group. TTX or donepezil was applied from 15 min or 1 day before the veratridine treatment, respectively. The right panel of Fig. 5 shows the area under the ratio value during exposure to veratridine for 163 s after subtraction of the basal level before veratridine treatment. Pretreatment of the cultures with donepezil for 1 day significantly decreased the rise of $[Ca^{2+}]_i$ in a concentration-dependent manner at concentrations higher than 0.1 μM. TTX (0.1 μM) decreased the rise of $[Ca^{2+}]_i$ with statistical significance. TTX did not have any direct effect on VDCCs, indicating the indirect inhibition via sodium channel.

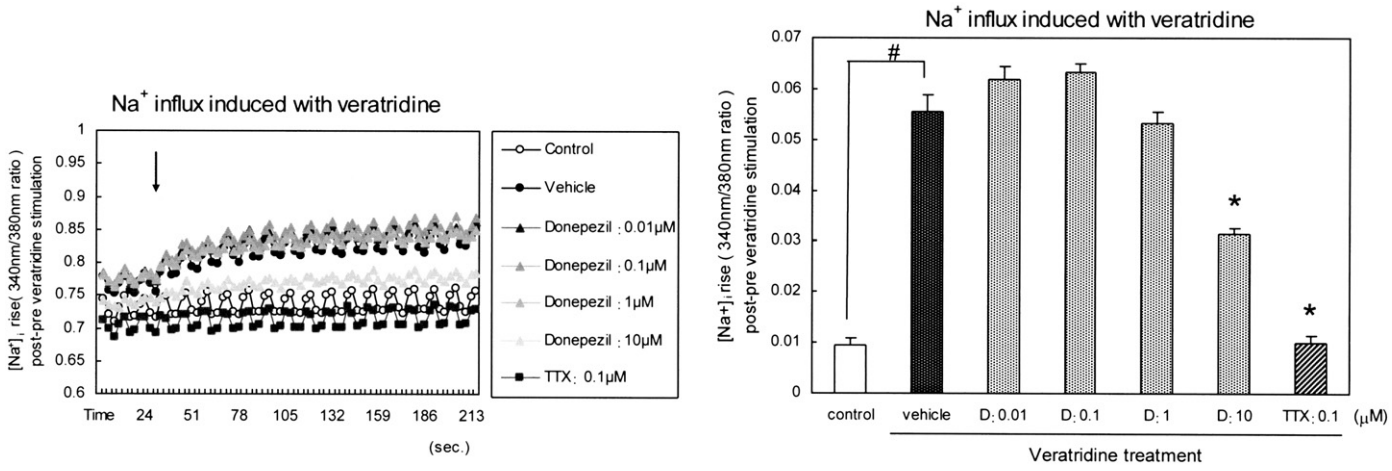

Fig. 4. Effect of donepezil on the increase of $[Na^+]_i$ in rat cultured cerebral cortical neurons induced by veratridine (10 μM) application. The increase of $[Na^+]_i$ was measured by using an SBFI-based ratiometric fluorescence method (excitation at 340 nm and 380 nm; emission at 505 nm). The left panel shows the change in the ratio of the fluorescence with veratridine stimulation. The fluorescence intensities were measured every 2.62 s. The black arrowhead indicates the addition of veratridine. The right panel shows the area under the ratio value during exposure to veratridine for 190 s after subtraction of the basal level before veratridine. Donepezil (D; 0.01, 0.1, 1, 10 μM) was applied from 1 day before and during 5-h SBFI-AM loading and during the veratridine treatment. TTX (0.1 μM), which was used as a positive control, was applied from 15 min before and during the veratridine treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means ± S.E. $N=5$. $^*P<0.05$, $^{\#}P<0.05$ compared with the vehicle group (Dunnett's multiple comparison).

3.6. Effects of various agents on $[Ca^{2+}]_i$ increase induced by veratridine

The effects of blocking concentrations of a combination of three subtype-specific calcium channel blockers (calcium channel blocker cocktail: 0.3 μM ω-conotoxin MVIIA for N-type, 1 μM ω-agatoxin IVA for P/Q type and 3 μM nifedipine for L-type), the Na^+/Ca^{2+} exchange inhibitor KB-R7943 (1, 3, 10 μM) (Fig. 6A), the NMDA antagonist MK801 (0.1, 1, 10 μM), donepezil (10 μM), and the combination of donepezil (10 μM) and MK801 (10 μM) (Fig. 6B) on the $[Ca^{2+}]_i$ rise induced by veratridine were investigated. As shown in the left panel of Fig. 6A, $[Ca^{2+}]_i$ in the vehicle control group increased sharply within 15 s after exposure to veratridine. In the calcium channel blocker cocktail group, the initial rise of $[Ca^{2+}]_i$ over about 50 s was strongly depressed, but the later plateau phase was not markedly affected. The Na^+/Ca^{2+} exchange inhibitor, KB-R7943 decreased the rise of $[Ca^{2+}]_i$ and the effect was statistically significant at the concentration of 10 μM (Fig. 6A right panel). The NMDA antagonist, MK801 (0.1, 1, and 10 μM) had no effect on the $[Ca^{2+}]_i$ rise, and did not affect the decrease of $[Ca^{2+}]_i$ in the presence of donepezil (Fig. 6B right panel).

3.7. Effect of various agents on glutamate and glycine release induced by veratridine

We examined the effect of various compounds on glutamate and glycine (an agonist and a co-agonist for NMDA receptors, respectively) release induced by veratridine in cortical neurons (Table 1). The incubation of cortical cell cultures with 10 μM veratridine for 30 min evoked substantial increases in the extracellular levels of glutamate and glycine. Donepezil at the concentration of 10 μM and TTX at 0.1 μM markedly inhibited the increase of extracellular glutamate and glycine. The combination of the three subtype-specific calcium channel blockers

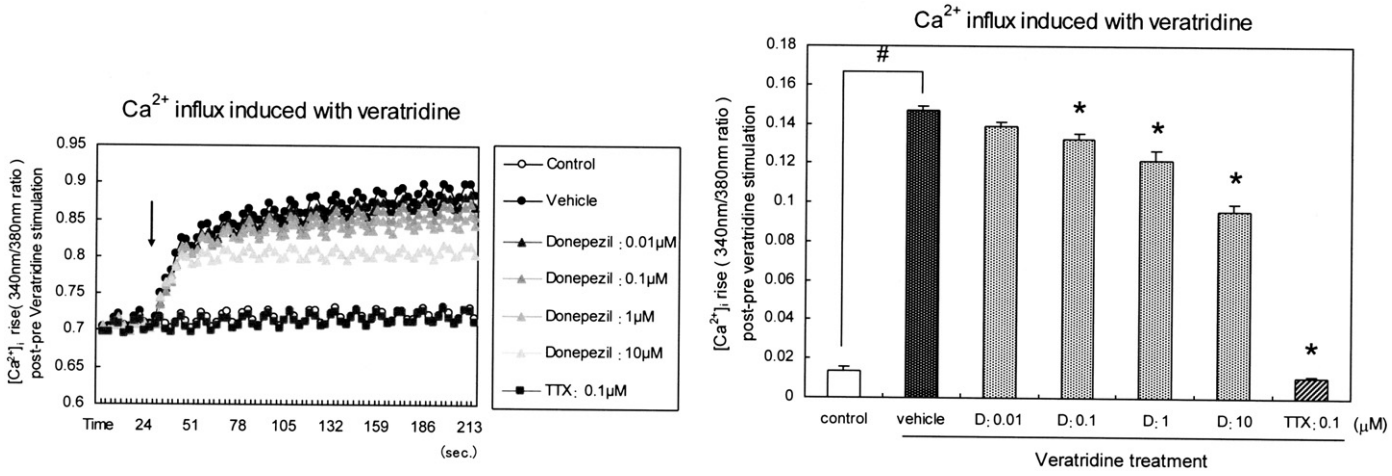

Fig. 5. Effect of donepezil on the increase of $[Ca^{2+}]_i$ in rat cultured cerebral cortical neurons induced by veratridine (10 μM) application. The increase of $[Ca^{2+}]_i$ was measured by using a Fura2-based ratiometric fluorescence method (excitation at 340 nm and 380 nm; emission at 505 nm). The left panel shows the change in the ratio of the fluorescence with veratridine stimulation. The fluorescence intensities were measured every 2.62 s. The black arrowhead indicates the addition of veratridine. The right panel shows the area under the ratio value during the exposure to veratridine for 190 s after subtraction of the basal level before veratridine. Donepezil (D; 0.01, 0.1, 1, 10 μM) was applied from 1 day before and during 5-h Fura2-AM loading, and during the veratridine treatment. TTX (0.1 μM), which was used as a positive control, was applied from 15 min before and during the veratridine treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means ± S.E. $N=5$. $^*P<0.05$, $^{\#}P<0.05$ compared with the vehicle group (Dunnett's multiple comparison).

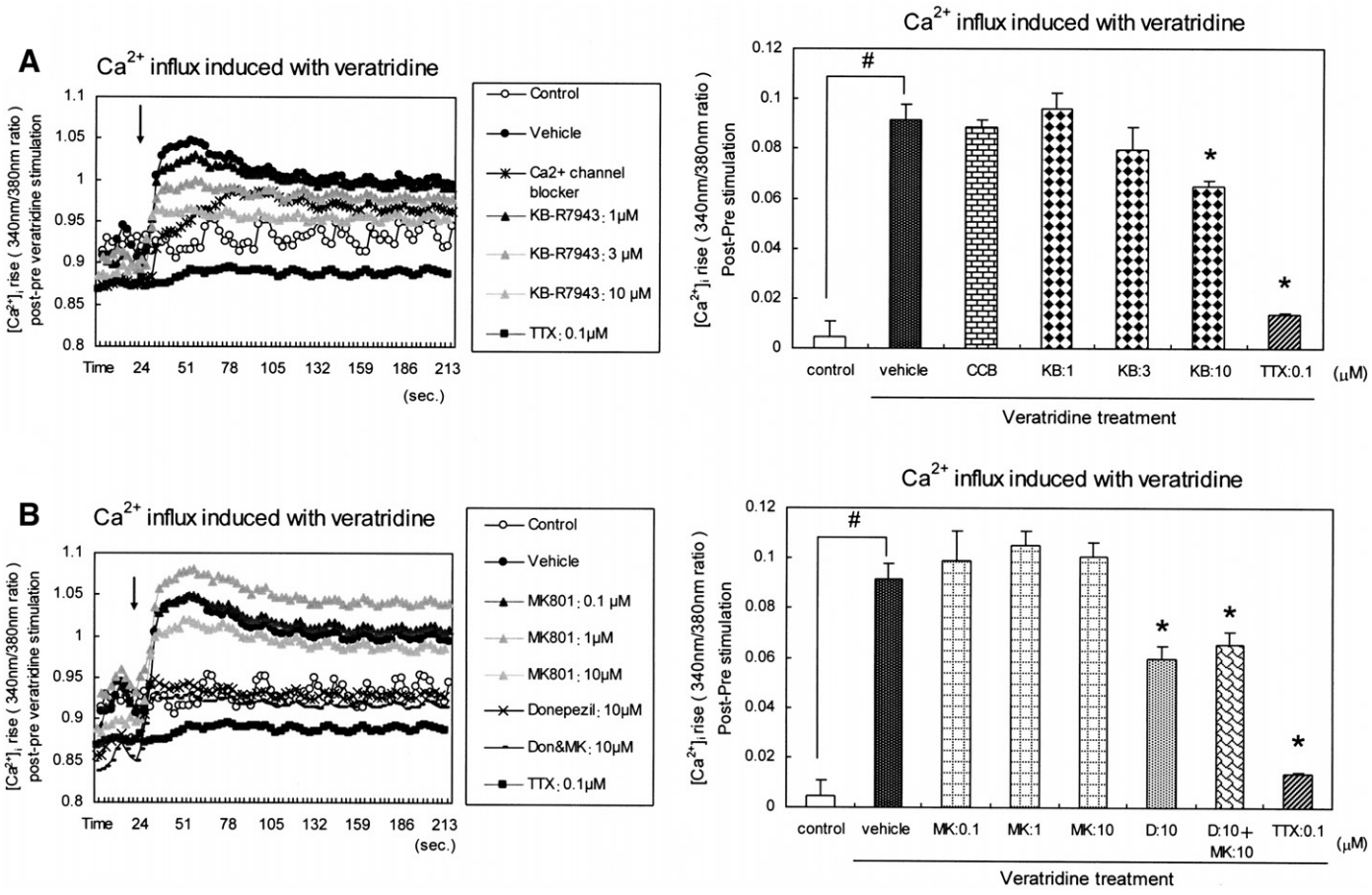

Fig. 6. Effect of Ca^{2+} channel blockers, Na^+/Ca^{2+} exchange inhibitor, and NMDA receptor antagonist on the increase of $[Ca^{2+}]_i$ in rat cultured cerebral cortical neurons induced by veratridine (10 μM) application. The increase of $[Ca^{2+}]_i$ was measured by using a Fura2-based ratiometric fluorescence method (excitation at 340 nm and 380 nm; emission at 505 nm). The left panels show the change in the ratio of the fluorescence with veratridine stimulation. The fluorescence intensities were measured every 2.62 s. The black arrowhead indicates the addition of veratridine. The right panels show the area under the ratio value during the exposure to veratridine for 190 s after subtraction of the basal level before veratridine. (A) Calcium channel blocker cocktail (CCB: ω-conotoxin MVIIA (0.3 μM), ω-agatoxin IVA (1 μM) and nifedipine (3 μM)), KB-R7943 (KB; 1, 3 and 10 μM), and TTX (0.1 μM) were applied from 15 min before and during the veratridine treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. (B) MK801 (MK; 0.1, 1 and 10 μM) and TTX (0.1 μM) were applied from 15 min before and during the veratridine treatment. Donepezil (D;10 μM) was applied from 1 day before and during 5-h Fura2-AM loading, and during the veratridine treatment. Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Values are means ± S.E. ($N = 5$). $^*P < 0.05$, $^{\#}P < 0.05$ compared with the vehicle group (Dunnett's multiple comparison).

described above moderately attenuated the veratridine-evoked extracellular glutamate and glycine releases, but the NMDA receptor antagonist MK-801 had no effect.

Table 1
Effects of veratridine treatment on the extracellular glutamate/glycine accumulation

Treatment	Drug	Glutamate release (pmol/mg protein)	Glycine release (nmol/mg protein)
Non-veratridine	Control	263.4 ± 13.5	25.5 ± 0.6
Veratridine	Vehicle	320.4. ± 7.6	55.6 ± 1.6
	Donepezil: 10 μM	266.9. ± 10.6	47.3 ± 1.2
	TTX: 0.1 μM	162.8 ± 5.8	34.5 ± 1.0
	MK801: 10 μM	327.8 ± 6.2	59.1 ± 0.4
	Ca^{2+} channel blockers cocktail	297.3 ± 12.8	55.1 ± 1.0

Cultured cortical neurons were incubated with or without veratridine in KR buffer solution for 30 min. The concentrations of glutamate and glycine accumulated in the KR buffer solution sampled at 30 min. Calcium channel blocker cocktail (ω-conotoxin MVIIAA (0.3 μM), ω-agatoxin IVA (1 μM) and nifedipine (3 μM)), TTX (0.1 μM), MK801 (10 μM) were applied from 15 min before and during the veratridine treatment. Donepezil (10 μM) was applied from 1 day before and during the veratridine treatment . Control: non-veratridine-treated control group. Vehicle: veratridine-treated control group. Each value represents the mean with S.E. ($N = 5$).

4. Discussion

The results of the present study indicate that donepezil shows a neuroprotective effect against depolarization-induced neurotoxicity induced by veratridine, and this effect is partially mediated through the reduction of glutamate release triggered by veratridine in rat cortical neuron cultures. It is widely accepted that Na^+ influx through voltage-sensitive Na^+ channels upon depolarization in situations such as ischemic insult or traumatic injury exacerbates neuronal cell damage and overstimulates ionotropic glutamate receptors (Lynch et al., 1995; Stys, 1998). Veratridine at 10 μM induced neuronal cell damage (Fig. 1) with increases of $[Na^+]_i$ (Fig. 4) and $[Ca^{2+}]_i$ (Fig. 5), and these effects were strongly inhibited by the application of 0.1 μM TTX, almost to the level of veratridine-untreated neurons. This concentration of TTX specifically inhibits TTX-sensitive neuronal Na^+ channels (Lu et al., 1998; Benjamin et al., 2006), suggesting that the neurotoxicity induced by veratridine was triggered by the activation of Na^+ channels. The mechanisms of veratridine-induced neurotoxicity are thought to be as follows (Fig. 7). Veratridine induces prolonged opening of Na^+ channels and causes membrane depolarization. The membrane depolarization in presynaptic terminals elicits glutamate release, and glutamate activates AMPA/kainate and NMDA receptors of the postsynaptic membrane. The postsynaptic membrane is also depolarized by veratridine, and this depolarization opens VDCCs,

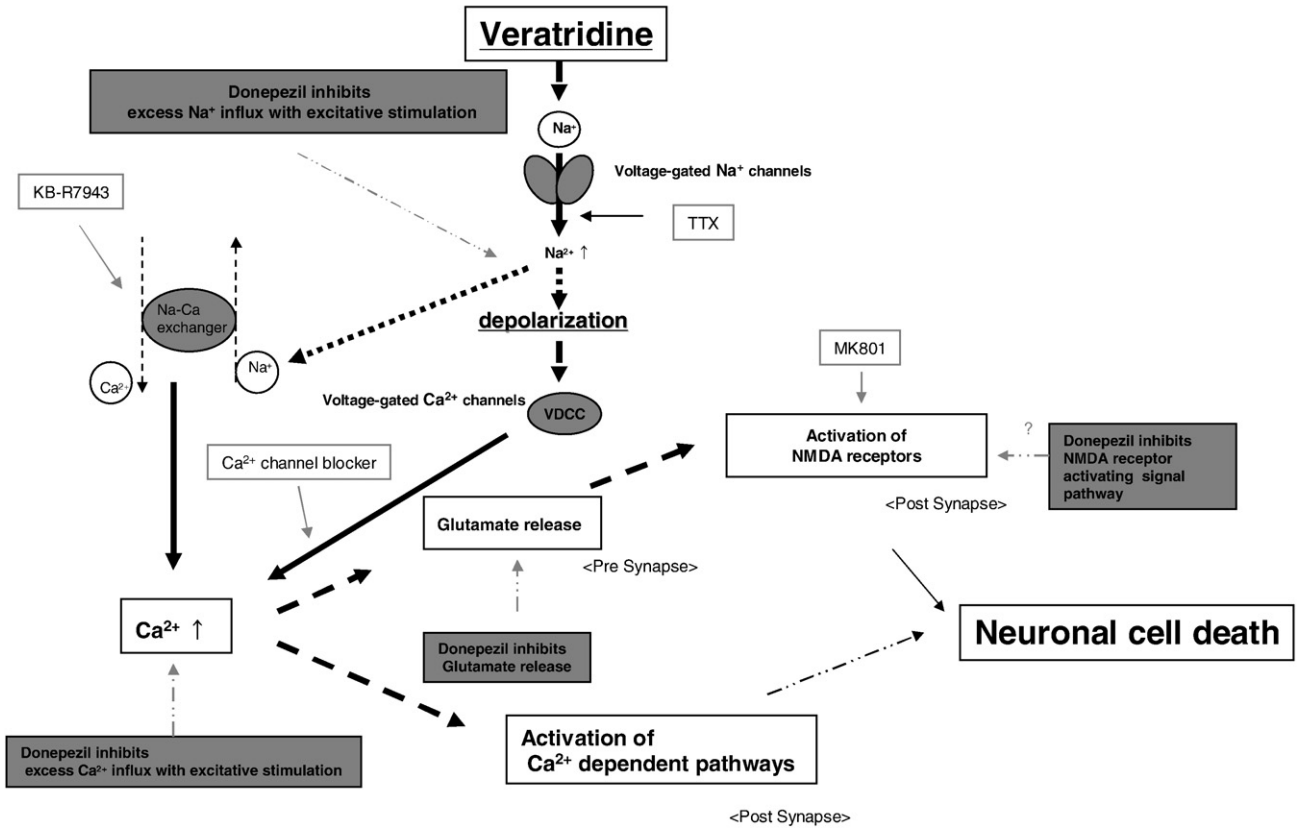

Fig. 7. Neurotoxic mechanisms subsequent to veratridine-induced depolarization. Veratridine induces prolonged opening of Na^+ channels, leading to increased $[Na^+]_i$ and membrane depolarization. Exchange of Na^+ and Ca^{2+} through Na^+/Ca^{2+} exchanger results in an increase of $[Ca^{2+}]_i$ due to the increase of $[Na^+]_i$. The membrane depolarization leads to an increase of $[Ca^{2+}]_i$ through the voltage-dependent Ca^{2+} channels (VDCCs), and elicits glutamate release in presynaptic terminals, and glutamate activates NMDA receptors of the postsynaptic membrane. The excessive increase of $[Ca^{2+}]_i$ through these mechanisms and the activation of NMDA receptors are considered to be the primary causes of neuronal damage by veratridine. The neuroprotective effect of donepezil may be mediated by blocking of voltage-dependent Na^+ channels and VDCCs, and inhibition of the Na^+/Ca^{2+} exchanger, thereby inhibiting excess glutamate release.

facilitating the activation of NMDA receptors. Increase of $[Ca^{2+}]_i$ is induced through the VDCCs and glutamate receptors, and also through exchange of Na^+ and Ca^{2+} via the Na^+/Ca^{2+} exchanger due to the increase of $[Na^+]_i$. The excessive increase of $[Ca^{2+}]_i$ via these mechanisms might be the primary cause of neuronal damage by veratridine.

Donepezil may exert its neuroprotective effect by interfering with the mechanisms mentioned above. Strong inhibition of the rise of $[Na^+]_i$ by donepezil at 10 μM (Fig. 4) may imply a direct effect on Na^+ channels. This idea is supported by the finding that donepezil exerted a weak blocking effect on Na^+ channels at hyperpolarizing membrane potential (IC_{50}=291±26 μM when neurons were held at −80 mV), but its potency was greatly increased at a holding potential near the resting membrane potential (IC_{50}=3.8±0.3 μM at the holding potential of −60 mV) (Yu and Hu, 2005). The rise of $[Na^+]_i$ leads to an increase of $[Ca^{2+}]_i$ through the Na^+/Ca^{2+} exchange system, and this mechanism was shown to be functioning to raise $[Ca^{2+}]_i$ in the present experiments, because KB-R7943, a Na^+/Ca^{2+} exchange inhibitor, significantly decreased the rise of $[Ca^{2+}]_i$. As the IC_{50} of KB-R7943 for the inhibition of Na^+-dependent Ca^{2+} uptake was reported to be around 1 μM (Soma et al., 2006; Namekata et al., 2006), 10 μM KB-R7943 is considered to be sufficient to block Ca^{2+} uptake through the Na^+/Ca^{2+} exchange system. The decrease of $[Ca^{2+}]_i$ produced by 10 μM donepezil was about 40%, which is somewhat larger than that by KB-R7943 (30% at 10 μM), suggesting that donepezil may also reduce $[Ca^{2+}]_i$ through other mechanism(s). As donepezil at 0.1 μM and 1 μM showed neuroprotective effects and a decrease of $[Ca^{2+}]_i$ without any change in $[Na^+]_i$, the neuroprotective mechanisms at these low concentrations are not likely to be related to Na^+ channel inhibition.

A combination of three subtype-specific calcium channel blockers (ω-conotoxin MVIIA for N-type, ω-agatoxin IVA for P/Q type and nifedipine for L-type) decreased the rise of $[Ca^{2+}]_i$ induced by veratridine, suggesting that VDCCs are activated in this model. Although we could not identify what types of VDCC contributed to the rise of calcium induced by the application of veratridine from the results of this study, López et al. (2001) reported that P/Q-, N- and L-type VDCCs mediate Ca^{2+} entry evoked by veratridine in rat cortical neurons. Further, Takano et al. (2003) reported that the L-type VDCC blocker nifedipine has a neuroprotective effect against veratridine-induced insult. Donepezil might have a neuroprotective effect by inhibition of Ca^{2+} channels, as it was reported to inhibit the high potassium-induced $[Ca^{2+}]_i$ rise at high concentrations (Dajas-Bailador et al., 2003), and it inhibited voltage-gated calcium channels in molluscan neurons with the IC_{50} value of 7.9 μM (Solntseva et al., 2007).

Activation of NMDA receptors in response to veratridine exposure is likely to be one of the causes of neuronal injury, as dizocilpine (MK801), a NMDA antagonist, was shown to reduce neuronal damage induced by veratridine in hippocampal cultures (Pauwels et al., 1989). It is known that Na^+ channel-mediated Ca^{2+} entry leads to glutamate secretion (Platel et al., 2005). In the present study, the levels of glutamate and glycine, an agonist and a co-agonist for NMDA receptors, respectively, increased after treatment of the cells with veratridine (Table 1), and MK801 showed a marked neuroprotective effect against veratridine-induced toxicity (Fig. 3), though it

did not affect the veratridine-induced rise of $[Ca^{2+}]_i$ (Fig. 6B), suggesting that NMDA receptor activation is involved in the neurotoxicity. It has been generally assumed that both activation of NMDA receptor and elevation of $[Ca^{2+}]_i$ contribute to neuronal excitotoxic injury, and that decrease in $[Ca^{2+}]_i$ is important for neuroprotection against excitotoxicity. However, recent studies show that interference with the downstream pathway through PSD-95 in NMDA receptor activation exerts a neuroprotective effect without decrease in $[Ca^{2+}]_i$ (Sattler et al., 1999; Aarts et al., 2002; Cui et al., 2007). These facts imply that NMDA receptor blockade can produce neuroprotection without a change in $[Ca^{2+}]_i$, although a high $[Ca^{2+}]_i$ level may be necessary, but not sufficient, for excitotoxicity. Our present results can be interpreted in line with this hypothesis. MK801 did not reduce the elevation of $[Ca^{2+}]_i$, but inhibition of the downstream pathway of NMDA receptor activation by MK801 leads to neuroprotective effects. In the presence of MK801, a high level of $[Ca^{2+}]_i$ is presumed to be maintained by Na^+/Ca^{2+} exchange and/or via voltage-gated calcium channels.

Donepezil reduced the veratridine-induced rise of glutamate and glycine levels, and this reduction might lead to reduced activation of NMDA receptors, resulting in a neuroprotective effect. Donepezil might also interfere with the downstream pathways from NMDA receptor activation, because our previous study showed that 12-h pretreatment with donepezil reduced the NMDA-induced neurotoxicity (Akasofu et al., 2006).

Moreover, inhibition of the Na^+/Ca^{2+} exchanger has been shown to afford neuroprotection in vivo and in vitro (Stys, 1996; Schroder et al., 1999; Breder et al., 2000; Matsuda et al., 2001), so there is a possibility that donepezil may affect the function of the Na^+/Ca^{2+} exchanger. Further study will be needed to clarify this possibility.

The neuroprotective effects of donepezil and TTX might involve similar mechanisms, at least in part. However, a further distinct mechanism might be involved in the case of donepezil, because donepezil plus TTX showed a greater neuroprotective effect than TTX alone (Fig. 2A). Taking into account that donepezil is an acetylcholinesterase inhibitor, an effect on the nicotinic acetylcholine receptor is one possible mechanism for the neuroprotective action of donepezil (Akaike et al., 1994). Therefore, we investigated whether the neuroprotective effect of donepezil is influenced by acetylcholine receptor antagonists or not. A nicotinic receptor antagonist, mecamylamine, and a muscarinic receptor antagonist, scopolamine, did not antagonize the neuroprotective effect of donepezil (Fig. 2B and C). The concentration (10 μM) used in this study is appropriate to analyze the antagonistic effect at acetylcholine receptors (Takada et al., 2003). There is no report indicating that nicotinic receptor agonists are neuroprotective in a veratridine-induced cell injury model. Moreover, carbachol does not show a neuroprotective effect (Fig. 2D). These results suggest that muscarinic and nicotinic activation induced by accumulated acetylcholine, which would result from acetylcholinesterase inhibition, is not related to the protective effect of donepezil. In sympathetic neurons, both necrotic and apoptotic cell deaths have been observed after exposure to veratridine; after 21 days of culture in vitro, most of the cells had died with necrotic features (Koike and Ninomiya, 2000). In the present experiments, the cell death induced by veratridine might predominantly occur through necrosis, because a caspase-3 inhibitor had only a slight neuroprotective effect (Fig. 3). As nicotinic acetylcholine receptor activation is thought to be involved in apoptotic cell death (Takada-Takatori et al., 2006), it is reasonable that mecamylamine did not affect the neuroprotection of donepezil in the veratridine-induced necrotic cell death model.

Although the neuroprotective mechanisms of donepezil cannot yet be clearly defined, the results of the present study with regard to neuroprotection by donepezil can be interpreted as follows. It is widely accepted that the Ca^{2+} overload induced by excitotoxic stimulation plays a crucial role in the pathogenesis of various kinds of neurodegenerative diseases. Intraneuronal Na^+ overload also seems to be profoundly involved in the pathogenesis of neurodegenerative diseases such as cerebral ischemia or epilepsy, since inhibition of extracellular Na^+ influx has been shown to reduce neuronal ischemic vulnerability (Tasker et al., 1992; Fried et al., 1995; Breder et al., 2000). In the case of either excitotoxicity or ischemic insult, the membrane potential will be depolarized, and Na^+ and Ca^{2+} enter neuronal cells through ion channels or Na^+/Ca^{2+} exchangers, resulting in $[Na^+]_i$ and $[Ca^{2+}]_i$ overload and neuronal injury (Schroder et al., 1999 and Matsuda et al., 2001). Therefore, the control of both $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis is very important for neuronal protection. A Na^+/Ca^{2+} exchanger inhibitor, KB-R7943, reduced veratridine-induced $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. 6A). Considering that donepezil inhibited not only the veratridine-induced rise of $[Na^+]_i$ and $[Ca^{2+}]_i$, but also the veratridine-induced glutamate and glycine release, it may reduce depolarization-induced neuronal cell injury through inhibiting voltage-sensitive Na^+ channels, Ca^{2+} channels and Na^+/Ca^{2+} exchangers, and consequently, suppress the release of excitatory amino acids and apoptotic cell death via the NMDA-activated pathway (Fig. 7).

In conclusion, donepezil showed a neuroprotective effect against veratridine-induced neurotoxicity in rat cortical neuron cultures. Although the mechanism(s) involved are not fully understood, the neuroprotective effect seems to be mediated via reductions of $[Na^+]_i$ and $[Ca^{2+}]_i$, with a consequent decrease of glutamate release. If neuronal damage occurs under depolarizing conditions such as ischemia, hypoglycemia, or epilepsy in the clinical situation, donepezil may be useful in treatment for the amelioration of various degenerative diseases, including Alzheimer's disease.

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